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An intact plant assay for estimating nitrogenase activity (C_2H_2 reduction) of sorghum and millet plants grown in pots[1]

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Summary A non destructive intact-plant assay for estimating nitrogenase activity (C_2H_2 reduction) of pot-grown sorghum and millet plants is described. Plants with intact shoots sustained more activity than plants whose tops were removed prior to the assay. With this technique individual plants can be assayed several times during their life cycle. The C_2H_2 reduction was linear up to 16 h incubation in this assay procedure. More rapid diffusion of C_2H_2 was achieved by injection through a Suba seal in the bottom of the pot. The equilibration of injected C_2H_2 in the gas phase of the pots filled with sand and sand:FYM media was completed within 1 h. Significantly higher nitrogenase activity and better growth of sorghum and millet plants occurred when plants were grown in a mixture of sand and farmyard manure (FYM) than when plants were grown in vermiculite, soil, or sand + soil medium. Nitrogenase activity and plant growth were greater in a mixture of sand with 2 and 3% FYM than with 0.5 and 1% FYM. Activity was higher when the plants were incubated at 33°C and 40°C than at 27°C. Activity also increased with increasing soil moisture. There were significant differences amongst 15 sorghum cultivars screened for associated nitrogenase activity. This new technique has good prospects for screening cultivars of millet, sorghum and other grain crops for their nitrogen-fixing ability.

Introduction

Nitrogenase activity associated with non nodulated plants has normally been estimated by the destructive acetylene reduction technique[9,10,20,23]. Nitrogenase activity (C_2H_2 reduction) associated with rice[3,4,6,24] and *Spartina alterniflora*[17], was estimated by an *in situ* intact-plant assay. Intact wheat and barley plants grown in a glasshouse have been assayed for C_2H_4 production by keeping them in a vessel but leaving the stem outside in the air[16]. Intact, glasshouse grown, potted plants have also been assayed in a desiccator or covered with a clear plastic dome[14,15]. It is tedious to enclose large intact plants in a

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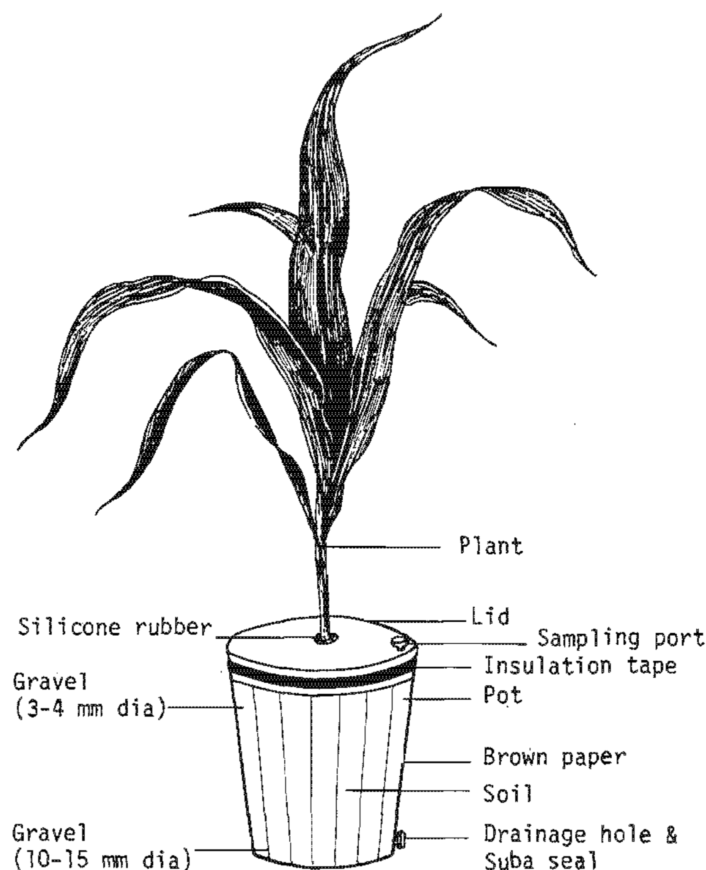

Fig. 1. Assembly for intact-plant assay.

desiccator with clear plastic domes because, if photosynthesis is to be maintained, the control of temperature, and carbon dioxide concentration requires expensive equipment. In this paper we report work on intact-plant assays for measuring nitrogenase activity (C_2H_2 reduction) associated with the roots of sorghum (*Sorghum bicolor* (L.) Moench) and pearl millet (*Pennisetum americanum* (L.) Leeke), and some of the factors affecting activity and the applicability of the technique for screening a large number of cultivars.

Materials and methods

Intact-plant assay technique

Sorghum or pearl millet plants were grown singly in 6 litre plastic containers (20.5 × 33.5 cm) in a glasshouse (Fig. 1). The bottom of the plastic containers had a drainage hole and a layer of 800 g of 10–15 mm diameter gravel to facilitate drainage, and the pots were filled with either 7 kg of Alfisol soil, 3 kg of vermiculite or a 7 kg mixture of sand plus 3% farmyard manure (FYM). The pots were wrapped with brown bituminised craft paper to prevent algal growth at the interface of the pot and the root medium. The pots were saturated with water to 100% water holding capacity (WHC) of the growth medium and, 1 day later, seeds were sown in the centre. Plants were thinned to one per pot 8 days after emergence, and a layer of 2.5 cm thick gravel of 3–4 mm diameter (1 kg) was spread on top of the root medium to prevent algal development. The lids were drilled with two holes, one at the centre for the

plant to grow through and the other at the periphery for watering and, during the assay, for gas sampling. When the seedlings had grown 10 cm tall they were carefully pulled through the central hole of the lid and the lid was replaced to close the container.

