Factors affecting nitrogenase activity (C_2H_2 reduction) associated with sorghum and millet estimated using the soil core assay[1]

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Factors affecting nitrogenase activity associated with sorghum and millet roots have been studied. Plants grown in iron cores in the field and then assayed had significantly higher activity than plants cored at the time of assay. Mechanical disturbance during transportation of the cores reduced the activity significantly. Any delay between cutting off the plant top and injecting C_2H_2 gas led to a reduction in the level of nitrogenase activity determined. Diurnal variation in nitrogenase activity was noted but was not correlated with soil temperature. Most activity occurred at the end of the photoperiod. Seasonal variation in nitrogenase activity of plants was observed and was correlated with the ontogenetic development of the host plant, being most at flowering. A low but significant correlation existed between soil moisture content and nitrogenase activity associated with the plant.

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Les facteurs qui affectent l'activité nitrogénase associée aux racines de sorgho et de millet ont été étudiés. Des plantes croissant en plein champ, dans une rhizosphère riche en fer, ont été testées *in situ* et ont présenté une activité significativement plus élevée que les plantes dont la rhizosphère est extraite du sol pour fins d'essais. Les perturbations mécaniques des rhizosphères lors du transport des échantillons réduisent cette activité de façon significative. Tout délai entre la séparation de la partie supérieure de la plante et l'injection du gaz C_2H_2 cause une réduction du niveau d'activité nitrogénase déterminé. Une variation diurne de l'activité nitrogénase a été notée, mais elle ne fut pas correlée avec la température du sol. La plus grande part d'activité est survenue à la fin de la photopériode. Une variation saisonnière dans l'activité nitrogénase des plantes a été observée et fut correlée avec le développement ontogénique des plantes hôtes, avec un maximum au temps de la floraison. Une corrélation faible, mais significative existe entre la teneur en eau du sol et l'activité nitrogénase associée aux plantes.

[Traduit par le journal]

Introduction

Nitrogenase activity associated with many tropical grasses and grain crops has been estimated by the C_2H_2 reduction assay technique (Döbereiner *et al.* 1972; Neyra and Döbereiner 1977; Dart and Wani 1982). In many of the earlier studies, nitrogenase activity was measured by an excised root assay, a technique which has been criticized because the 8–18-h preincubation period prior to the injection of acetylene (Neyra and Döbereiner 1977) allows for considerable fermentation and bacterial proliferation (Okon *et al.* 1977; P. van Berkum. 1978. Ph.D. thesis, University of London, London) and resulted in an overestimation of nitrogenase activity. *In situ* assays have also been used but are cumbersome and the measurements are difficult to interpret (Balandreau and Dommergues 1973; Tjepkema and van Berkum 1977). Greenhouse-grown, potted plants (Harris and Dart 1973; Hirota *et al.* 1978) have also been assayed for nitrogenase activity, but special precautions must be taken to control growth of blue-green algae if one is measuring heterotrophic N_2 fixation.

Soil cores containing plant roots, removed from the field at harvest, have been used to measure C_2H_2 reduction activity of both grasses and grain crops (Day, Harris *et al.* 1975; van Berkum and Day 1980; Subba Rao and Dart 1981). However, large plant-to-plant variability has been reported using this method (Dart and Wani 1982). In this paper we report studies of the factors affecting nitrogenase activity associated with roots of sorghum (*Sorghum bicolor* (L.) Moench) and pearl millet (*Pennisetum americanum* (L.) K. Schum) estimated by soil core assays, in an attempt to understand the reasons for the variability.

Materials and methods

Effect of growing plants in planted cores and in plastic buckets

Pearl millet cv. NHB-3 was planted during the 1980 rainy season in an Alfisol soil at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Centre, Hyderabad, India at a spacing of 50 cm between rows and 15 cm between plants within the row and supplied with a basal dose of 20 kg N and 20 kg P_2O_5 per hectare.

