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INFLUENCE OF SOME ENVIRONMENTAL FACTORS ON NITROGEN FIXATION IN THE RHIZOSPHERE OF RICE

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SUMMARY

Nitrogen fixers make up a large percentage of the total microflora in the rhizosphere of lowland rice. There are more aerobic nitrogen fixers than there are anaerobic ones.

When soil crumbs from the root zone were placed on a nitrogen free agar medium and inoculated at 0, 5, 10, and 21 percent oxygen concentration, colonies of aerobic nitrogen fixers reached their greatest diameter at 5 and 10 percent oxygen.

In acetylene reduction assays rice plants grown in paddy fields and in solution culture were tested for the nitrogenase activities of their roots at different oxygen tensions. Nitrogenase activity was highest at 3 percent oxygen, lower at 0 percent, and far lower at 21 percent.

When rice was grown in solution culture the redox potential of the nutrient solution strongly influenced nitrogenase activity. With declining redox potential, nitrogenase activity increased to a maximum value but dropped sharply as redox potential further decreased.

Ten ppm of combined nitrogen as urea depressed nitrogenase activity on excised roots. Combined nitrogen applied to one part of the root system affected, to some extent, nitrogen fixation on other roots kept in a solution without nitrogen.

Nitrogenase activity in a fertility trial with lowland rice, examined at several dates, showed no inhibitory effect of fertilizer nitrogen, however, presumably because the nitrogen concentration in the soil solution rapidly decreased. Instead, an overall stimulating effect of nitrogen dressing was noticeable.

Diurnal fluctuations of nitrogenase activity in the rhizosphere, with a peak in the afternoon and low fixation rates after low solar radiation, suggest a photosynthetic effect on nitrogen fixation.

INTRODUCTION

As previously reported[20] excessive microbial activity in the rhizosphere seems to be more detrimental to than favorable for, growing

rice in flooded soil because the organisms compete with roots for oxygen and aggravate reduction. Such metabolic products of anaerobic decomposition as organic acids, as well as excessive ferrous iron and hydrogen sulfide, are toxic to rice.

Recently, however, the rhizosphere of rice was found to be an ideal location for another beneficial reduction process, the microbial reduction of molecular nitrogen to ammonia[12 15 22]. The fixation of molecular nitrogen, according to Yoshida and Ancajas[23] is much higher under lowland conditions than under upland conditions. Obviously, submergence provides especially suitable conditions for N_2-fixation in the rhizosphere of rice.

It seemed worthwhile to examine in more detail some environmental factors that might play a major role in nitrogen fixation in the rhizosphere of rice.

METHODS

1. *Enumeration of rhizosphere bacteria*

At milk ripening stage, rice plants were dug out from a lowland plot not fertilized with nitrogen. The roots were washed free of soil. Then 10 g of roots were macerated in 100 ml sterilized de-ionised water in a Waring blendor for 5 min at high speed. Subsequently serial dilutions were prepared up to 10^{-8}.

Total saprophytic bacteria. By the plate count technique the number of bacteria was determined on nutrient agar: 2 g nutrient broth (Difco), 10 g agar, 1,000 ml de-ionized water.

Aerobic nitrogen fixers. The number of aerobic nitrogen-fixing bacteria was determined on a medium with 1 percent mannitol described by Allen[1] for isolating Azotobacter. The medium was poured into Petri dishes at least 1 day in advance and the Petri dishes were stored at 37°C to allow the agar surface to dry. From suitable dilutions 0.1 ml aliquots were uniformly distributed on the agar surface with a bended sterile glass rod. Thus the liquid was soaked in rapidly. The inoculated Petri dishes were incubated for 1 week at 30°C.