Ten day old sorghum and millet seedlings were inoculated with 50 ml of rhizosphere soil extract prepared by suspending 300 g of sorghum or millet rhizosphere soil in 5 litres of deionised water. Throughout the experiment plants were kept in a glasshouse where the temperature was maintained at 30–35°C during the day and 25–28°C during the night. If tillers were present they were pulled in, then bent, and were held in the closed container until the assay period, before the start of which they were cut, leaving the main shoot out. During the growth period the moisture content in the pots was maintained at 60–70% WHC with water or nutrient solution[7]. One day before the assay the moisture content of all pots was adjusted to the same WHC by weighing individual pots. The gap between the plant stem and the lid was plugged with cotton wool and sealed with silicone rubber sealant (No 790, Dow Corning, Australia Pty, Ltd, N.S.W. 2148). Several silicone rubber sealants were tried for sealing the plants but only Dow Corning No. 790 was found to be non toxic to plants. It cured in 5–6 h and the seal was elastic, to hold enough positive pressure required to test the airtightness of the system. After the assay the seal was removed.

The joint between lid and container was sealed by applying polyvinyl chloride (PVC) electrical insulation tape (Fig. 1). On the assay day, before injection of C_2H_2, plants were again watered and Suba seals (No. 29, William Freeman and Co. Ltd., Barnsley, Yorks) were fixed in the top and bottom sampling and injection ports. Acetylene gas (320 ml) was injected into each container through the bottom Suba seal by using a 'syringe' system based on two-way valves and operated by cylinder pressure[11]. Acetylene flow in to the soil was promoted by injecting C_2H_2 through the bottom seal and venting excess pressure through the top Suba seal. Three ml gas samples were collected from each container through the top Suba seal at 1 and 6 h intervals and stored in pre evacuated 'Venoject' tubes. Gas samples vacutainers were brought to normal atmospheric pressure by puncturing with a syringe needle. After thorough mixing a 0.5 ml gas sample was withdrawn with 1 ml syringe and injected onto a Pye Unicam 104 GC fitted with a flame ionization detector (f.i.d.) and a 0.6 cm o.d. × 150 cm glass column, packed with Porapak N. The oven temperature and carrier gas (N_2) flow rate were 100°C and 45 ml/min respectively.

Time-course of C_2H_4 production in the intact-plant assay

Sorghum hybrid cv CSH-5 and millet cv Ex-Bornu plants were grown in containers filled with either Alfisol soil or sand:FYM (97:3 w/w). Ten days after planting, 10 kg N/ha equivalent as ammonium sulphate was added in solution and every seven days, each pot received 100 ml N-free nutrient solution[7]. Twelve days after sowing each plant was inoculated with 100 ml sorghum and millet rhizosphere soil extract, and 100 ml napier bajra root extract (NBRE), prepared by macerating 160 g of roots and rhizosphere soil of napier bajra (*P. Americanum* × *P. purpureum*) in 2 litres of N-free nutrient solution incubated for 24 h at room temperature before inoculation, and supernatant was obtained after passing the solution through double-layered cheese cloth. The drain holes of the pots were plugged with Suba seals to prevent entry of water in the pots. The pots were placed in temperature-controlled water baths, maintained at 40 ± 0.5°C 10 days prior to the assay. Acetylene gas was injected and gas samples were collected at 1, 3, 5, 7, 10, 13, 16, 22, 25 and 27 h after injection and analysed for C_2H_4 production. Each treatment was replicated 5 times.

Diffusion of C_2H_2 in different growth media

Sorghum hybrid cv CSH-8R and millet cv Gam-73 plants replicated 15 times were grown in pots filled with either 6.5 kg Alfisol soil, 7 kg washed sand, 3 kg vermiculite, 7.5 kg sand: soil mixture (60:40 w/w) or 7 kg sand:FYM (97:3 w/w). Seven days old seedlings received 15 kg N/ha equivalent as ammonium sulphate in solution and each plant was inoculated with 50 ml of rhizosphere soil suspension. Every seven days plants were fed with N-free nutrient solution[7] required to adjust the pots to 60–70% WHC. Diffusion of C_2H_2 in different growth media was studied with millet plants 63 days old, and sorghum plants 67 days old. Acetylene

gas was injected through the bottom Suba seal and, at 0, 1 and 2 h intervals, gas samples were taken through both the bottom and top Suba seals, and after 3 and 6 h, through the top only. Gas samples were analysed for C_2H_2 and C_2H_4 concentrations. Plant tops were harvested 1 day after assay, dried at 70°C for 72 h in a hot air oven, and dry weights were recorded.

Effect of root medium on activity

Sorghum and millet plants grown in different rooting media for studying gas diffusion were assayed for nitrogenase activity.

The effect of amount of FYM on nitrogenase activity was examined using 0, 1, 2 and 3% mixtures with washed sand. Sorghum hybrid cv CSH-5 and millet cv Ex-Bornu were grown in a glasshouse with 10 replicate plants per treatment. Plants were assayed at 44 and 71 days after planting (DAP) for nitrogenase activity, and plant dry weights were recorded.