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Nitrogenase activity of cores of field-grown plants sampled in the normal way (regular core assay) at 55, 66, and 69 days after planting (DAP) was compared with that of plants grown with their roots enclosed by the core from 15 days onward (planted cores) and plants grown in soil in plastic buckets sunk into the soil to the rim. At sampling nitrogenase activity of both the regular cores and planted cores was estimated by the method described below. Plants grown in buckets were cut off at ground level, the buckets dug out from the field, and further processed for C_2H_2 reduction activity.

Nitrogenase activity of the plants was estimated by the soil-core assay technique (Anonymous 1978). The surface layer of soil around the plant to be sampled was first scraped to remove any algal growth, and the plant top was cut off. A metal core of 15 cm diameter and 22 cm length was placed around an individual plant and driven in to ground level (regular core). Soil cores were removed from the ground, placed in 6-L plastic containers (20.5 cm diameter and 23.5 cm tall), transported to the laboratory, and sealed by applying polyvinyl chloride (PVC) electrical insulation tape to the joint between the container and
sampling through Suba seals (No. 29). Acetylene (320 mL) was injected in each container using a "syringe" system using two-way valves and operated by cylinder pressure (Day, Harris *et al.* 1975). After injecting the unscrubbed C_2H_2, the gas phase in the container was brought to atmospheric pressure by bleeding excess gas through a hypodermic needle inserted through the Suba seal. The assay containers were incubated at room temperature (30 ± 2°C). Three-millilitre gas samples were taken from each container after 1 and 6 h of incubation time and stored in preevacuated "Venoject" tubes before gas chromatographic analysis.

A similar experiment was conducted using nine cultivars of sorghum grown during the irrigated summer season of 1981 in an Alfisol soil. The cores were driven into the soil around 15-day-old plants. Planted cores and regular cores were compared by assaying the plants 54 DAP. For each cultivar four cores were used for each treatment and all cultivars were replicated four times. Sampling and processing of cores was done as detailed earlier.

Effect of the mechanical disturbance

The effect of mechanical disturbance during transportation on nitrogenase activity of plants was studied by assaying field-grown millet plants cv. NHB-3 from the previous experiment at flowering stage (52 DAP). Two treatments were employed (*i*) regular core assay in which all the operations were done in the usual way and (*ii*) regular core assay sampled and transported with special precautions during all aspects of handling the cores. Each treatment involved 50 replicate cores which were processed as detailed earlier.

Effect of the time lag between cutting off the plant top and injecting C_2H_2

One hundred randomly selected, field-grown millet plants from the first experiment were assayed at grain-filling stage (66 DAP), and cores were taken as in the regular assay technique but were then held in the laboratory for 0.5, 1, 2, 3, and 4 h after the plant tops were cut off in the field and before the containers for the cores were sealed and C_2H_2 injected with 20 replicate cores in each time set.

Diurnal variation in nitrogenase activity

The effect on nitrogenase activity of time of sampling during the day was examined by sampling field-grown millet plants from the first experiment which were approaching physiological maturity (98 DAP). The plants were assayed by the regular soil core assay technique. The sampling was started at 0915 and continued until 0715 of the next day, with 10 sampling times, each with 20 replicate cores. Soil temperature at a depth of 20 cm was recorded at each sampling.

Seasonal pattern for nitrogenase

The seasonal pattern of the nitrogenase activity was examined for sorghum hybrid CSH-1 and pearl millet cv. IP2787 sown on 2 February 1980 during the dry season in polythene bags filled with 4 kg of an Alfisol soil. The soil surface in each bag was covered with a 5-cm-thick layer of gravel (5–10 mm size) and the bags were kept immersed in a sand bed to avoid algal growth at the soil surface and on the sides of the bag. Plants were watered as required with tap water and 1 day before assay the moisture content of the bags was adjusted to 65–70% water-holding capacity (WHC). Five randomly selected plants of sorghum and millet were assayed at 16, 30, 45, 62, 79, 94, and 111 DAP with the assay starting each day at 1000. At harvest the plant tops were cut off, then the bags containing the undisturbed roots were put in 6-L plastic containers, and processed further as for the soil-core samples.

Effect of moisture on nitrogenase activity

Effect of moisture on nitrogenase activity was studied by manipulating the moisture regime in the soil by two different methods.