Anaerobic nitrogen fixers. The 'Most Probable Number' technique was used on a medium recommended by Allen[1]. To record the gas evolved Durham tubes were inserted into the tubes containing the medium. After 1 week of anaerobic incubation, the number of anaerobic nitrogen fixers was evaluated from the tubes that produced gas. An additional incubation of the tubes in an argon atmosphere for 1 day with 10 percent acetylene in the gas phase followed by an acetylene reduction assay, showed that gas production coincided with nitrogenase activity. In a recent paper, however, Villemin *et al.*[21] reported that decrease of redox potential in inoculated tubes, indicated by change in color of phenosafranine – a method proposed by Brouzes *et al.*[6] – better agrees with nitrogenase activity than gas formation.

2. *Acetylene reduction assay*

Roots from solution-cultured plants. The solution culture of rice is described elsewhere[20]. Entire plants with their roots placed in nutrient solution were brought into the laboratory, where 2 g fresh roots were placed in 180 ml dilution bottles and covered with a rubber needle puncture stopper. In most experiments the air was replaced by an oxygen-argon mixture containing 3 percent oxygen. To study the influence of oxygen on nitrogenase activity in a further treatment the air remained in the flasks and in a third set the flasks were filled with argon. Then 10 percent of the gas volume of the flasks was replaced by acetylene. After a period of incubation at 30°C, the acetylene reduction assay as described by Yoshida and Ancajas[22] was conducted.

Rhizosphere of lowland rice. A plant with adjacent soil was dug out from a lowland plot. A core about 250 to 350 ml in volume was cut out from the center of the root system with a pair of scissors and placed in a 1 liter glass jar. The jar was taken to the laboratory, sealed, 10 percent of the gas phase replaced by acetylene, and incubated at 30°C.

In contrast with that on roots from plants grown in solution culture, where ethylene formation was almost linear up to 1 day of incubation (see below), the ethylene formation on washed roots from lowland plants and soil cores from the rhizosphere was accelerated during incubation (Fig. 1). No attempt

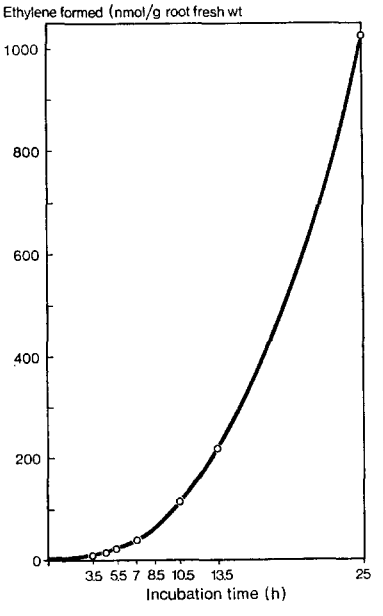

Fig. 1. Effect of incubation time on acetylene reduction assay with root-soil cores.

was made to clarify the different behaviours. Several authors[5 11] discuss the effect of length of incubation on rates of ethylene formation and recommend shortterm incubation. With rhizosphere soil from rice, Rinaudo[15] also reported accelerated ethylene formation with continuous incubation. Therefore, ethylene formation was measured after 3 and 5 hours of incubation and the rate was calculated from the difference. The gas phase volume of the jars containing the soil cores was determined by water displacement. The roots were collected by washing the soil cores and the ethylene found in the gas phase was related to 1 g of fresh roots.

3. *Effect of redox potential on nitrogenase activity*

For several weeks rice plants were grown in nitrogen-deficient nutrient solution and, 78 and 91 days after transplanting, were assayed for redox potential of the solution as described by Trolldenier[20]. Although the experimental pots received the same treatment and contained the same number of plants, their redox potentials differed. Four aliquot root samples were taken from each pot and assayed for acetylene reduction at a pO_2 of 0.03 atm. The ethylene formed per hour was calculated from the difference between values obtained after 3 and after 5 hours of incubation.

