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Nitrogen Fixation by Photosynthetic Bacteria in Lowland Rice Culture

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Propanil (3',4'-dichloropropionanilide) was a potent inhibitor of the nitrogenase activity of blue-green algae (cyanobacteria) in flooded soil, but the herbicide at comparable concentrations was not toxic to rice, protozoa, and nitrogen-fixing bacteria. Ethanol-amended flooded soils treated with propanil exhibited higher rates of nitrogenase activity than those not treated with the herbicide. The enhanced nitrogenase activity in propanil-treated soils was associated with a rise in the population of purple sulfur bacteria, especially of cells resembling *Chromatium* and *Thiospirillum*. By employing propanil and a means of excluding light from the floodwater to prevent the development of phototrophs during rice growth under lowland conditions, the relative activities of blue-green algae, photosynthetic bacteria, and the rhizosphere microflora were determined. The results suggest that the potential contribution of photosynthetic bacteria may be quite high.

The knowledge that lowland rice not fertilized with nitrogen could be grown on the same soil year after year without a decrease in yield or a decline in the soil nitrogen level has stimulated investigations on biological nitrogen fixation. Moreover, the marked increase in recent years in the price of nitrogen fertilizer has made the need for this research more acute. It is now believed that biological nitrogen fixation can contribute a considerable portion of the nitrogen needed by lowland rice (11).

A number of microorganisms have been identified as agents responsible for nitrogen fixation in lowland rice culture. The major groups appear to be heterotrophic bacteria in the root zone, free-living blue-green algae (cyanobacteria), symbiotic blue-green algae associated with the fern *Azolla*, and photosynthetic bacteria (7, 11). Numerous studies have been conducted to determine the role played by these organisms in the nitrogen nutrition of rice grown under lowland conditions, and some information is now available on the relative importance of nitrogen fixation by free-living blue-green algae, the blue-green algae–*Azolla* association, and the rhizosphere microflora (2, 4, 10, 12). On the contrary, although the potential role of photosynthetic bacteria in the nitrogen nutrition of lowland rice is often recognized (7, 8), their actual contribution to the quantity of nitrogen fixed is unknown.

The objective of this investigation was to assess the nitrogenase activity of photosynthetic bacteria in lowland rice culture in relation to the nitrogen-fixing activity of blue-green algae and the rhizosphere microflora.

MATERIALS AND METHODS

Inoculum. *Anabaena* MH was isolated from Maahas clay, a Philippine rice soil, and was grown in a nitrogen-free modification of Stanier's medium (14). The medium contained 40 mg of K_2HPO_4, 36 mg of $CaCl_2 \cdot 2H_2O$, 76 mg of $MgSO_4 \cdot 7H_2O$, 1.0 mg of tetrasodium ethylenediaminetetraacetate, 4.0 mg of sodium citrate, 20 mg of $Fe_2(SO_4)_3 \cdot xH_2O$, 20 mg of Na_2CO_3, 2.9 mg of H_3BO_3, 1.8 mg of $MnCl_2 \cdot 4H_2O$, 0.22 mg of $ZnSO_4 \cdot 7H_2O$, 0.39 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.079 mg of $CuSO_4 \cdot 5H_2O$, and 0.049 mg of $Co(NO_3)_2 \cdot 6H_2O$ in 1.0 liter of distilled water. The pH was adjusted to 7.0 before the mixture was autoclaved.

The inocula for the experiments were prepared by adding 10-ml portions of a washed suspension from a stock culture into 200-ml portions of medium contained in 500-ml Erlenmeyer flasks. The flasks were incubated at 26°C on a rotary shaker (200 cycles per min) illuminated by fluorescent lamps at approximately 100 μEinsteins/s per m^2. After 12 to 14 days of growth, the contents of the flasks were collected by centrifugation at 8,000 × *g* for 10 min at 4°C. The pellet was washed twice in a sterile solution containing 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.1 g of NaCl in 1.0 liter of distilled water, and the cells were suspended to volume in the same solution. The inoculum was applied to soil at a rate of 2 ml/100 g of soil.

Chemicals. The herbicide propanil (98.5% pure) (3',4'-dichloropropionanilide) was obtained from Monsanto Chemical Co., St. Louis, Mo. Ethylene (chemically pure) and prepurified acetylene were purchased from Matheson Gas Products, East Rutherford, N.J.