Effect of incubation temperature

Sorghum hybrid cv CSH-5 and millet cv Ex-Bornu plants were grown in pots containing 7 kg Alfisol soil or sand:FYM (97:3 w/w) and inoculated with respective rhizosphere soil extract and NBRE culture. Ten day old seedlings were given 10 kg N/ha equivalent as ammonium sulphate in solution and, at 7 day intervals, plants received 100 ml N-free nutrient solution[7]. Ten days prior to the assay, five replicated plants grown in both media were placed in temperature-controlled water baths set at 27, 33 and 40°C. Millet (56 DAP) and sorghum (59 DAP) were assayed for nitrogenase activity at the same temperatures by intact-plant assay with gas samples collected at 5 and 7 h after the addition of acetylene gas. Temperatures in the pot at 4 and 16 cm depth were recorded, using an automatic temperature recorder. The growth-medium temperature recorded was the same as that of the water bath in which the pots were kept.

Effect of soil moisture

Sorghum hybrid cv CSH-5 and millet cv Ex-Bornu plants were grown in Alfisol soil and 10 kg N/ha equivalent as ammonium sulphate was added. Every seven days each plant received 100 ml nutrient solution. Different moisture regimes at assay times were established by watering one set of pots to 60–70% WHC on each of the 5 days before the assay with no further watering. Another set was adjusted to 80% WHC on the assay day. Sorghum was assayed at 65 and 73 DAP and millet at 66, 74 and 97 DAP. Gas samples were collected 1, 6 and 22 h after the injection of C_2H_2. Soil moisture percentages were calculated by weighing the pots. Each treatment was replicated 8 times and regression analyses were performed for nitrogenase activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) and soil moisture content.

Effect of the shoot on activity

Fifteen cultivars of sorghum grown in pots filled with Alfisol soil were assayed at 49 and 76 days for nitrogenase activity using the intact-plant assay and destructive assay (shoot cut-off) techniques. The second set of plants was treated identically except that, just before assay, the shoot was cut off at soil level. Each treatment was replicated 5 times.

Screening of cultivars

The intact-plant assay technique was used to see if differences in associated nitrogenase activity could be detected amongst 15 cultivars of sorghum grown in Alfisol soil. Each pot received 20 kg N/ha equivalent as ammonium sulphate. Each cultivar was replicated 10 times.

Results

Time-course C_2H_4 production in the intact-plant assay

Fig. 2 shows the pattern of cumulative nitrogenase activity of sorghum hybrid cv CSH-5 plants against the incubation time. A small