4. *Root split technique for studying the effect of combined nitrogen on nitrogenase activity*

Plants of IR26, grown for 75 days in a complete nutrient solution, were divided into two equal parts. Two 800-ml vessels were arranged close together. A plant was placed on top of the trucking walls with the roots dipping into the two vessels. One vessel contained a complete nutrient solution with 40 ppm N, the other a solution deficient in nitrogen. In the control plants both root halves were placed in a nitrogen-free solution. One week later nitrogenase activity was assayed.

RESULTS

1. *Number of nitrogen-fixing bacteria on rice roots*

The number of nitrogen fixers found on washed rice roots that were cut into small pieces and shaken in water with sand for 30 min was much lower than that found on roots that were completely macerated. At one date on roots of the variety IR20, in addition to 18 million total saprophytic aerobic bacteria, 8 million aerobic and 1 million anaerobic nitrogen fixers per gram fresh root were counted. At another sampling date, on roots of the variety IR8 there were 255 million total bacteria, 59 million aerobic nitrogen fixers, but only 23,000 anaerobic nitrogen-fixing bacteria. Although bacterial counts are likely to fluctuate greatly, provisional conclusions may be drawn.

Nitrogen fixers make up a large percentage of the total microflora in the rhizosphere of lowland rice and there are more aerobic nitrogen fixers than anaerobic ones.

2. *Effect of oxygen concentration on nitrogen fixation*

Aerobic nitrogen fixers fix nitrogen more efficiently at sub-atmospheric oxygen tension[7 17]. With time, however, they may adapt to higher pO_2 values. Fresh isolates grown under different pO_2 values, therefore, reflect, by their different growth rates at different oxygen concentrations, the oxygen tension that prevails in their natural environment. This can be seen when rice roots or soil crumbs from the root zone are placed on the mentioned nitrogen-free agar medium and incubated at different oxygen concentrations. After 5 days colonies on plates incubated at 5 and 10 percent oxygen reached a greater diameter than colonies on plates incubated at atmospheric oxygen tension (Fig. 2). With 0 percent oxygen, where only traces of O_2 eventually might have been present, the growth of aerobic nitro-

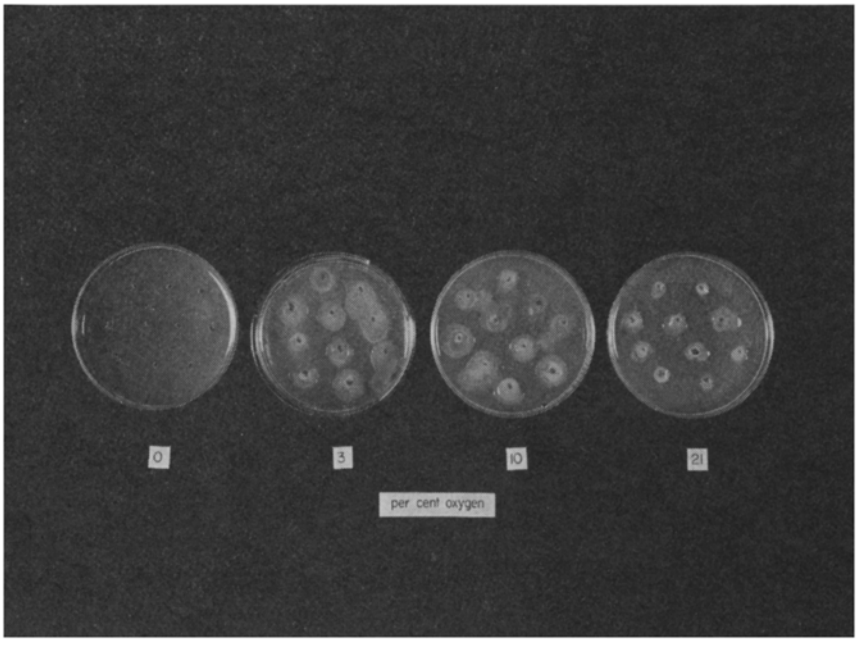

Fig. 2. Effect of oxygen concentration during incubation on growth of aerobic nitrogen fixing bacteria on soil crumbs taken from the vicinity of lowland rice roots.

gen fixers was negligible. This illustrates the importance of an oxidized rhizosphere in a submerged soil for efficient nitrogen fixation. On the other hand a habitat as well aerated as the rhizosphere of rice grown under upland conditions is less suitable for nitrogen fixation[23].

