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BIOLOGICAL NITROGEN FIXATION

IN PADDY FIELD STUDIED BY IN SITU ACETYLENE-REDUCTION ASSAYS

I. WATANABE, K. K. LEE, B. V. ALIMAGNO,
M. SATO, D. C. DEL ROSARIO, AND M. R. DE GUZMAN

The International Rice Research Institute
P.O. Box 933, Manila, Philippines

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BIOLOGICAL NITROGEN FIXATION IN PADDY FIELD STUDIED BY IN SITU ACETYLENE-REDUCTION ASSAYS[1]

ABSTRACT

To assess the contribution of blue-green algae and the rice root zone to nitrogen fixation in the submerged rice soil, an in situ acetylene-reduction assay technique was developed. Assays were conducted for 11 months in the International Rice Research Institute (IRRI) plots at Los Baños, Philippines.

Assay chambers consisted of plastic bags attached to metal cylinders, which were inserted into the soil. Because the evolved ethylene was oversaturated in its soil-water phase within the assay chamber, it was necessary to stir the soil before gas sampling to release the ethylene into its gas phase. This assay method could not detect nitrogen fixing activity occurring in the bulk of anaerobic soil.

In unfertilized plots, highest peaks of in situ acetylene-reduction activity appeared late in the growing season, in both wet- and dry-season crops, when the activity of blue-green algae in flood water was highest. By replacing flood water with distilled water, to eliminate the bulk of algal activity, we assessed the contribution of blue-green algae to the nitrogen fixing process. When algal growth was visible in the field, nitrogenase activity of the assay system decreased sharply after water replacement. Nitrogenase activity estimated by the in situ assay technique fluctuated greatly with the activity of blue-green algae. Nitrogenase activity in the rice root zone was at an almost constant but low (0.05 kg N/ha per day) rate.

Ratoons from rice stubble showed higher nitrogen fixing activity than intact plants before harvest or than stubble in which ratooning was prevented.

Facultative anaerobic, nitrogen fixing bacteria, including *Enterobacter*, and unidentified, gram-negative rods were isolated from lowland rice roots.

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[1]by I. Watanabe, soil microbiologist, K. K. Lee, postdoctoral fellow, B. V. Alimagno, senior research assistant, M. Sato, postmaster fellow, D. C. del Rosario, research assistant, and M. R. de Guzman, research aide, International Rice Research Institute, Los Baños, Philippines. This paper was originally submitted to the "Somiplan" symposium held 13-17 August 1976, Kuala Lumpur, and revised and submitted to the IRRI Research Paper Series Committee 8 October 1976.

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BIOLOGICAL NITROGEN FIXATION IN PADDY FIELD STUDIED BY IN SITU ACETYLENE-REDUCTION ASSAYS

It has long been observed that a mechanism for maintaining nitrogen fertility exists in submerged rice soils. In IRRI field experiments 23 successive rice crops were grown during 12 years without nitrogen fertilizer addition, and without any decline in rice yield, which was 3 to 4 tons/ha per crop (IRRI unpublished, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976). Nitrogen absorbed by the above-ground parts of the rice plant is estimated at 40 kg - 60 kg N/ha. Fertilizer experiments in Asia show that one rice crop can take from 37 kg to 113 kg N/ha from sources other than mineral nitrogen fertilizers (Table 1).

Table 1. Nitrogen uptake by lowland rice from sources other than fertilizer.

Region	N observed by crop (kg N/ha)	Method	References
Hokkaido-Kyushu, Japan	64 (47-113)[a]	Non-N plot[b]	Yanagisawa and Takahashi (1964)
Bangkhen, Thailand	37	Non-N plot	Koyama *et al.* (1972)
	52	Isotope[c]	"
Los Baños, Philippines	76	Non-N plot (1973 wet season)	IRRI (1975)
	97-113	Isotope (1973 dry season)	IRRI (1974)
Muara, Indonesia	47	Non-N plot	Ismunadji *et al.* (1973)
Pusakanegara, Indonesia	65	Non-N plot	"

[a]Average (maximum-minimum) among 15 Provincial Agricultural Experiment Stations.