Two sorghum hybrids CSH-6 and CSH-8 were planted in an Alfisol soil on 31 October 1980 with a basal dose of 60 kg N and 60 kg P_2O_5 per hectare. Thirty days after planting a top dressing of 40 kg N per hectare was given. All the plots received three furrow irrigations to recharge the soil profile and to ensure good crop establishment. Thereafter at 30 DAP a differential moisture gradient in the field was created using a line-source sprinkler irrigation system (Hanks *et al.* 1976) to produce a water application pattern which was uniformly variable across the plot. At the grain-filling stage (97 DAP), plants 2, 4, 6, and 8 m away from the sprinkler were sampled for nitrogenase estimation taking eight plant–soil cores from each of two replicate plots at each distance from the line source. Nitrogenase activity was estimated as previously described. A soil sample ws collected near each sampling spot and the moisture content determined by weighing before and after drying at 110°C for 48 h.

In a separate experiment, sorghum hybrid CSH-1 was grown without added nitrogen in an Alfisol soil which had been kept fallow during the previous season. At the vegetative stage (47 DAP), 50 plants selected for uniform size were randomly arranged into five groups and sampled for nitrogenase activity by the soil core assay technique. Different levels of moisture were obtained in soil cores by adding either 0, 100, 200, 300, or 400 mL per core to a particular group of cores. After 1 h the plastic containers were sealed, C_2H_2 injected, and C_2H_4 production by the cores measured after 1 and 6 h of incubation. After assay, the soil moisture content was measured for each core.