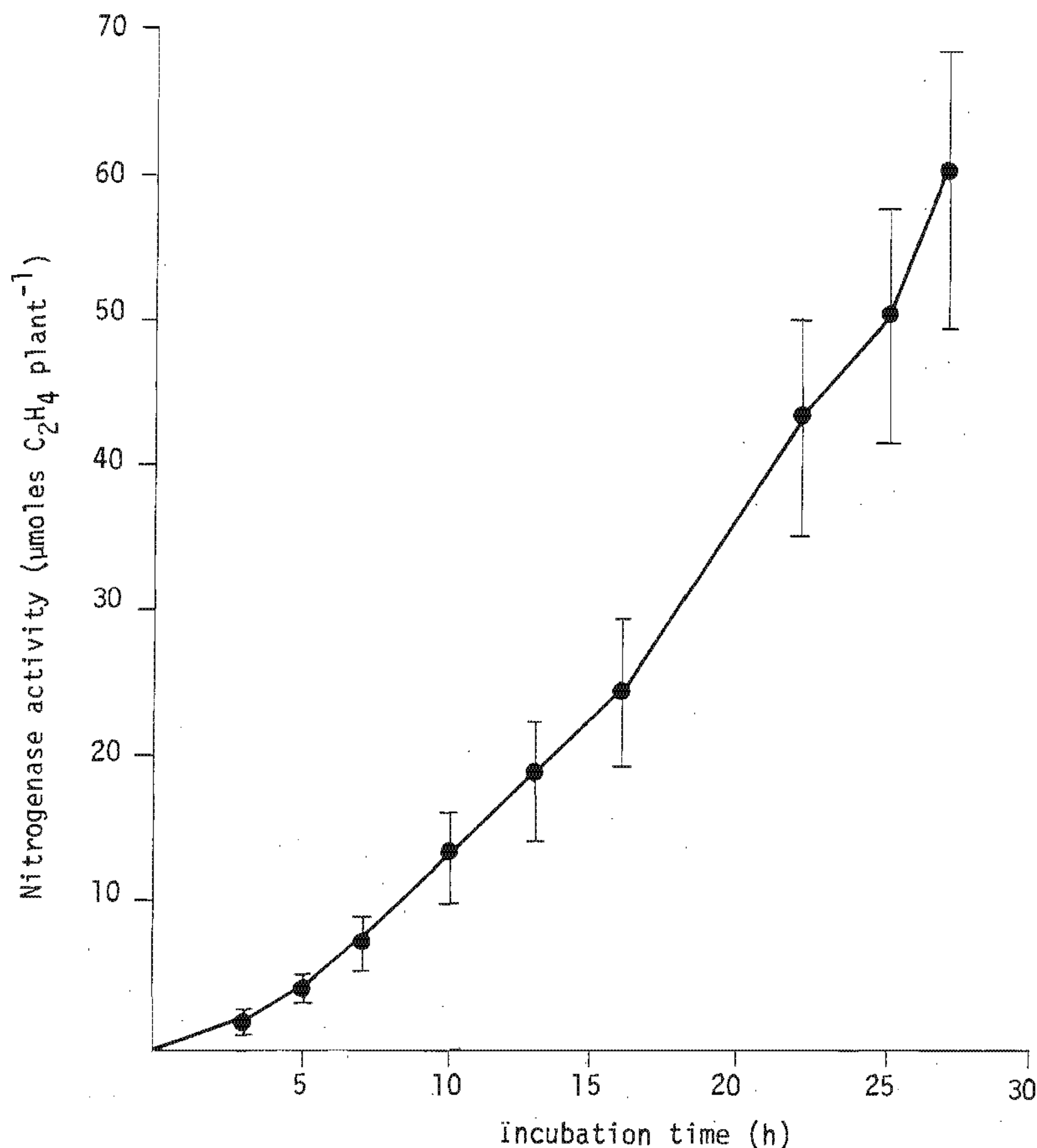

Fig. 2. Relationship between ethylene production and incubation time (h) during the assay of intact sorghum plants. Bars represent ± SEM.

initial lag for C_2H_4 production in these systems was observed. Later the production of C_2H_4 was linear up to 16 h of incubation, and then slightly increased, as shown in Fig. 2. Regression analysis indicated a linear relationship ($Y = 1.85x$, $P \leqslant 0.01$). Similarly, with millet, intact-plant assays also an initial lag for C_2H_4 production was observed and, after 16 h, the production increased slightly as did that with sorghum. A linear relationship ($Y = 0.823x$) for cumulative C_2H_4 production (umoles C_2H_4 plant^{-1}) against incubation time (h) was observed ($R = 0.839; P \leqslant 0.01$).

Diffusion of C_2H_2 in different growth media

Quicker and better diffusion of C_2H_2 was observed when it was injected through the bottom Suba seal as compared with top injection. Fig. 3 shows the patterns of C_2H_2 diffusion (injected through the

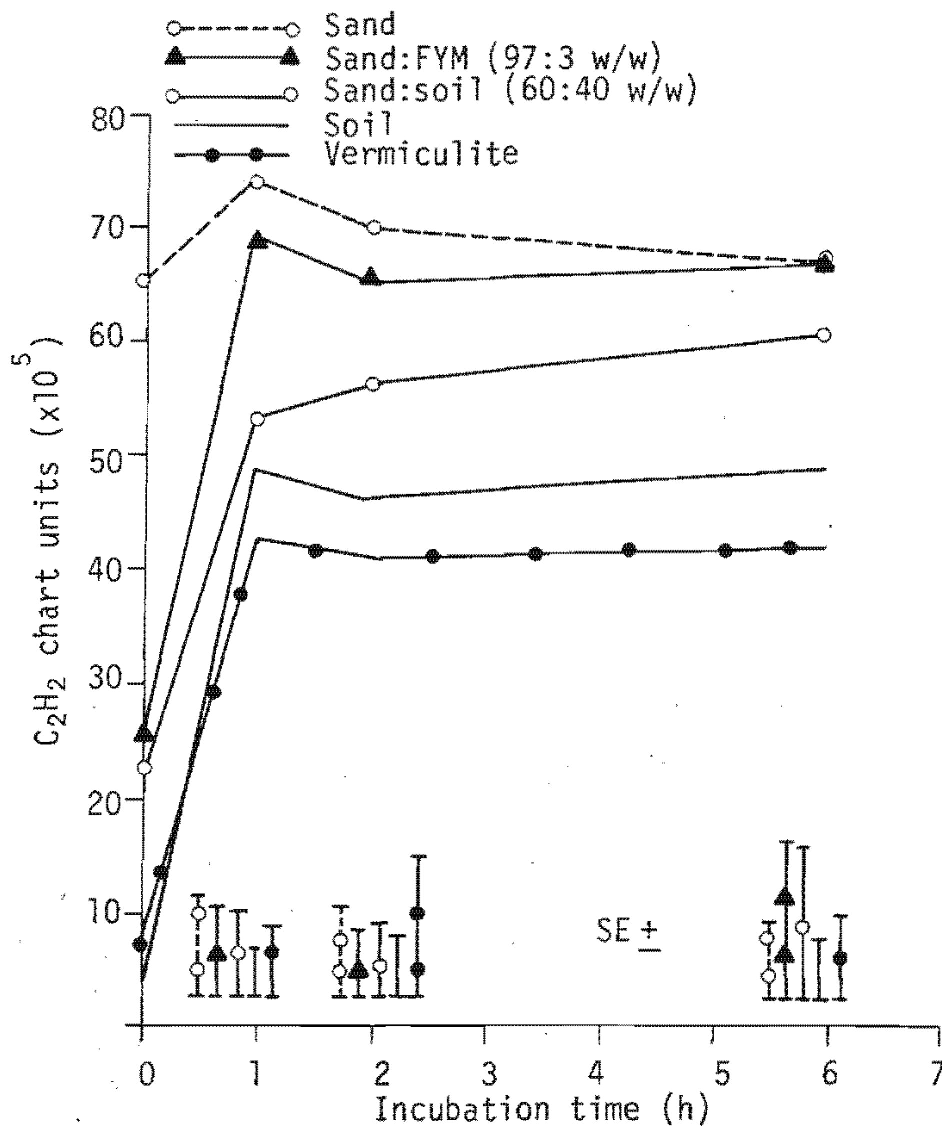

Fig. 3. Concentration of bottom-injected C_2H_2 in the top gas phase at different time intervals in the pots filled with different media.