[b]N-absorption in non-nitrogen fertilizer plots.

[c]Estimated by N^{15}-labeled nitrogen fertilizer.

There is a dearth of data explaining the source of nitrogen that supports continuous growth in the absence of nitrogen fertilizer. Biological nitrogen fixation may partly explain long-term nitrogen fertility of flooded rice soils. Principal agents of biological nitrogen fixation for rice are

1. blue-green algae,
2. a water fern, *Azolla*, in association with blue-green algae (*Anabaena azolla*),
3. nonsymbiotic nitrogen fixing bacteria around the rice plant's root zone, and
4. nonsymbiotic bacteria in the bulk of anaerobic soil.

Nitrogen fixation in the root zone of rice plants in comparison with nitrogen fixation by blue-green algae is described in this paper.

MATERIALS AND METHODS

In situ assay of nitrogen fixing activity

The acetylene reduction technique (Hardy et al., 1971, 1973; Burris, 1972) is useful in studying nitrogen fixation because of its simplicity, low cost, and extreme sensitivity, provided that the results are carefully understood in view of indirect measure. Significant nitrogen fixation in the root zone of several *Graminaceae* is reported based on acetylene reduction assay of the excised root (Yoshida and Ancajas, 1971, 1973; Dobereiner et al., 1973; Day et al., 1975; Von Bulow Joachim and Dobereiner, 1975). However, assay of the excised roots has only limited implication for the nitrogen fixation rate of plants growing in soil. The in situ technique, enclosing field- or pot-grown plants in an atmosphere of acetylene and other gases, has been used (Balandreau and Dommergues, 1973; Burris, 1974).

Lee et al. (1975) developed the in situ acetylene-reduction assay technique for use in paddy fields, using plastic bags and bottomless metal frames (Fig. 1). The plastic bag chamber is convenient for addition and removal of gas.

The plastic bag method was used to measure the nitrogenase activity in various rice field soils. Mixed gases were prepared and regulated by passing