The inhibitory effect of atmospheric oxygen concentration on nitrogen fixation is revealed when roots are incubated at different O_2 pressures during acetylene reduction assay.

Assays were done with gently washed roots of paddy rice and with roots from rice grown in solution culture. In experiments set up in triplicate least ethylene was formed with an atmosphere of 21 percent oxygen, most with 3 percent oxygen; an intermediate amount was formed under anaerobic conditions. The solution culture plants (Table 1) and the lowland plants gave similar differences between

TABLE 1

Effect of oxygen concentration on acetylene reduction of roots from plants grown in solution culture

	Ethylene formed ($nmol \cdot g^{-1}$ fresh root) at		
	21% O_2	3% O_2	0% O_2
After 3 hours	1.4	100.3	61.8
After 14 hours	11.9	654.8	549.0

treatments. From these results it can be concluded that intermediate oxidative conditions in the rhizosphere are more favorable for acetylene reduction than is excessive aeration or absence of oxygen.

3. *Effect of redox potential on nitrogenase activity*

Additional support for the significance of an intermediate oxidative status in nitrogen fixation was obtained from rice plants grown in solution culture with different redox potentials. The higher redox potentials in the first experiment indicate the presence of oxygen in the nutrient solution. With falling redox potential, *e.g.* decreasing O_2 concentration, nitrogen fixation increased in the first experiment (Table 2). The lower potentials in the second experiment indicated the absence of oxygen and therefore a higher sink for oxygen excreted by the roots. At the lowest redox potential (+39

TABLE 2

Acetylene reduction assay with rice roots from culture solutions of different redox potentials

Eh (mV)	Ethylene formed ($nmol \cdot h^{-1} \cdot g^{-1}$ fresh root)
1st Experiment	
+609	16.1
+504	73.0
+296	143.0
*2nd Experiment**	
+381	9.0 a
+350	34.5
+ 66	146.3
+ 39	8.8 a

* Means followed by letter a are not significantly different at 5% level.

mV), apparently the optimum for nitrogen fixation under these experimental conditions was exceeded. Acetylene reduction was significantly lower in this treatment than at the next higher redox potential. The relationship between nitrogen fixation on rice roots and redox potential in the culture solution does not apply directly to the conditions found in submerged soil and may give only an approximate idea of the effect of soil reduction on nitrogen fixation. In solution culture, such processes leading to lower redox potentials (lower pO_2) as nutrient deficiency may even stimulate nitrogen fixation. This could be said of an assay done in the previously described

TABLE 3

Interaction between mineral nutrition, redox potential and nitrogenase activity at high Eh values

Nutrient solution without		Eh (mV) of nutrient solution*	Ethylene formed ($nmol \cdot h^{-1} \cdot g^{-1}$ fresh root)
Nitrogen	(–N)	+566	44.7
Nitrogen and phosphorus	(–N–P)	+139	70.4
Nitrogen and potassium	(–N–K)	+ 86	127.0

* Measured 1 day after assay.

solution culture experiment[20] in which at 73 days after transplaning, nitrogenase activity was highest in the treatment with the lowest redox potential, *e.g.* the treatment in which, potassium besides nitrogen was deficient (–N–K). Nitrogenase activity decreased with increasing redox potentials of the treatments (Table 3).

4. *Effect of combined nitrogen on fixation of atmospheric nitrogen*

Since combined forms of nitrogen control nitrogenase activity in living organisms, it would be interesting to know whether fertilizer nitrogen counteracts nitrogen fixation in the rhizosphere.