Soils. The Maahas and Maligaya soils were obtained as slurries from the fields of the International Rice Research Institute in Los Banos, Philippines. Moist Crowley soil was obtained from Stuttgart, Ark. These three soils are used in lowland rice cultivation. The Hudson soil was from Ithaca, N.Y. The soils were

passed through 2-mm sieves and maintained under water during storage and in the experiments unless otherwise stated.

Measurement of acetylene reduction. Acetylene reduction assays involving soils or plants or both were performed using (i) serum-stoppered glass tubes (32 by 200 mm or 32 by 300 mm) or (ii) glass tubes (70 by 150 mm) joined at the open ends to glass tubes (70 by 300 mm) with rubber tubing. In the latter instance, the large tube was at the top in an inverted position, and the rubber connecting the two glass tubes was fitted with a glass tube (10 by 90 mm) closed with a serum stopper; the latter tube served as a gas port. The chambers contained 10 kPa of acetylene and 91 kPa of air in the gas phase. Analysis for acetylene and ethylene was performed with a Perkin-Elmer 3920B gas chromatograph equipped with flame-ionization detectors and a 1.0-m column packed with 2.0 g of phenyl isocyanate-porasil C (Waters Associates, Milford, Mass.). The column was operated at 24°C, and the detector was operated at 200°C. The flow rate of the carrier gas, N_2, was 40 ml/min. The retention times of ethylene and acetylene were 20 and 40 s, respectively.

Effect of propanil on blue-green algae. To 50-g portions of flooded Hudson soil (equivalent to 25 g of dry soil) contained in glass tubes (32 by 200 mm) were added 1.0-ml portions of a washed suspension prepared from a 12-day-old culture of *Anabaena* MH or 1.0 ml of the suspending medium. The acetylene reduction activity was determined in the dark or in the light (70 μEinsteins/s per m^2) at 30°C after the soil was treated with distilled water or a solution of propanil to yield concentrations of 20, 25, or 40 μg/ml in the flooded soil-floodwater mixture. The propanil was applied immediately after inoculation or 30 days after the algae were added. The soil was maintained under water. Each treatment was replicated five times.

The effect of propanil on *Anabaena* MH was tested by inoculating 5.0-ml portions of a washed suspension from a 12-day-old culture into 250-ml portions of Stanier's medium with or without propanil. The herbicide concentrations were from 15 to 50 μg/ml. The flasks were incubated at 26°C on a rotary shaker as described above before analysis for chlorophyll content.

Effect of propanil on rice. Rice plants (variety IR2037) were grown from seed in Hudson silty clay loam (moisture content of 40%) contained in 500-ml polystyrene pots. The pots were incubated at 30°C under 12 h of illumination per day at about 150 μEinsteins/s per m^2. When the seedlings were 10 days old, the soil was flooded with distilled water or with a solution of propanil to yield various final concentrations. Each treatment was replicated four times. After 90 days, the panicles were harvested and dried at 60°C for 72 h. The seeds were removed from the panicles, and then the dry weight of the filled grains from the different treatments was measured.

Effect of propanil on heterotrophic nitrogen-fixing bacteria and protozoa. Portions (50 g) of flooded Maahas soil containing the equivalent of 20 g of dry soil were dispensed in glass tubes (32 by 200 mm). The soil was amended with ethanol (to stimulate nitrogenase activity) and propanil to yield final concentrations of 0.2% and 25 μg/ml, respectively, in the soil and floodwater. Each treatment was replicated four times. The tubes were then incubated at 26°C under continuous light at about 70 μEinsteins/s per m^2. Samples were taken daily to determine the acetylene reduction activity and the numbers of protozoa and nitrogen-fixing bacteria.

The number of heterotrophic nitrogen-fixing bacteria was determined by the most-probable-number technique by using acetylene reduction as the criterion. For this purpose, a glucose-yeast extract semisolid medium (I. Watanabe, personal communication) was used. The medium contained 5.0 g of glucose, 0.05 g of yeast extract, 0.15 g of $CaCl_2 \cdot 2H_2O$, 0.005 g of Na_2MoO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of K_2HPO_4, 1.0 g of $CaCO_3$, and 1.75 g of Noble agar (Difco Laboratories, Detroit, Mich.) in 1.0 liter of distilled water. The pH of the medium was adjusted to 7.0 before $CaCO_3$ was added. Dilutions of soil were inoculated into 20-ml test tubes containing 5.0-ml portions of the sterilized medium. The tubes were incubated for 48 h in the dark at 30°C. Ten percent of the headspace in the tubes was then replaced with acetylene. After an additional 24-h incubation period under the above conditions, the contents of the tubes were analyzed for ethylene production. Protozoa were enumerated by employing a modification of the Singh ring method (6).