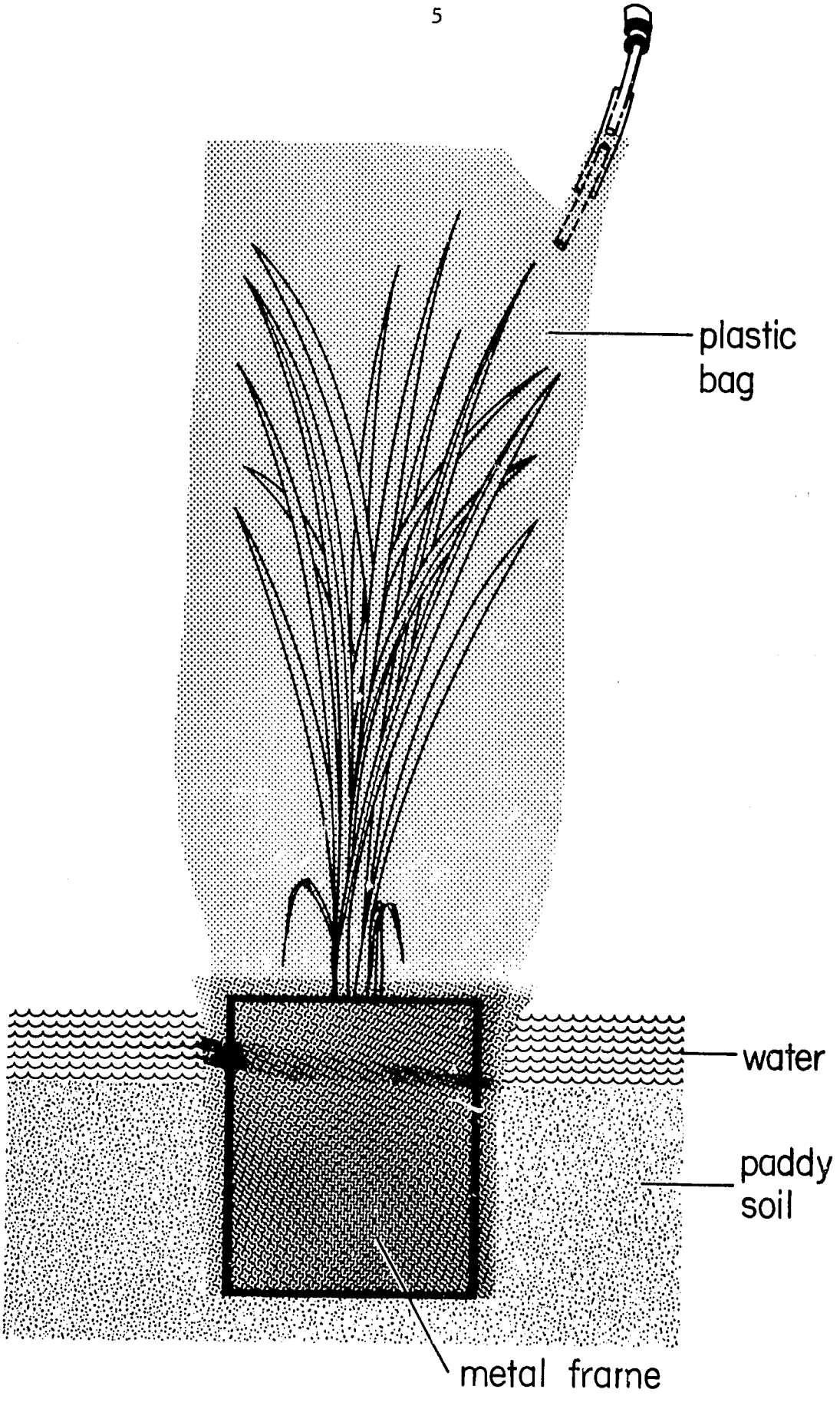

Fig. 1. Diagram of acetylene reduction assay using a plastic bag.

other to a second gas tank. The plastic bag was evacuated until it collapsed and clung to the sample. Acetylene and other gases were introduced through polyethylene tubing, which was then clamped, and the bag was disconnected from the apparatus. Finally, the tubing was plugged with a needle-puncture stopper. The change in bag volume during 24-hour incubation was not significant. The amounts of acetylene and ethylene lost through permeation during the 24-hour period had only a negligible effect on the acetylene reduction assay.

Method modification because of gas transfer

The diffusion of gas in water is far slower than in air-filled space. Therefore, in flooded soils the transfer of acetylene to the nitrogen fixing site in the soil-water system, and the release of the evolved ethylene to its gas phase in the assay chamber, may limit the apparent acetylene reducing activity. In laboratory assays, we suggest that samples be shaken before start of the assay to dissolve the acetylene in the water, and after assay to release the evolved and oversaturated ethylene from water phase to gas phase within the assay vial.

In our field assay, a small piece of bamboo was placed inside the assay chamber. The bamboo was manipulated from outside the plastic bag to stir the soil. In the field, disturbing the soil before assay to enhance the transfer of the acetylene into the water had a negligible effect, particularly when a rice plant was in the chamber.

The amount of ethylene dissolved in the soil-water system after the assay period was studied by taking soil core samples and determining their ethylene content (Table 2). However, it was tedious to determine the amount of ethylene in a chamber when a large number of assay chambers were installed in the field. Soil disturbance by a stick was quite effective, though not complete, in releasing the dissolved ethylene into the assay chamber (Table 3). The amount of ethylene dissolved in the water-soil phase within the assay chamber ranged from about 10 to 20 μmol C_2H_4/chamber (0.28-0.55 m mole/m^2). In terms of agricultural significance of nitrogen fixation around the rice root, this amount would be insignificant.

We have evidence that the rice plant has a system for rapidly transporting ethylene to the atmosphere. It is probable that the technique described in

Table 2. Ethylene remaining in paddy water and soil after acetylene reduction assay for one day.