In vitro experiments. Experiment 1. Two-gram root samples were taken from the variety IR26 grown for 7 weeks in a nitrogen-deficient nutrient solution. The roots were placed in 180-ml flasks to which 10 ml of a solution with different concentrations of urea were added. The control received an aliquot of water. During acetylene reduction assay the roots were submerged in the solution. Ten ppm of combined nitrogen decreased nitrogenase activity significantly (Table 4).

Experiment 2. Available soil nitrogen and fertilizer nitrogen are not equally distributed in the soil. The supply of nitrogen to single roots of the same plant can vary under field conditions. Therefore by using the root split technique, it was studied whether combined nitrogen applied to one part of the root system affects nitrogen fixation on other roots of the same plant. The result (Fig. 3) reveals that when nitrogen fixation on some roots of the same plant is inhibited by high concentrations of combined nitrogen on other roots in a

TABLE 4

Effect of adding combined nitrogen (urea) to excised roots on C_2H_2-reduction

N (ppm)	Ethylene formed* ($nmol \cdot h^{-1} \cdot g^{-1}$ fresh root)
0	54.6 a
5	29.8 ab
10	13.2 b
20	3.1 b

* Any two means followed by a common letter are not significantly different at 5% level.

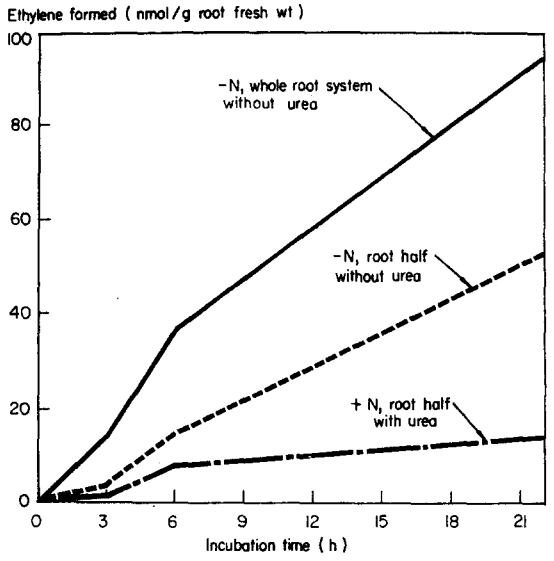

Fig. 3. Effect of combined nitrogen (urea) application to one part of the roots on nitrogenase activity on both root halves (root split technique).

nitrogen-free medium, nitrogen fixation still continues. The nitrogen fixation on those roots was, however, lower than on plants whose entire root system was growing in a nitrogen-free solution.

In situ experiments. Assays of nitrogen fixation with rice grown in solution culture are comparatively easy to accomplish. They may give preliminary information on some influencing factors. Additional experiments were made to study the effect of nitrogen fertilization under field conditions.

For duplicating natural conditions as closely as possible – to cover aerobic and anaerobic N_2-fixation simultaneously – we assayed undisturbed root-soil cores. Soil cores had been used to study another nitrogen-fixing system, the rhizosphere of *Paspalum notatum*[8]. In soil cores, aerobic nitrogen fixers are protected from damaging excessive oxygen and the external pO_2 has little effect on nitrogenase activity[9].

a. Diurnal variation of nitrogen fixation in the rhizosphere of rice.