Influence of propanil on photodependent nitrogenase activity. Portions (50 g) of flooded Maligaya, Crowley, or Hudson soil (equivalent to 20 g of dry soil) were transferred into separate glass tubes (32 by 200 mm). The flooded soils were then amended with both 0.2% ethanol and propanil to yield a concentration of 25 μg/ml of herbicide in the slurry. The tubes were incubated at 26°C in the dark or in the light at about 50 μEinsteins/s per m^2. At regular intervals, the acetylene reduction activity of the soils was tested.

Effect of propanil on nitrogenase activity of planted soils. Portions (100 g) of a slurry of Hudson soil containing the equivalent of 50 g of dry soil were transferred to several glass tubes (32 by 300 mm). Three 9-day-old rice seedlings (variety IR32) were transplanted into each tube. The tubes were then incubated at 30°C and illuminated for 12 h per day at about 150 μEinsteins/s per m^2. After 24 h, the level of the floodwater was adjusted to a depth of 3 cm. In some of the tubes, the light coming vertically to the soil-water surface was excluded by means of 1.3-cm-thick polystyrene disks cut to loosely fit in the 32-mm-diameter glass tubes. Each disk had a hole at its center through which the seedlings could emerge. The disks were placed immediately above the floodwater. Light striking the soil-floodwater from the sides was excluded by wrapping the tubes with dark tape from the bottom to 1.3 cm above the level of the floodwater. After 21 days of incubation, half of the tubes that did not have polystyrene disks were treated with a solution of propanil to yield a final concentration of 50 μg/ml in the soil plus floodwater. Each treatment was replicated four times. The acetylene reduction activity of the soils was tested 48 h after propanil application.

In a similar experiment, 550-g portions of a slurry of Hudson silty clay loam containing the equivalent of 250 g of dry soil were transferred to glass tubes (70 by 150 mm). Two 21-day-old rice seedlings (variety IR26)

were transferred into each tube. Polystyrene disks (12.5 by 70 mm) placed on the lips of some of the glass tubes and held in place with aluminum foil prevented light from reaching the surface of the soil and water. Each disk had a hole at its center through which the seedlings emerged. Light reaching the soil and floodwater from the sides was excluded by means of polystyrene disks as described above. The tubes were incubated at 30°C with 12 h of illumination at an intensity of 25,000 lx. After 20 days, the tubes without polystyrene disks were inoculated with 5 ml of a washed suspension derived from a 12-day-old culture of *Anabaena* MH. After 30 additional days, half of the tubes that had no polystyrene disks were amended with propanil to a final concentration of 50 μg/ml in the flooded soil-floodwater mixture. The acetylene reduction activity of the soils was determined 48 h after treatment with propanil. Each treatment was replicated four times.

RESULTS AND DISCUSSION

Effect of propanil on the rice plant. Rice was grown from seed in flooded Hudson silty clay loam. When the seedlings were 10 days old, the soil was flooded with either distilled water or distilled water amended with propanil to yield concentrations ranging from 25 to 100 μg/ml in the soil slurry. After 90 days, the weight of the filled grains was measured for each treatment. The results indicate that propanil was not toxic, even at the highest concentration used (Table 1). Although the mean yield suggested that the highest concentration was phytotoxic, a statistical analysis failed to show a significant difference at the 5% level. Propanil is generally applied to lowland rice at concentrations not exceeding 30 kg/ha (9). The lack of phytotoxicity even at the highest herbicide concentration may be related to the site of application. Since propanil is degraded in soil (4) and is also metabolized in rice tissue (3), the extent of detoxication may have been appreciable so that toxic amounts were not translocated from soil to the site of action, the photosynthetic system of the plant (3).