Replication	C_2H_4 (µmol/chamber[a])	
	In gas	Remaining in water and soil
	Tillering stage	
1	9.7 (59)[b]	6.8
2	18.5 (54)	15.6
3	12.3 (48)	13.2
4	20.2 (52)	18.7
	Heading stage	
1	22.0 (78)[b]	6.2
2	21.0 (69)	9.6
3	11.6 (59)	7.9
4	15.9 (52)	14.6
	One week after harvest (ratoon)	
1	67.4 (94)[b]	4.6
2	86.2 (96)	3.7
3	74.5 (93)	5.4
4	36.1 (90)	4.1
5	24.3 (90)	2.6

[a]Chamber size, 19 cm x 19 cm.

[b]Figures in parentheses are percentage of the total.

this paper can detect this activity in flood water, at the soil surface, and around rice roots, but not in the bulk of anaerobic soil.

RESULTS

Seasonal changes of nitrogen fixing activity

Using the in situ acetylene-reduction technique, a field assay was conducted from June 1975 to May 1976 in IRRI rice fields where rice had been grown twice a year (23rd and 24th crops) for 12 years as long-term fertilizer

Table 3. The effect of stirring the soil on the release of ethylene remaining in the soil.

Replication[a]	C_2H_4 (μmol/chamber[b])			
	In gas phase	Released after stirring	Remaining after stirring	Total
1	18.5 (60)[c]	4.4 (14)	7.9 (26)	30.8
2	5.3 (49)	3.3 (30)	2.2 (21)	10.8
3	8.8 (34)	11.4 (45)	5.3 (21)	25.5
4	17.6 (69)	3.5 (14)	4.4 (17)	25.5

[a]Rice plants (IR26) were at panicle initiation stage.

[b]Chamber size, 19 cm x 19 cm.

[c]Figures in parentheses are percentages of the totals.

experiments. Treatments without nitrogen, potassium, and phosphorus fertilizers, and treatments with nitrogen (basal and top dressing), potassium, and phosphorus fertilizers were examined (Fig. 2).

After assay, the flood water, the floating algae, the weeds growing on the surface of the soil, and 5 cm of surface soil were taken from the assay chambers for laboratory assay of acetylene reduction under light (water, algae, and weeds under 4,000 lux) and dark conditions (soil).

During the 1975 wet season, soil stirring to release ethylene after the assay was not performed. Figure 2a shows the seasonal changes of dinitrogen fixing activity in the rice soil around the rice hills. Activity was higher in unfertilized plots than in fertilized plots except during the early growth stage of the 1975 wet season. Two peaks of activity in unfertilized plots were observed, both during the late stage of rice growth. The changing amounts of algae collected from flood water by passing it through cheese cloth are shown in Figure 2b. Figure 2c shows the sum of photodependent, in vitro nitrogen fixing activity for flood water, for the algal mass collected from the water, and for weeds to which algae firmly adhered. Algal activity was higher in the dry season. The photodependent activity, which we ascribe