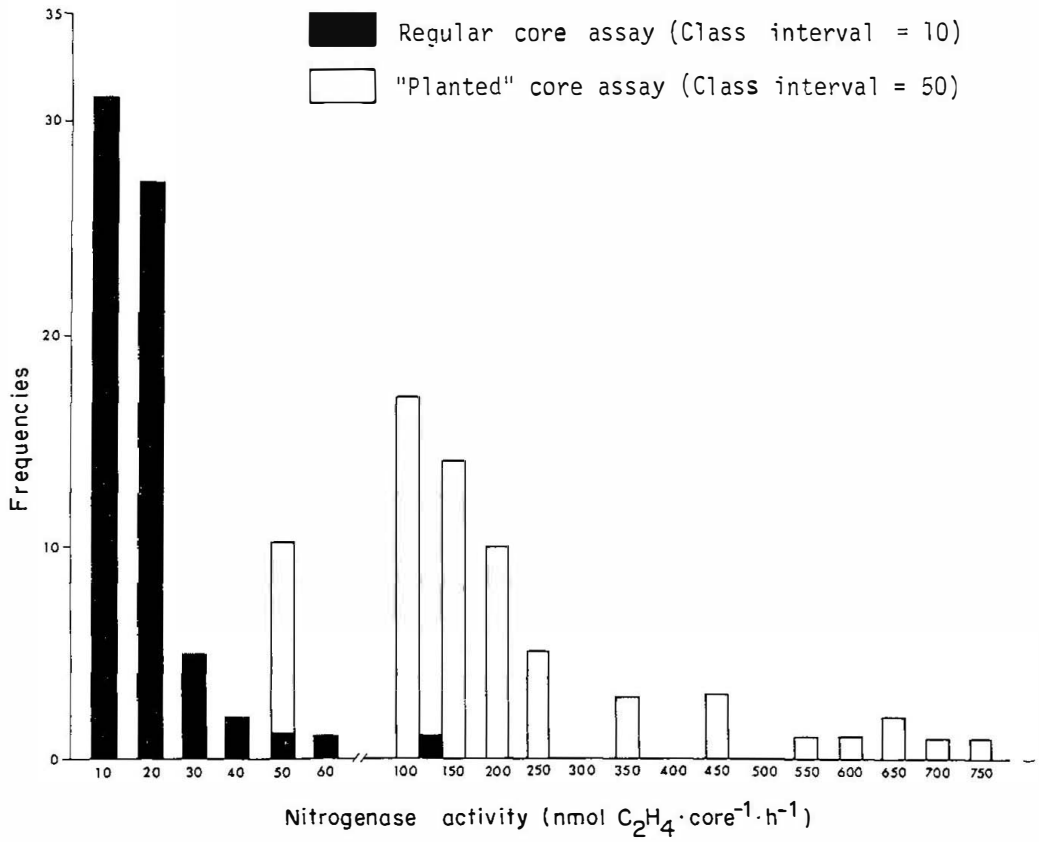

FIG. 1. Frequency distribution of nitrogenase activity of millet hybrid NHB-3, expressed as nanomoles per plant per hour, for planted cores and for disturbed cores. The mean nitrogenase activity was 17.6 and 167 nmol $C_2H_4 \cdot core^{-1} \cdot h^{-1}$ for the regular and "planted"core assays, respectively.

Results

Nitrogenase activity of plants grown in planted cores, in buckets, and by the regular coring method

Nitrogenase activity of millet plants cv. NHB-3 grown in planted cores and estimated by taking cores in the regular way is shown in Fig. 1 as frequency distributions of different levels of activity. The majority of the plants estimated by the regular, "disturbed" core assay fell in the range of 0–22 nmol $C_2H_4 \cdot plant^{-1} \cdot h^{-1}$, whereas for the planted core method the majority fell in the range of 100–250 nmol $\cdot plant^{-1} \cdot h^{-1}$. The pooled results of all the samples taken at 55, 66, and 69 DAP indicated significantly higher nitrogenase activity for the planted cores than for plants grown and sampled by the regular core assay. The mean activity recorded with planted cores was 10, 14, and 23 times higher than that of disturbed cores for harvests at 55, 66, and 69 days after planting, respectively. With increasing plant age, the activity of the regular cores relative to that of planted cores declined. Plants grown in plastic buckets had higher activity than disturbed cores but the increase was not significant.

Similar observations were recorded with nine genotypes of sorghum. Plants grown in cores had significantly higher activity ($p < 0.01$; 259 nmol $\cdot plant^{-1} \cdot h^{-1}$) than the regular cores (78 nmol $\cdot plant^{-1} \cdot h^{-1}$).

TABLE 1. Effect of disturbance during transport on the nitrogenase activity of millet cv. NHB-3

Treatment	nmol $C_2H_4 \cdot plant^{-1} \cdot h^{-1}$
Regular core assay	8[a]
Regular core assay with special precautions	25
SEM	±2.7

NOTE: Percent coefficient of variation (% C.V.) = 113.
[a]Mean of 50 replicate cores.

Effect of mechanical disturbance

Mechanical disturbance caused during transportation significantly reduced the nitrogenase activity associated with the soil cores (Table 1). Even though the general level of activity recorded in this experiment was low, cores sampled and transported with special precautions had threefold greater activity than the regular cores.

Effect of the time lag between cutting off the plant top and injecting C_2H_2

The most activity (104 nmol $C_2H_4 \cdot plant^{-1} \cdot h^{-1}$) was obtained where 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 ($r = -0.421$, $p < 0.01$). The linear