Effect of propanil on blue-green algae. Experiments were conducted to assess the influence of propanil when applied to soil or culture medium immediately after inoculation with *Anabaena* MH and when applied to a soil containing a full bloom of the blue-green alga. *Anabaena* MH exposed to propanil in culture at concentrations as low as 20 μg/ml lost more than 85% of its chlorophyll in 48 h, and no visible sign of recovery was apparent 10 days after exposure to the herbicide. Applying the herbicide at 25 μg/ml immediately after inoculation suppressed the activity of the algae at least 98% (Table 2). The inhibition extended for at least 30 days. The data presented in Table 2 were derived from tests in which the inoculated soil was incubated in the light for 24 h. No activity was detected in soils incubated for the same time period in the dark.

Propanil was also effective even when applied after the full bloom had been established, that is, after 30 days (Table 3). The nitrogenase activity of the bloom was suppressed by more than 95% by 20 μg of propanil per ml in 24 h, and inhibition by the herbicide was still evident at 72 h. In both propanil-treated and untreated soil incubated in the dark, nitrogenase activity was detectable, suggesting a role for heterotrophic bacteria; however, propanil had no influence on this activity. The activity in the dark of the inoculated soil incubated in the light without propanil was approximately 10 times greater than in soil with the herbicide or in uninoculated soil kept in the dark. These observations suggest the functioning of algal nitrogenase in the dark. Such activity, however, was less than 7% of the bloom activity in the light. Although photophosphorylation supplies the bulk of the adenosine triphosphate required for nitrogen fixation by blue-green algae, the algae are known to assimilate N_2 in the dark by utilizing heterotrophically derived adenosine triphosphate (5).

Effect of propanil on nitrogen-fixing bacteria and protozoa. Flooded Maahas clay untreated or treated with 25 μg of propanil per ml was amended with 0.2% (vol/vol) ethanol to increase the nitrogenase activity. The soils were incubated in the light, and samples were taken at daily intervals for a determination of nitro-

TABLE 1. *Effect of propanil applied to soil on rice*

Concn (μg/ml)	Grain dry wt/pot (g)
0	1.9
25	2.0
50	1.9
100	1.5

TABLE 2. *Effect of propanil on the development of Anabaena MH inoculated into flooded Hudson silty clay loam incubated in the light*

Days	nmol of C_2H_4 formed/cm^2 of soil surface per day	
	Propanil	No propanil
0	0.6 ± 0.1	51 ± 1.6
2	ND[a]	176 ± 14
4	ND	197 ± 14
6	ND	251 ± 18
12	3.3 ± 0.8	179 ± 24
30	0.3 ± 0.1	1,190 ± 130

[a] ND, Not detectable.

TABLE 3. *Effect of incubating an established bloom of Anabaena MH for various periods with propanil in a flooded Hudson silty clay loam*

Incubation period (h)	nmol of C_2H_4/cm^2 of soil surface per day					
	Propanil			No propanil		
	20 μg/ml, light	40 μg/ml, light	40 μg/ml, dark, uninoculated[a]	Light	Dark	Dark, uninoculated[a]
24	29.9 ± 15.7	6.4 ± 2.5	4.9 ± 0.6	732 ± 38	45 ± 10	3.9 ± 0.5
48	12.2 ± 3.1	10.9 ± 3.3	4.8 ± 0.6	733 ± 37	50 ± 8	3.8 ± 0.5
72	16.7 ± 4.9	4.8 ± 1.0	4.9 ± 1.0	728 ± 31	46 ± 8	4.4 ± 0.7

[a] Soil was incubated and assayed in the dark. All other treatments were incubated in the light after inoculation, and the nitrogenase activity of the bloom was assayed either in the dark or in the light, as indicated in the table.

genase activity and of the numbers of nitrogen-fixing bacteria and protozoa. The numbers of N_2-fixing bacteria and protozoa were similar in soils treated and not treated with propanil in the 4-day period (Table 4). The nitrogenase activity of the propanil-treated soil, however, was different from that of the untreated soil. On the first day, the activity in the treated soil was less than that in the untreated soil, but it rose to substantially higher values during the next 3 days. Since propanil had no detectable influence on the number of heterotrophic N_2-fixing bacteria or protozoa (which presumably graze on these bacteria), the greater nitrogenase activity in the light in the presence of propanil was probably a result of the stimulation of photodependent nitrogenase activity by herbicide-resistant organisms. The validity of this hypothesis was then tested, as described below.