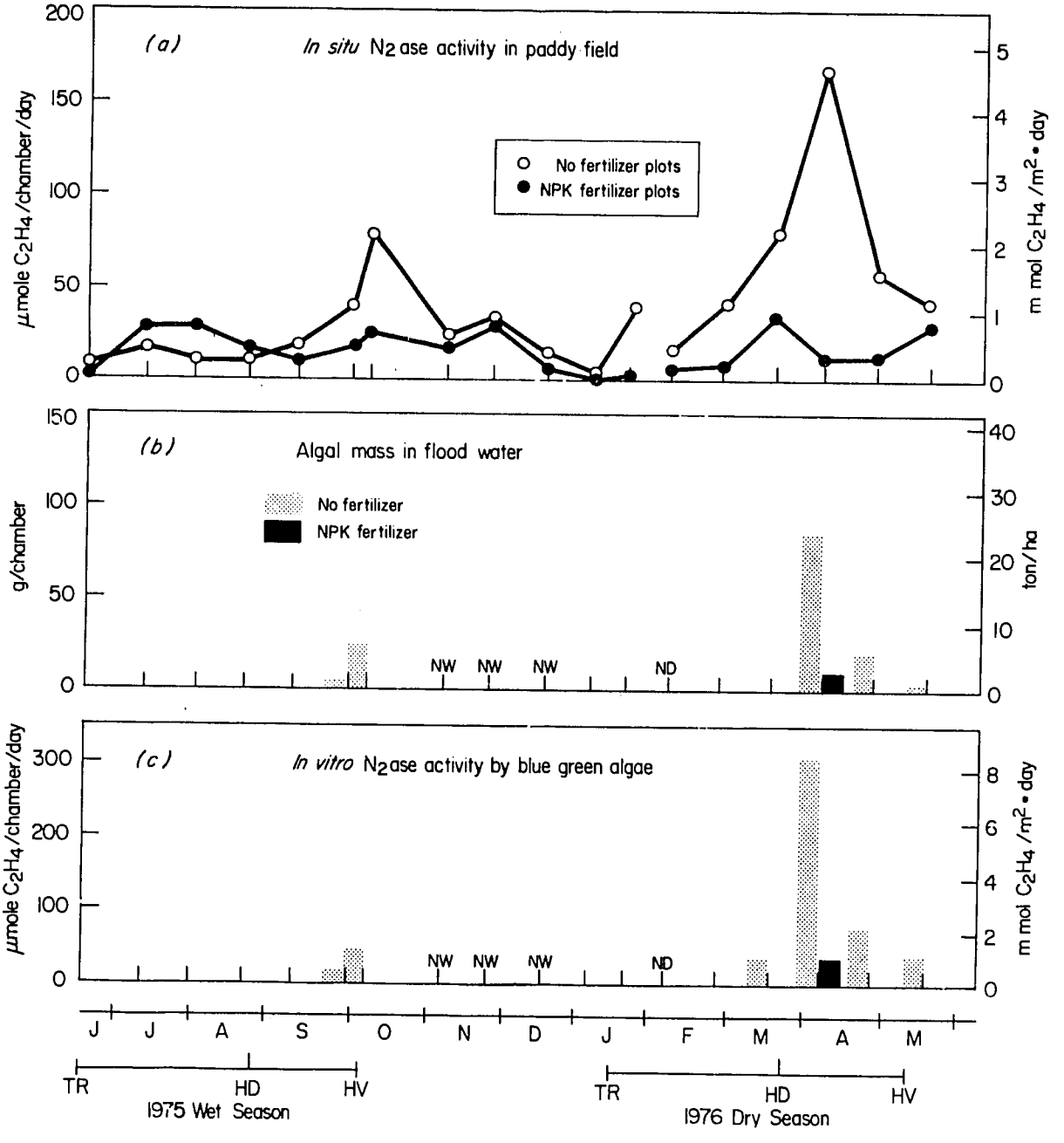

Fig. 2. (a) Seasonal variation of in situ nitrogenase activity in IRRI long-term fertilizer experiments plots, (b) algal biomass in flood water, (c) in vitro nitrogenase activity by blue-green algae taken from assay chamber. In the assays after February 1976, soil in the assay chamber was stirred after one day's assay to release the ethylene gas to its gas phase. Because the amount of the ethylene increases after stirring, lines connecting the values are discontinuous. Growth periods of rice (IR26) from transplanting are TR: transplanting; HD: heading; HV: harvesting; NW: no flood water; ND: no data. In NPK fertilizer plots, 30 kg P_2O_5 and 30 kg K_2O were applied before transplanting; 40 kg N (a-monium sulfate) in wet season or 60 kg N in dry season were applied at the time of transplanting and 20 kg N in wet season or 40 kg N in dry season were applied at panicle stage. 100 μmol C_2H_4/chamber corresponds to 256 g N/ha (3:1 conversion) and 100 g/chamber algal mass corresponds to 27.7 ton/ha.

to blue-green algae, and the biomass of algae, also showed peaks at the late stage of rice growth in both seasons, and coincided with the period when in situ assay revealed the highest activity in each season. Endogenous ethylene formation from soil and plants was negligible in these fields.