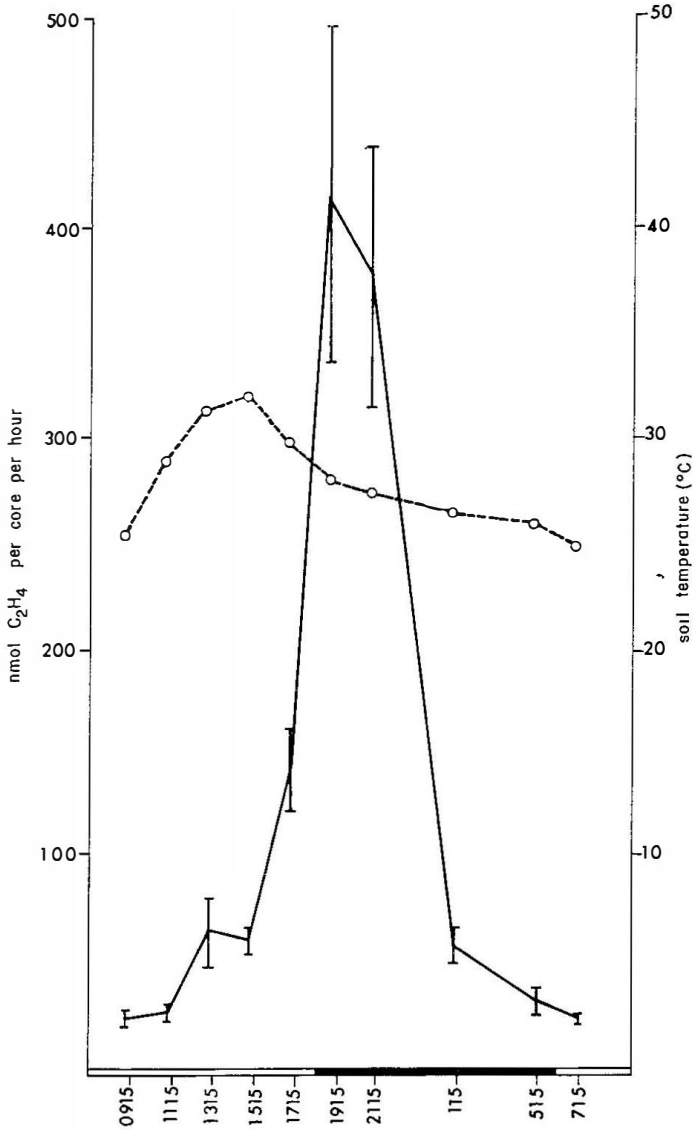

FIG. 2. Diurnal variation in nitrogenase activity (—) of millet cv. NHB-3 assayed in metal cores and incubated under C_2H_2 for 6 h. The calculated activity per hour is recorded against the time when the core was taken in the field. The night period is indicated by the shaded region on the X-axis. Bars represent ± SEM. Soil temperature at 20 cm depth (---) is also plotted.

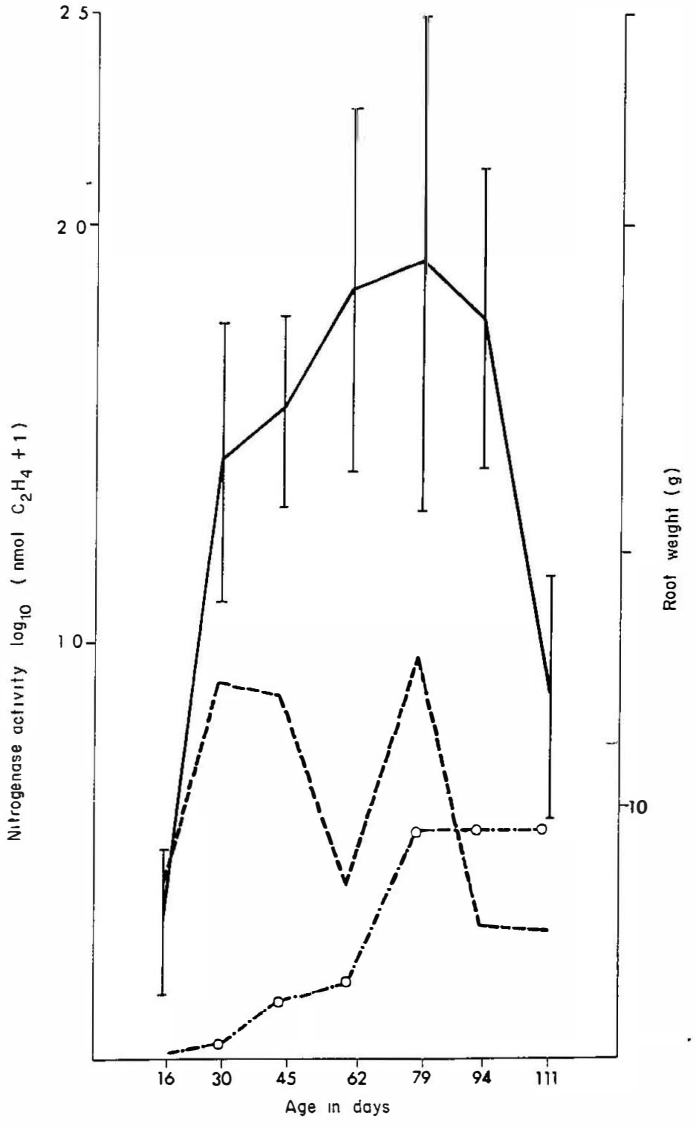

FIG. 3. Seasonal pattern for nitrogenase activity of sorghum cv. CSH-1 (—) grown in an Alfisol soil in plastic bags in the summer season. Root dry weight (○—·—○) and nitrogenase activity of unplanted soil per bag (---) are also recorded. Bars represent ± SEM.

regression between nitrogenase activity and the time taken to inject C_2H_2 is expressed by the equation $Y = 48.6 - 11.19X$.