Influence of propanil on photodependent nitrogenase activity. Two rice soils and one upland soil were flooded and amended with both 0.2% ethanol and 25 μg of propanil per ml, the concentrations being expressed in terms of the volume of the soil-water mixture. Samples of the soils were then incubated in the dark or in the light. At intervals, the nitrogenase activity was tested to see if the greater activity in the presence of propanil was dependent on the presence of light. No significant activity was detected on the first day in flooded Hudson soil under both light and dark conditions (Table 5). The two rice soils, however, exhibited substantial activity at this time, and the activity in the light was significantly greater than that in the dark. On the third day, all of the flooded soils showed higher activity in the light than in the dark; however, the differences observed in the Crowley and the Maligaya soils were not statistically significant. The activities in the rice soils were lower on the sixth day, and the values obtained in the light were less than those in the dark at this time. The propanil-dependent stimulation of nitrogenase activity in the light noted earlier was confirmed with these three soils. The speed of initiation, the extent, and the duration of the stimulation varied among the soils, suggesting an influence of soil type.

TABLE 4. *Effect of propanil on the number and activity of nitrogen-fixing bacteria and protozoa in ethanol-amended Maahas clay*

Days	Propanil-treated soil			Untreated soil		
	No. × 10^3/g of dry soil		nmol of C_2H_4/cm^2 of soil surface per day	No. × 10^3/g of dry soil		nmol of C_2H_4/cm^2 of soil surface per day
	Protozoa	N_2 fixers		Protozoa	N_2 fixers	
1	0.41	240	11 ± 0.4	0.49	170	15 ± 1
2	2.4	240	199 ± 16	2.4	240	129 ± 4
3	2.4	240	135 ± 28	2.4	240	62 ± 10
4	2.4	240	213 ± 26	3.3	170	94 ± 10

Since propanil is a potent suppressor of the nitrogenase activity of blue-green algae, the light-dependent nitrogenase activity in the presence of the herbicide is probably attributable to photosynthetic bacteria. None of the soils treated with propanil and ethanol evolved ethylene unless they were incubated in acetylene. In the absence of ethanol, propanil did not stimulate nitrogenase activity in any of the soils. Examination of a suspension of the ethanol- and propanil-amended unplanted soil (2 to 4 days after the amendment) under the phase-contrast microscope revealed that the dominant forms were purple sulfur bacteria closely resembling *Chromatium*. In contrast, microscopic examination of a suspension of samples taken from the soil planted with rice and amended with propanil but no ethanol showed the predominance of cells closely resembling photosynthetic bacteria of the genera *Chromatium* and *Thiospirillum* at 3 to 6 days after amendment with propanil. Cells of the purple sulfur bacteria contained sulfur granules. Propanil probably acts by eliminating the oxygen-generating algae, in whose presence the obligately anaerobic photosynthetic bacteria

TABLE 5. *Photodependent nitrogenase activity in three flooded soils amended with propanil*

Days	nmol of C_2H_4/cm^2 of soil surface per day					
	Hudson silty clay loam		Crowley silt loam		Maligaya clay	
	Dark	Light	Dark	Light	Dark	Light
1	0.2 ± 0	0.3 ± 0.2	9 ± 1	34 ± 3	98 ± 5	157 ± 13
3	38 ± 6	231 ± 34	107 ± 15	123 ± 14	1,043 ± 22	1,127 ± 43
6	181 ± 38	127 ± 22	33 ± 8	6 ± 1	84 ± 3	48 ± 9

may not be active. The need for a source of readily available organic carbon is likely related to the requirement of these organisms for H_2S, the production of which would be promoted by an energy source such as ethanol. Under conditions of lowland rice cultivation, on the other hand, the energy needed for this purpose can be derived from root excretions.

Significance of nitrogen fixation by photosynthetic bacteria. The relative importance of nitrogen fixation by photosynthetic bacteria as compared with the activity of blue-green algae and the rhizosphere microflora was assessed by growing rice for 30 days in flooded Hudson silty clay loam under the following conditions: (i) with light being excluded at the soil-floodwater level by use of disks, (ii) in the presence of light, and (iii) in the presence of light but with the indigenous algae suppressed by 50 μg of propanil per ml before the acetylene reduction test was made. The activity in the light (which presumably came from metabolism of blue-green algae as well as photosynthetic bacteria and rhizosphere bacteria) was not significantly different from that obtained when disks were employed to eliminate phototrophic activity (Fig. 1). Since the soil was well colonized with indigenous algae at the time of the acetylene reduction assay, the low activity in the light indicates either the absence of highly active blue-green algae or the presence of some limitation on the fixation. The much greater activity when these indigenous algae were exposed to propanil suggests that the algae must be suppressed for the nitrogenase activity of the photosynthetic bacteria to become appreciable.