bottom Suba seal and sampled from the top) through millet pots filled with different media, *viz.* sand, sand:FYM (97:3 w/w), sand:soil (60:40 w/w), Alfisol soil and vermiculite. Gas samples collected from the top of the media soon after the injection of C_2H_2 from the bottom varied significantly for C_2H_2 concentration amongst the media. The highest C_2H_2 concentration was observed in the samples collected from the top of the sand and lowest from Alfisol soil. One hour after injection, C_2H_2 concentration above the sand and sand: FYM media was significantly higher than that from above other media. The concentration was the same as that in samples collected from the bottom of the same pots, indicating an equilibration of the gas phase. The equilibration of C_2H_2 through all the media, except sand:soil, was observed within 2 h after injection. Similar results for C_2H_2 diffusion were recorded with the sorghum pots filled with different media.

Table 1. Growth and nitrogenase activity associated with sorghum hybrid CSH 8 grown in different root media

Media	Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1})	Plant dry matter (g/plant)
Soil	7^{a*}	40
Vermiculite	49^{c}	35
Sand : soil (60 : 40 w/w)	22^{b}	24
Sand : FYM (97 : 3 w/w)	270^{d}	43
SE		± 1.5
CV%		20

* = Log transformation of (nmoles C_2H_4 + 1) were used for analysing data. Values with different letters vary significantly ($P \leq 0.05$) from each other.

Effect of root medium on activity

Use of the intact-plant assay demonstrated differences between root media supporting nitrogenase activity. Significantly higher nitrogenase activity (270 nmoles C_2H_4 plant^{-1} h^{-1}) was observed with plants of sorghum hybrid CSH-8 grown in sand : FYM mixture than in other media (Table 1). In this experiment the overall activity recorded with plants was considerably low in comparison with other experiments. Highest plant dry matter (43 g/plant) was recorded with the plants grown in sand : FYM (3%), which was equal to the dry weight of the plants grown in Alfisol soil. However, lowest activity (7 nmoles C_2H_4 plant^{-1} h^{-1}) was recorded with plants grown in soil. Generally higher levels of nitrogenase activity have been observed with the plants grown in Alfisol soil *e.g.* refer values in Table 5. Dry matter of plants of millet cv Gam-73 grown in soil (28 g/plant) and sand : FYM (27 g/plant) was at par and significantly higher than dry matter of plants grown in sand : soil (12 g/plant) or vermiculite (20 g/plant). At assay time millet plants grown in different media had low nitrogenase activity.

Significantly higher activity was recorded with plants of sorghum hybrid CSH-5 grown in sand with 2 and 3% FYM than with 1 and 0.5% FYM (Table 2). A maximum nitrogenase activity of 1920 nmoles C_2H_4 plant^{-1} h^{-1}) was recorded with 44 day old plants grown in sand with 3% FYM and highest activity of 1460 nmoles C_2H_4 plant^{-1} h^{-1}) was recorded with 71 day old plants grown with 2% FYM. An increasing amount of dry matter was produced with the increasing FYM concentration. Results of nitrogenase activity and growth of millet cv Ex-Bornu plants were similar to those of sorghum (Table 3). Significantly higher activity was recorded with the plants grown in sand with 3 and 2% FYM than with 1 and 0.5% FYM. Increased FYM concentration resulted in increased plant dry matter production.

Table 2. Effect of different levels of farmyard manure (FYM) on total dry matter and nitrogenase activity associated with sorghum hybrid CSH 5

Medium	Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1})		Plant dry weight (g/plant)
	44 DAP	71 DAP	
Sand:FYM (97:3)	1920 ± 480*	1340 ± 360	35.6 ± 1.13
Sand:FYM (98:2)	1340 ± 280	1460 ± 360	29.7 ± 1.06
Sand:FYM (99:1)	490 ± 85	680 ± 84	13.9 ± 0.82
Sand:FYM (99.5:0.5)	150 ± 34	430 ± 140	7.7 ± 0.50

* ± SE. Each value is average of 10 replications

Table 3. Effect of different levels of farmyard manure (FYM) on total dry matter and nitrogenase activity associated with millet cv Ex-Bornu

Treatment	Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1})		Total plant dry wt. (g/plant)
	44 DAP	71 DAP	
Sand:FYM (97:3 w/w)	593 ± 101*	483 ± 193	32.6 ± 1.28
Sand:FYM (98:2 w/w)	542 ± 154	745 ± 234	27.7 ± 1.43
Sand:FYM (99:1 w/w)	86 ± 24	137 ± 38	10.4 ± 0.6
Sand:FYM (99.5:0.5 w/w)	106 ± 23	276 ± 72	5.2 ± 0.46

* ± SE. Each value is average of 10 replications

Table 4. Effect of incubation temperature on nitrogenase activity associated with millet cv Ex-Bornu plants

Temperature (°C)		Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1}) Medium		
		Sand:FYM		Soil
40		750		510
43		1060		530
27		240		150
	SE		± 220	
	Mean	685		330
	SE		± 104	

Effect of incubation temperature

Millet cv Ex-Bornu plants incubated at 33 ± 1°C and 40 ± 0.5°C gave significantly higher activity than plants incubated at 27 ± 1°C in both media (Table 4). Plants grown in sand:FYM, incubated at 33 ± 1°C showed the highest activity (1060 nmoles C_2H_4 plant^{-1} h^{-1}) whereas, with plants grown in soil, highest activity (510 nmoles C_2H_4 plant^{-1} h^{-1}) was recorded at 40 ± 0.5°C. The activity results for interaction between media and incubation temperature were statistically nonsignificant. Significantly higher activity was recorded with plants grown in sand:FYM than with plants grown in Alfisol soil.