Diurnal variation in nitrogenase activity

The time of sampling during the day significantly affected nitrogenase activity of field-grown millet cv. NHB-3 (Fig. 2). Nitrogenase activity increased from 0915 to 1815 during the photoperiod and then declined during the night until 0715 the next morning. The activity changed little between 0915 and 1500. However, significantly higher nitrogenase activity was recorded for the plants sampled between 1715 and 2115 than at 1115.

Maximum nitrogenase activity of 416 nmol·plant^{-1}·h^{-1} was observed with the plants sampled at 1915, with a slight but not significant reduction in activity at the sampling at 2115.

Soil temperature recorded at 20-cm depth at each sampling time varied between 25 and 32°C with a maximum at 1515. There was no correlation between nitrogenase activity and soil temperature.

Seasonal pattern for nitrogenase

Figure 3 shows the variation in nitrogenase activity plotted as log (nanomoles C_2H_4 per plant per hour + 1) of sorghum hybrid CSH-1 during the crop growth period. The activity changed significantly, increasing continuously from 16 DAP to 79 DAP. After reaching a maximum at 79 DAP (late flowering – early grain-filling

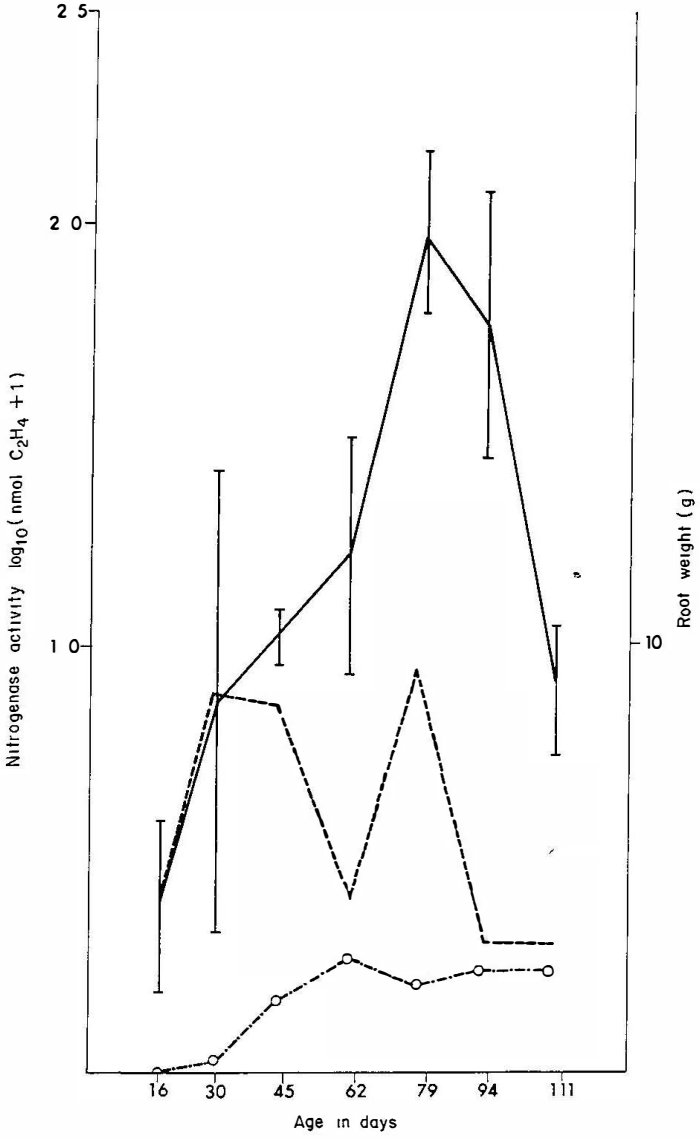

FIG. 4. Seasonal pattern for nitrogenase activity of millet cv. IP 2787 grown in an Alfisol soil in plastic bags (—) during the summer season. Root dry weight (○—·—○) and nitrogenase activity of unplanted soil per bag (---) are also recorded. Bars represent ± SEM.