In a similar experiment, rice was grown for 55 days in flooded soil inoculated with *Anabaena* MH. Under these circumstances, both photosynthetic bacteria and blue-green algae were substantially more active than the rhizosphere microflora (Fig. 2); that is, the activity was greater in the presence of propanil (photosynthetic bacteria) or with neither propanil nor disks being present (algae) than in tubes with the disks. The highest value was associated with the development of blue-green algae. This is not surprising because the soil was inoculated with *Anabaena*. The results of this and the preceding experiment

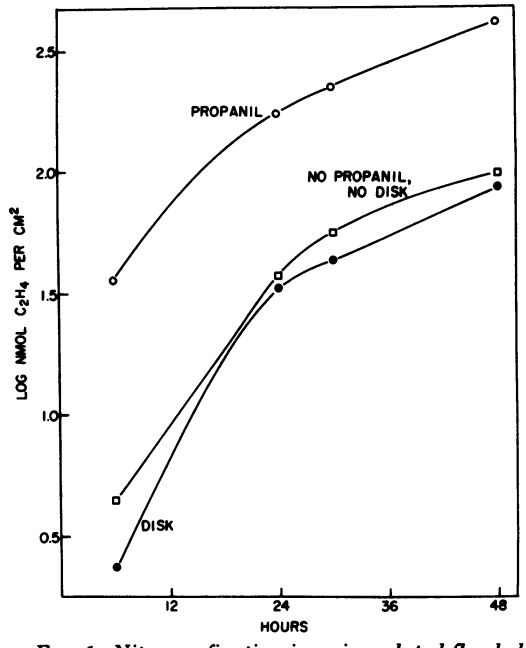

FIG. 1. *Nitrogen fixation in uninoculated flooded Hudson silty clay loam planted to rice.*

suggest that sometimes the contribution of photosynthetic bacteria may be significantly higher than that of the rhizosphere microflora and that they may contribute as much nitrogen as blue-green algae.

The data suggest that nitrogen fixation by photosynthetic bacteria and blue-green algae may not occur simultaneously in lowland rice culture. To realize the benefits of nitrogen fixation by photosynthetic bacteria, it may thus be necessary to inhibit the development of algae. Whether or not this is feasible or represents good crop management remains to be determined. On the other hand, nitrogen fixation by photosynthetic bacteria is attractive because they may grow faster than the blue-green algae (13), they are able to utilize light energy in the far red and infrared regions of the spectrum (which are regions of the spectrum not utilized by the plant) (1), they are insensitive to at least certain herbicides used in rice culture, and they detoxify H_2S by oxidizing it to sulfate (1).

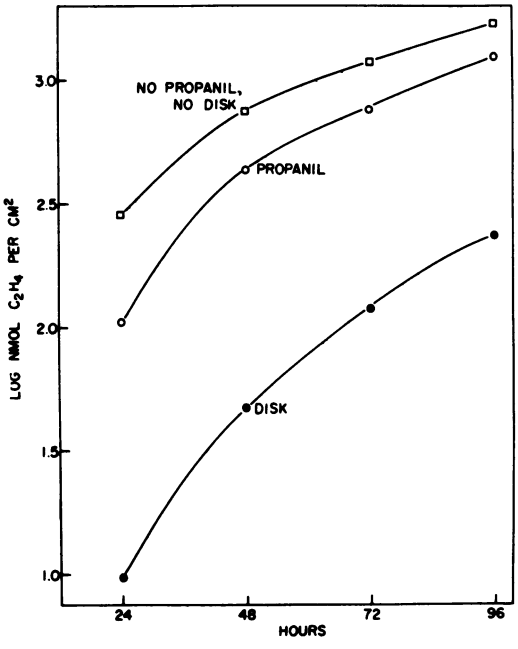

FIG. 2. *Nitrogen fixation in flooded Hudson silty clay loam planted to rice and inoculated with Anabaena MH.*

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