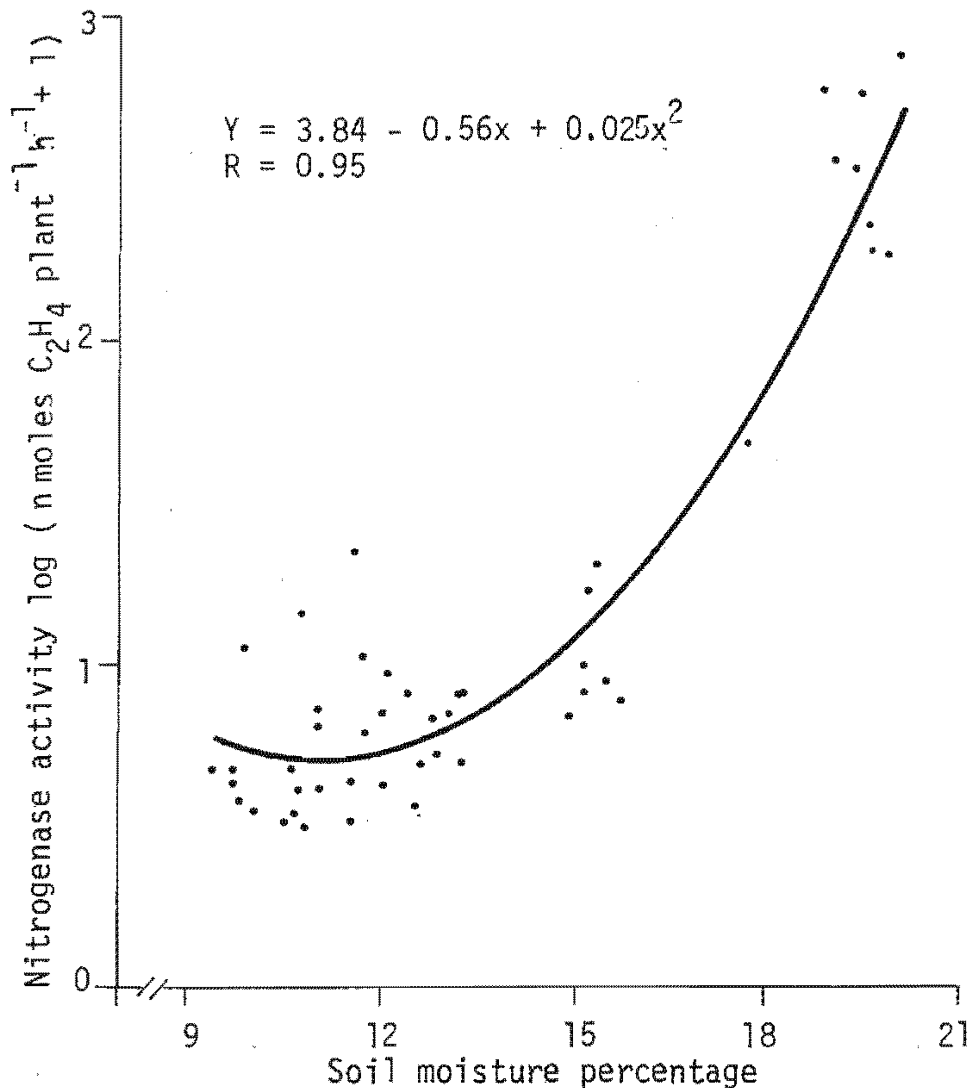

Fig. 4. Relationship between nitrogenase activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) associated with sorghum hybrid CSH 5 plants and soil moisture content when assayed by the intact-plant method.

A similar trend of activity for sorghum hybrid cv CSH-5 plants was also observed.

Effect of soil moisture

The watering regime established a range of soil moisture varying between 10.6 and 19.8% (42–79% WHC). Nitrogenase activity of 73 day old sorghum hybrid cv CSH 5 plants increased with increasing soil moisture content. Regression analysis indicated a significant ($R = 0.95$) ($P \leqslant 0.01$) curvilinear relationship ($Y = 3.84–0.56x + 0.025x^2$) between the activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) and soil moisture content. Fig. 4 shows the fitted regression curve and spread of points of nitrogenase activity against varying soil moisture levels. A curvilinear relationship ($Y = 1.08–0.16x – 0.01x^2$) was