stage), activity declined slightly at 94 DAP with a much greater decline by 111 DAP. Plant top and root weight also increased up to 79 DAP but then remained static.

For pearl millet cv. IP 2787, nitrogenase activity was also significantly affected by crop age (Fig. 4). There was an increasing trend in activity up to 45 DAP, and by 62 DAP, activity was significantly greater than that at 62 DAP, but then declined.

The log transformed values for nitrogenase activity associated with sorghum and millet plants were significantly greater than those for unplanted soil. The mean nitrogenase activities associated with sorghum and millet plants were 171 and 59 nmol·plant^{-1}·h^{-1}, whereas the activity recorded for the same amount of unplanted soil was 6 nmol·h^{-1}. The activities for sorghum hybrid CSH-1 and millet cv. IP 2787 did not vary significantly from each other.

TABLE 2. Effect of soil moisture on nitrogenase activity of the sorghum hybrids CSH-6 and CSH-8

Distance from sprinkler (m)	Moisture content (g·100 g dry soil^{-1})	nmol C_2H_4·core^{-1}·h^{-1}		
		CSH-6	CSH-8	Mean
2	8.9(71)[a]	34[b]	45[b]	39.5
4	7.9(63)	10	14	12
6	5.7(45)	7	9	8
8	3.2(25)	7	10	8.5
SEM		±5.7		±4.0

NOTE: % C.V. = 47.

[a]Figures in parentheses indicate the percentage of the maximum water-holding capacity.

[b]Mean of 16 values, eight replicate plants from two replicate line sources.

Effect of soil moisture on nitrogenase activity

Table 2 shows the response of nitrogenase activity of sorghum cv. CSH-6 and cv. CSH-8 grown under different soil moisture conditions induced by a line-source irrigation system. Most activity with both the cultivars was recorded nearest to the line source where the soil moisture content was greatest (8.9% dry soil basis) at the time of sampling. As the distance from the sprinkler increased from 2 to 4 m, nitrogenase activity of both the cultivars was significantly reduced along with soil moisture content. The activity for plants at 4, 6, and 8 m distance away from the line-source sprinkler was similar, however, even though the moisture contents decreased from 7.9 to 3.2%. Regression analysis of nitrogenase activity (nanomoles C_2H_4 per plant per hour) and soil moisture content indicated a significant correlation between these two variables ($r = -0.66$; $p \leq 0.01$, $Y = 1.76 + 2.32X$). Nitrogenase activity in this experiment was not related to the dry weight of the plant tops.

In another experiment, addition of different quantities of water to the soil in the core just before assay produced a gradient of soil moisture ranging from 5.75 to 14.9% (field capacity = 17%) (Table 3). The nitrogenase activity of the sorghum plants was significantly affected by soil moisture, with no change in activity between 5.75 and 8.3% soil moisture, but activity increased along with soil moisture content above 8.3%. Regression analysis indicated a significant correlation ($r = -0.61$; $p \leq 0.01$; $Y = 6.67X - 21.51$) between soil moisture and nitrogenase activity.

Discussion

Nitrogenase activity associated with sorghum and millet plants as estimated by a soil core assay was sensitive to several factors, resulting in an underestimate

TABLE 3. Effect of soil moisture[a] on nitrogenase activity of sorghum hybrid CSH-1

Quantity of water added per core (mL)	Moisture content (g·100 g dry soil^{-1})	nmol C_2H_4·core^{-1}·h^{-1}
0	5.75(33)[b]	24[c]
100	8.3(48)	25
200	10.4(61)	42
300	12.5(73)	59
400	14.9(88)	88
SEM		±8.4

[a]Different soil moisture levels in the assay cores obtained by adding water to the cores just before assay.
[b]Figures in parentheses indicate percentage of the maximum water-holding capacity.
[c]Mean of 10 cores.

of the genuine field activity. That the nitrogenase activity is a plant-related phenomenon is clearly shown by the large rhizosphere effect. The activity associated with sorghum roots in the seasonal profile experiment was 171 nmol·plant^{-1}·h^{-1}, while that for an equivalent amount of unplanted soil was only 6 nmol·h^{-1}.