Contribution of algae to the nitrogen fixing process

In situ acetylene-reduction assays were conducted after removing flood water, floating algae, and weeds to which algae adhered; scraping the surface several millimeters soil, and replacing the flood water with distilled water in the assay chamber. That procedure eliminated the bulk of algal activity. Table 4 shows data on the acetylene reduction activity of soil that contained rice plants, with and without the distilled-water treatment. When algal growth was visible to the naked eye, the removal of algae sharply reduced nitrogen fixing activity in the field soil. The activity, after eliminating the bulk of algae, is ascribed to the root zone of the rice. It remained at an almost constant and comparatively low figure, regardless of algal activity. If we assume the root-zone acetylene reduction rate is 20 μmol/chamber per day

Table 4. Effect of removal of water and algae on acetylene reduction in paddy field containing rice plants.

Growth stage and variety	Location	Observation of algae[a]	Acetylene reduction (μmol/chamber)	
			With flood water and algae	With flood water replaced
40 days after transplanting (IR2071-588-5-43)	IRRI Site I	+	117.0 ± 4.2 (4)[b]	18.6 ± 2.6 (4)[b]
Flowering (IR2071-588-5-43)	IRRI Site I	-	11.2 ± 2.6 (5)	10.9 ± 2.1
Heading (IR26)	IRRI Site II	-	15.9 ± 2.7 (5)	15.9 ± 2.4 (5)
Heading (IR26)	IRRI Site II	+	221.0 ± 30.0 (5)	12.2 ± 1.5 (5)[c]
Ripening (IR26)	IRRI Site II	+	202.0 ± 44.0 (5)	17.5 ± 4.1 (5)[c]
Ripening (IR26)	IRRI long-term fertilizer	+	206.0 ± 8.9 (6)	23.9 ± 4.9 (6)[c]

[a] + algae were visible; - invisible.

[b] Average ± error of the average (number of replicates).

[c] Soil was stirred by bamboo sticks before sampling gas from assay chamber.

and that the conversion rate (N_2-$2NH_3$:C_2H_2-C_2H_4) is theoretically 1:3, root-zone nitrogen fixation would be 0.052 kg/ha per day. It is difficult to say whether this value is significant.

Nitrogen fixing activity of stubble

Rice stubble is usually left in the field water after harvest and incorporated into the soil as the field is plowed for the next crop. When soil is moist and temperature is high, ratooning occurs. We examined nitrogen fixing activity in the stubble.

Rice (IR26) was grown in a flooded field without fertilizers during the wet season. At harvest, the plants were cut 10 cm above the base. Rice in adjacent plots was left uncut but bundled around stakes to prevent lodging and to facilitate the penetration of sunlight to the paddy water. All plots were kept flooded. Before setting up the assay chamber, floating algae, weeds, and surface soil were removed to eliminate the contribution of algae to nitrogen fixation. Assays continued for 24 hours.

Ratoons sprouted both from stubble and from the uncut rice. The number and height of sprouts emerging from uncut hills were much lower than those from stubble. The difference in acetylene reducing activity of uncut hills and of stubble is shown in Figure 3. Immediately after harvest the stubble's nitrogenase activity began to increase. Uncut hills did not show as rapid an increase in nitrogenase activity. The nitrogenase activity of the ratoons was higher than that in the rice root zone during plant growth.[1]

In a similar experiment after the dry-season harvest, an additional treatment inhibited sprout growth to isolate the activity of the disintegrating stubble. Stubble was covered by a brown glass pot to prevent growth. Change in nitrogenase activity is shown in Table 5.