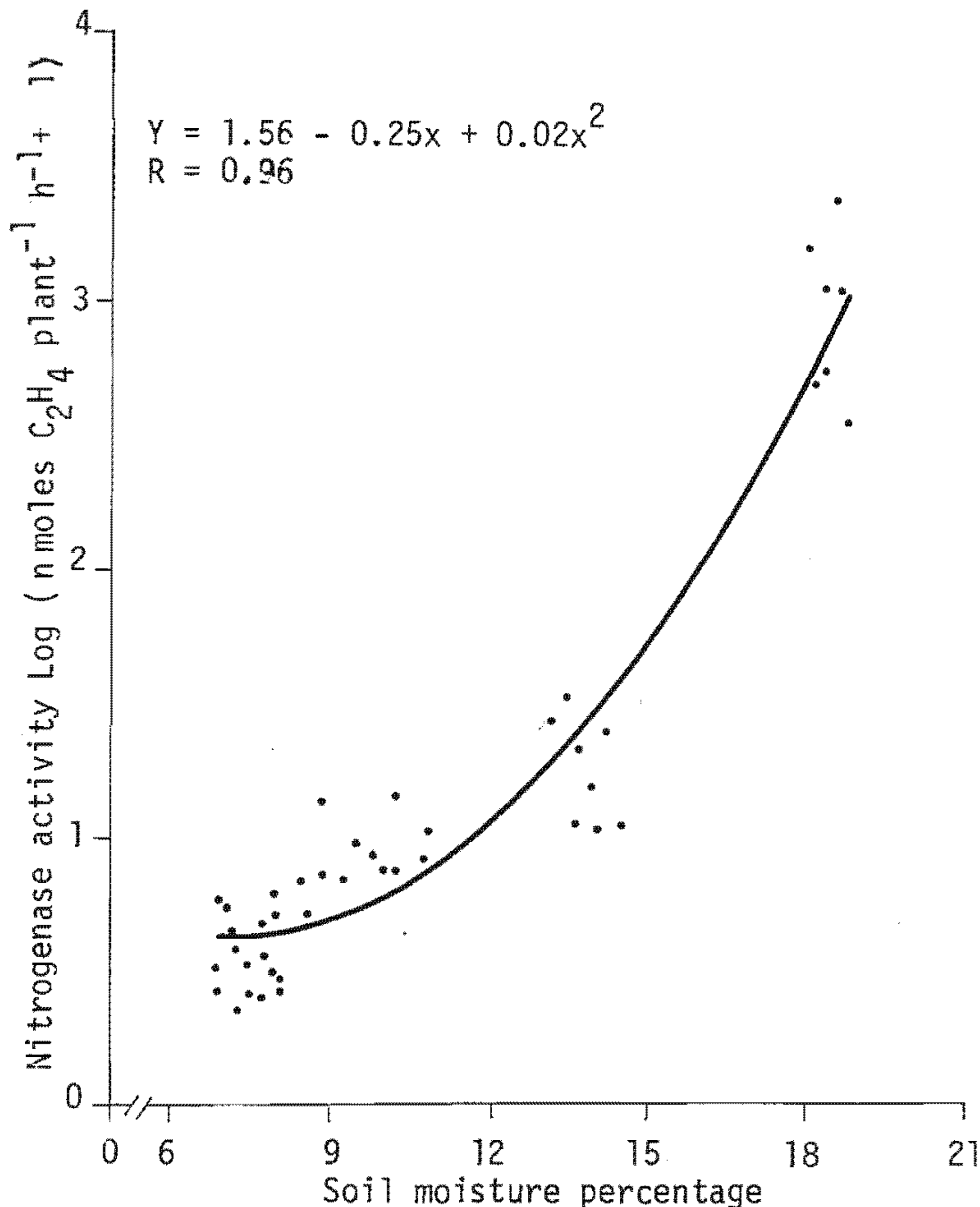

Fig 5. Relationship between nitrogenase activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) associated with millet cv Ex-Bornu plants and soil moisture content when assayed by the intact-plant method.

observed between nitrogenase activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) of 61 day old plants and soil moisture contents varying from 7.1 to 19.6% (28.6–78.4% WHC). The activity of 74 day old millet plants also increased as soil moisture increased from 7.1 to 19.6% (28.6–78.4% WHC) with a curvilinear relationship ($Y = 1.56 - 0.25x + 0.02x^2$) ($R = 0.98$, $P \leqslant 0.01$) between the activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) and soil moisture percentage (Fig. 5). Similarly, increased activity with increasing soil moisture was observed with 66 day old millet plants. However, the activity was low as compared with that of 74 day old plants. A linear relationship ($Y = 0.11x - 0.32$)

Table 5. Nitrogenase activity of intact and decapitated sorghum plants

Cultivar	Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1})*	
	Intact plant assay	Decapitated plant assay
IS-84	3595 ± 982***	1920 ± 800
CSV-5	3065 ± 1645	1370 ± 1354
IS-1256	1655 ± 944	25 ± 2
IS-5108	480 ± 310	80 ± 31
Dobbs	375 ± 267	15 ± 5
IS-2261	290 ± 270	30 ± 8
2077 B	195 ± 97	270 ± 134
IS-2190	65 ± 37	15 ± 8
IS-1324	60 ± 38	40 ± 10
IS-2267	50 ± 15	45 ± 12
IS-2980	40 ± 13	35 ± 7
IS-801	35 ± 5	35 ± 12
IS-2207	30 ± 6	15 ± 2
IS-1398	35 ± 2	20 ± 5
IS-5218	25 ± 3	20 ± 6
Soil	15 ± 2	25 ± 6
Mean**	625[b]	247[a]

* Average of 5 replications

** Log transformation of (nmoles C_2H_4 + 1) used for analysing data. Figures with different letters vary significantly ($P = \leqslant 0.05$) from each other.

*** ± Standard error.

was observed between activity (log (nmoles C_2H_4 plant^{-1} h^{-1} + 1)) and soil moisture percentage.