Ten to 23-fold higher nitrogenase activity was found for plants grown in cores in the field than for the plants assayed by the regular disturbed cores taken at the time of assay. Such an increase in the activity might arise from the larger proportion of roots contained by the planted cores compared with the disturbed cores. Earlier results (Anonymous 1978; Subba Rao and Dart 1981) indicated that there can be considerable lateral spread of nitrogenase activity in the soil away from the central core over the crown of the plant. Activity of the central core ranged from 14 to 50% of the total activity associated with the plant.

Nitrogenase activity increased with root growth up to 79 DAP and decreased after this when root growth ceased. At peak activity both sorghum and millet plants were in the late flowering – early grain-filling stage. For sorghum plants grown in Brazil, most activity occurred at flowering (P. van Berkum. 1978. Ph.D. thesis, University of London, London).

A second factor that may contribute to the smaller activity of the disturbed cores is physical disturbance of the soil–root interface. Relatively minor mechanical disturbances associated with sampling and transport from the field to the laboratory can seriously reduce nitrogenase activity. Extracting preplanted cores from the soil would obviously lead to considerably less disturbance of the plant–soil interface than the intense shearing action on the roots resulting from coring at the time of assay.

Another variable which needs to be considered in large-scale samplings associated with germplasm screening, etc., is the large reduction in nitrogenase activity associated with any delay in injecting C_2H_2 gas following decapitation of the plants.

Soil moisture is another important factor over which the experimentor often has only limited control. Our results support the earlier findings (Vlassak *et al.* 1973; Day, Neves *et al.* 1975; Tjepkema and Burris 1976; Anonymous 1978; Subba Rao and Dart 1981) that plant-associated nitrogenase activity increases as the soil moisture content increases. One of the problems associated with understanding how soil moisture affects nitrogenase activity is that many plant processes that may influence this activity are affected by soil moisture levels. For example, changes in plant growth rates with change in soil moisture will result in different amounts of root tissue, and hence sites for bacterial activity, as well as different rates of photosynthesis, and hence energy materials translocated to the roots to drive the nitrogenase activity. The wetting and drying pattern of the soil may also influence the amount of root exudation. amount of root exudation.

As soil moisture increases, the oxygen partial pressure in the soil atmosphere is likely to decrease as the soil pore continuity with the aerial atmosphere is broken, with a concomitant large reduction in the rate of diffusion into discontinuous soil pores. Root and microorganism respiration uses the oxygen held in such soil pores at a faster rate than diffusion can replenish the oxygen. The lower resultant oxygen partial pressure should favour increased nitrogenase activity. Our experiment adjusting the soil moisture tension of soil–plant cores after the plant tops have been removed, just before the nitrogenase assay was commenced, suggests that changes in soil moisture and presumably soil oxygen tension can have a fairly immediate effect on nitrogenase activity. If periods of low soil oxygen tension persist, root growth may decrease (Stolzy 1974), and this might decrease nitrogenase activity.

The soil core assay for nitrogenase activity of field-grown plants developed by Day, Harris *et al.* (1975) and at ICRISAT (Anonymous 1978), although giving comparative qualitative data, can be improved. Sampling, especially for germplasm comparison, should be done when the soil is wet as this will maximize expression of treatment differences. Activity can be increased by minimizing mechanical disturbance in handling the sample cores. This can be achieved by driving the core around the plants at an early age and sampling at a later date, lifting the cores carefully, sampling quickly to reduce variation owing to the diurnal pattern of nitrogenase activity, injecting the C_2H_2 immediately after decapitation of the plant and preferably in the field, and wherever possible eliminating transporting of the cores.

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