Mature rice stems in unharvested hills began to disintegrate immediately and sprouts emerged. At the end of the experiments there was no difference in the number and height of sprouts in harvested and unharvested hills, and nitrogenase activity did not differ greatly between the two treatments. However, nitrogenase activity was much lower in the stubble where regrowth

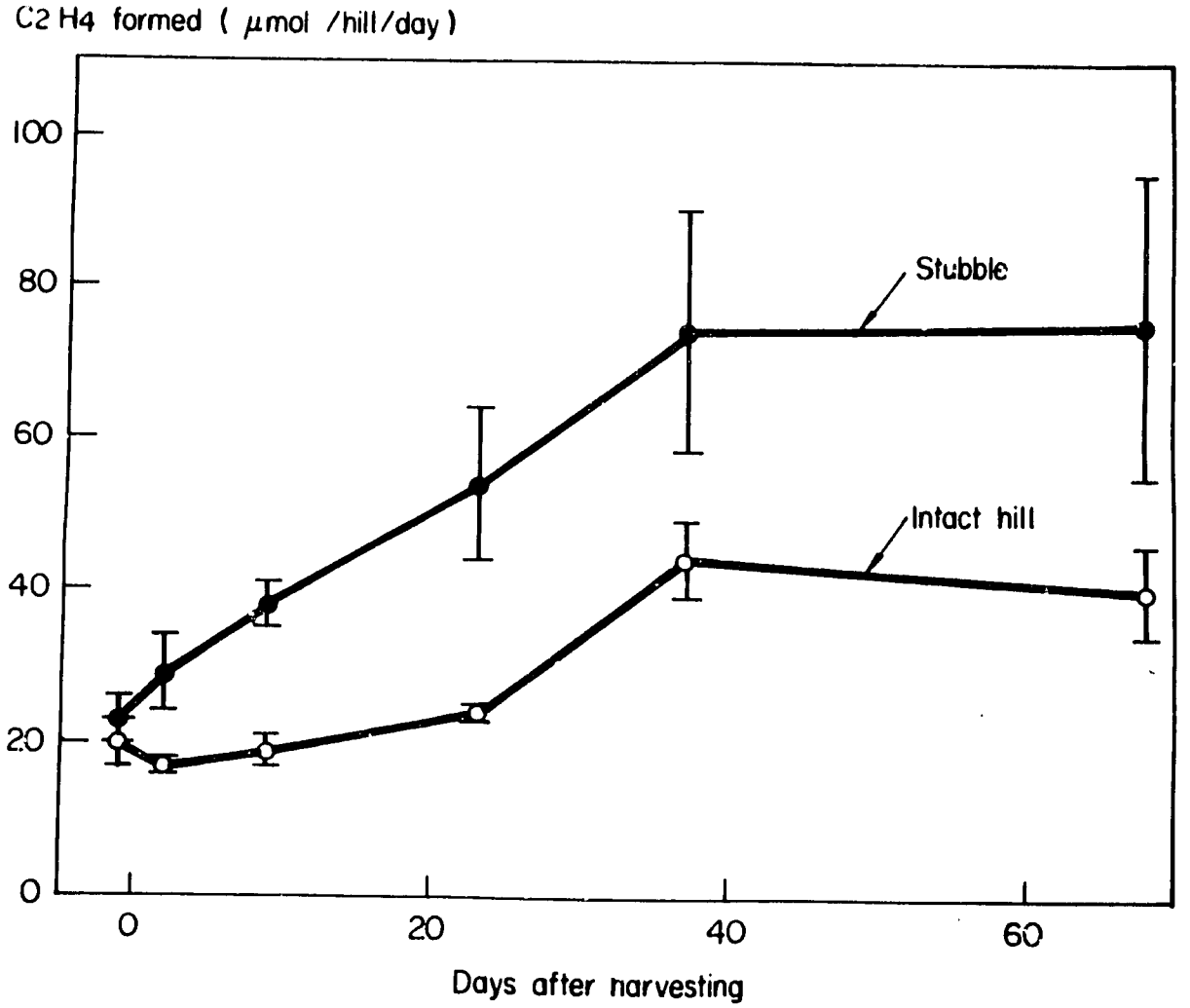

Fig. 3. Nitrogenase activity in stubble after harvesting. The bars indicate the standard deviation of the mean.

Table 5. Nitrogenase activity of rice ratoons.