Effect of the shoot on activity

The intact plants of 15 sorghum cultivars showed higher activity than that estimated with decapitated plants (Table 5). Mean nitrogenase activity of 49 day old plants across the cultivars with intact shoots was 625 nmoles C_2H_4 plant^{-1} h^{-1} which was significantly higher ($P \leqslant 0.05$) 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. Reduction in the activity of these cultivars due to decapitation of their shoots was considerably more (26 times) with 76 day old plants as compared with those of 49 day old plants (2.5 times). Activity of these cultivars 76 DAP by destructive (decapitated) assay was negligible, whereas intact-plant assay showed high activity with four cultivars. Mean activity of 76 day old plants across the cultivars by intact and decapitated plant assays was 291 and 11 nmoles C_2H_4 plant^{-1} h^{-1} respectively.

Screening of cultivars

By using the intact-plant assay technique, the same plant could be assayed at several stages during the growth period. Unplanted soil pots treated in the same way as that of planted ones had very low activity (15 nmoles C_2H_4 pot^{-1}), suggesting that plants stimulated notably high nitrogenase activity and that the measures taken for preventing the development of algae were successful. Activity associated with sorghum cultivars varied from 25 to 3595 nmoles C_2H_4 $plant^{-1}$ h^{-1}, permitting the differentiation of the cultivars with high and low associated activity (Table 5).

Discussion

The intact-plant assay technique for measuring nitrogenase activity associated with the roots of pot grown sorghum and millet plants is non destructive, and permits several assays to be made with the same plant. This technique gives higher nitrogenase activity and less plant-to-plant variability than soil core or excised root assays because it is possible to maintain optimum temperature and moisture. Using the technique also obviates such factors as mechanical disturbance, sampling time, diurnal variation, cutting of the plant top, *etc.*, that have been reported[23] to affect the estimation of nitrogenase activity when the soil core assay technique is used. Some of the problems associated with other acetylene reduction assay methods, such as the destruction of the plant in soil core or excised root assays[1,9,10,11,18,23] or enclosing the leaves in plastic, myler, nylon bags or clear plastic domes[2,3,13,14,15,17], with the possible associated problems of low radiation, low CO_2 concentration and high temperatures, are not faced with this technique.

Equilibration of the gas phase in pots filled with sand and sand : FYM mixture took upto 1 h whereas other media required 1–3 h. It was observed that equilibration of C_2H_2 and C_2H_4 across a Brazilian soil took 3 h, whereas Woburn or Rothamsted soils (without plants) needed 30 h[21]. Such a differential time requirement for diffusion of C_2H_2 across the different media was probably due to the difference in textures of the media and moisture content in the soil during assay[16,21]. In the pot assay system described, diffusion of C_2H_2 injected with pressure through the bottom Suba seal, permitting excess gas to move out through the needle inserted through the top Suba seal for equilibrating with atmospheric pressure, resulted in the early diffusion of C_2H_2.

Intact-plant assays with sorghum and millet showed linear rates

of C_2H_4 production up to 16 h 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 slightly increased, suggesting that the incubation period for estimating nitrogenase activity. The initial lag period for C_2H_4 production was possibly because of the time required for C_2H_2 diffusion throughout the root medium in the pot. Similarly, soybeans inoculated with *R. japonicum* grown in pots filled with soil and assayed by enclosing them in saran bags exhibited an initial lag of 1 h and then onwards rate of C_2H_4 production was linear up to 24 h[5]. Sorghum plant-soil cores estimated for C_2H_2 reduction by enclosing cores in plastic bags but leaving the stem outside showed a 6 h lag for C_2H_4 production, due to the time required for equilibration of gases between the top and bottom of the soil cores[18].

Higher activity was associated with the intact plants than with decapitated 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 detailed studies will be required for an understanding of this effect.

With the plants grown in sand higher nitrogenase activity was observed with increasing FYM concentration. The increased activity may be due to better plant growth, with increased FYM concentration providing more root surface area and more root exudates (derived from an increased root mass) for colonization for N_2-fixing bacteria. Further experimentation is required to understand the role of FYM in stimulating nitrogenase activity associated with plants. However, selection of an appropriate growth medium and standardization of the related conditions must be done for a given crop.

A further factor affecting nitrogenase activity of intact sorghum and millet plants is the incubation temperature during assay. Increased activity was recorded with the temperature increasing from 27°C to 33°C. Other workers have observed a relationship between soil temperature and nitrogenase activity with *Brachiaria mutica, Sorghum vulgare*, maize and *Thalissia testudinum*[8].

Soil moisture had a large effect on nitrogenase activity of sorghum and millet in the intact-plant assay. Our results support the earlier findings[11,19,22,23] that plant associated nitrogenase activity increases with the increasing soil moisture content. The problems associated with understanding the role of soil moisture affecting many plant processes that may influence the nitrogenase activity have been discussed[14,16,22,23].

The intact-plant assay described earlier was tested for estimating nitrogenase activity associated with 15 sorghum cultivars, and we could differentiate cultivars with high and low associated activity.

This suggested the possibility of using this technique for screening cultivars of sorghum and millet for their potential to fix atmospheric nitrogen biologically, after studying the relationship between intact-plant assay and field assay methods. This new technique has good prospects for screening cultivars of millet, sorghum and other grain crops for their nitrogen-fixing ability. 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. The maximum expressions of the activity could be obtained by growing plants in 3% farmyard manure mixed with sand or Alfisol soil, by watering the plants to 60–70% WHC, by maintaining the plant growth medium temperature around 33°C, and by not decapitating the plant shoot.

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