Days after cutting	Nitrogenase activity (μmol C_2H_4/day per chamber)		
	Mature plants left uncut	Stubble with regrowth permitted	Stubble with regrowth prevented
1	26.1± 5.9 (5)[a]	23.1± 3.9 (5)	19.6±2.2 (5)
18	79.0±10.8 (5)	70.0± 5.3 (5)	30.7±5.5 (5)
33	12.9± 3.9 (5)	9.7± 1.4 (5)	2.7±0.4 (5)
41	75.0±24.0 (5)	121.0±21.0 (5)	19.4±3.5 (5)

[a]Average ± standard deviation of the average (number of replicates).

was prevented (Table 5). The nitrogen fixing activity of the ratoons at 33 days after cutting was affected by cloudy, rainy weather.

Nitrogen fixing bacteria isolated from rice roots

Rice roots growing in flooded soil were collected, washed with water, and cut into small segments. These segments were placed in a nitrogen-free, mineral-glucose medium under aerobic (shaking) conditions, semi-anaerobic (0.03% O_2) conditions, and strict anaerobic (roll tube method) conditions.

Nitrogen fixers were not isolated under aerobic conditions. All of the isolates under strict anaerobic conditions were considered to belong to *Enterobacter*. *Enterobacter* were also identified in the isolates grown under semi-anaerobic conditions. Some unidentified gram-negative rods also were obtained from semi-anaerobic cultures. No *Beijerinckia* or *Azotobacter* were isolated. The highest nitrogenase activity among isolates in malate-added, nitrogen-free, glucose media was 1.4 μmol C_2H_4/ml per 20 hours. The number of semi-anaerobic fixers in washed rice roots at the panicle initiation stage was estimated to be 2 x 10^6/g of fresh roots after maceration.

DISCUSSION

Although many papers on nitrogen fixation or acetylene reduction in the rice root zone have been published (Rinaudo et al., 1971; Yoshida and Ancajas, 1971, 1973a, b; Balandreau and Villemin, 1973; Dommergues et al., 1973; Balandreau et al., 1974, 1975), there is a scarcity of data showing the importance of nitrogen fixation in or near the rice root to the supply of nitrogen in paddy rice soil. Rinaudo et al. (1971), Balandreau et al. (1974, 1975), Balandreau and Villemin (1973), Dommergues et al. (1973), reported high acetylene reduction rates in rice plants grown in the laboratory or in paddy fields, but the rates reported were restricted mainly to rice in the early seedling stage or in the paddy field with algal growth. Further studies are therefore needed to elucidate the agricultural importance of nitrogen fixation in the rice root zone.

Yoshida and Ancajas (1973a) argued that 63-57 kg N/ha per crop would be fixed in the flooded-planted soil, and 28-22 kg N/ha per crop would be fixed in

flooded-unplanted soil based on extrapolation of data obtained by the laboratory incubation (5 h) of well mixed paddy soils, using a 3:1 conversion (C_2H_4:N_2) rate. The well mixed soil including rice roots might give a higher nitrogen fixing rate than an undisturbed soil.

In another paper, Yoshida and Ancajas (1973b) point out that "despite the higher N_2 fixing activity of the roots, the amount of N_2 that they can fix per hectare in the field is small because on area basis their total weight is so much less than the soil and water." A large amount of nitrogen fixed in flooded-planted soil, they reported, was not simply due to the nitrogen fixation occurring within or in close vicinity of rice roots. Although their data clarified the importance of flooding and the presence of rice roots, and a level of nitrogen fixing activity of the excised roots, they have not presented an estimate of the nitrogen fixing rate occurring in or around the intact rice roots, the importance of which was suggested by them.

This paper describes experimental results that clarify the importance of root-zone nitrogen fixation in paddy soil. Although the nitrogen fixing rate in the paddy fields detectable by our in situ assay technique was controlled mainly by the activity of blue-green algae, or by the bacteria which may be closely associated with the biomass of algae in the flood water, the rice root or the rhizosphere had a consistent, although low, nitrogen fixing activity, except for the ratoon. Because our assays could not detect the nitrogen fixing activity occurring in the bulk of anaerobic soil, we do not contradict earlier findings by IRRI microbiologists who studied and estimated nitrogen fixation in the bulk of paddy soil.

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