To obtain information as to whether the time of sampling influences nitrogenase activity, root-soil samples were collected from the variety IR26 at booting stage every 3 hours. At each sampling time

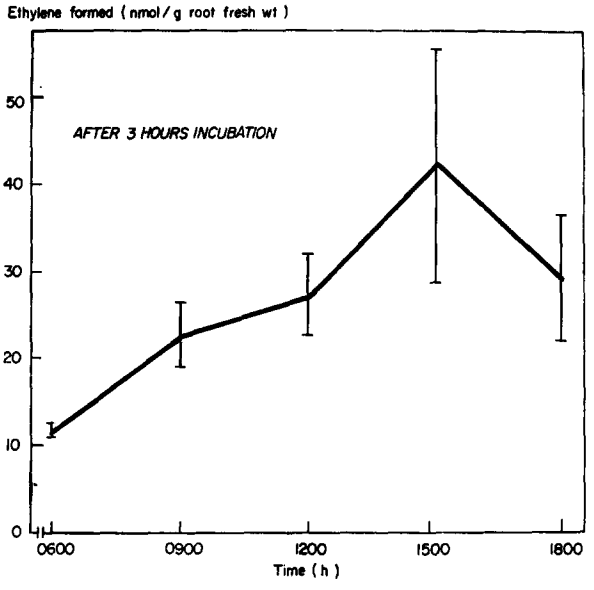

Fig. 4. Diurnal rhytm of nitrogen fixation in the rhizosphere of lowland rice. Vertical lines represent the limits of the standard error of the means.

two plants were dug out and from each plant two replicate root-soil cores were prepared. After 3 hours of incubation acetylene reduction was assayed. Nitrogenase activity obviously showed a diurnal rhythm (Fig. 4). The statistical analysis revealed a correlation between means and standard errors, justifying a logarithmic transformation of the data for statistical analysis. Based on this transformation nitrogenase activity was significantly higher at 3 pm than at 6 am. To eliminate the influence of day time and to ensure high nitrogenase activity, sampling in the subsequent experiment was done between 1300 and 1430 hours.

b. Nitrogenase activity in a fertilizer experiment.

The nitrogenase activities in plots with and without nitrogen fertilizer application were compared. In the nitrogen treatment 100 kg N was applied before transplanting and 40 kg N was topdressed 56 days after transplanting. Obviously nitrogen fixation was not impaired by heavy dressing of mineral nitrogen: on the contrary, it even tended to be higher in this treatment than in the no-fertilizer treatment (Table 5). The high variation between replicate samples,

TABLE 5

Nitrogenase activity (in terms of ethylene formation) in the rhizosphere of lowland rice 41 to 77 days after transplanting as affected by nitrogen application

N (kg/ha)	Ethylene formed ($nmol \cdot h^{-1} \cdot g^{-1}$ fresh root)			
	41 days	47 days	65 days	77 days
0	2.0	11.9	4.4	21.6
140*	5.0	81.5	7.1	36.2

* Includes 40 kg/ha N 56 days after transplanting.

reflected by coefficients of variation that consistently ranged from 41 to 64 percent, required a joint analysis of variance for all four sampling dates after log transformation. The statistical analysis revealed significantly higher nitrogenase activity in the treatment with nitrogen fertilizer.

The repression effect of fertilizer nitrogen probably decreases rapidly with the decline of ammonia concentration in the soil. Exchangeable ammonia in the nitrogen treatment amounted to only 6 ppm 2 days after the last assay, which corresponded to a still smaller amount in the soil solution. The low nitrogenase activity 65 days after transplanting coincides with a short sunshine duration and low solar radiation on the day before the assay (Table 6). This, as well as the diurnal variation, indicates a strong dependence of nitrogenase activity upon photosynthesis of the rice plant.

TABLE 6

Sunshine and solar radiation

	Days after transplanting			
	41	47	65	77
Sunshine				
One day before assay	10:48	9:00	6:39	10:33
On day of assay	10:48	7:27	10:18	11:15
Solar radiation ($g\text{-}cal \cdot cm^{-2} \cdot day^{-1}$)				
One day before assay	391	586	456	662
On day of assay	586	423	620	680

DISCUSSION

In a submerged paddy field where the bulk of the soil is reduced, somewhat surprisingly most nitrogen obviously is fixed by aerobic nitrogen fixers. Nitrogen fixation occurs, however, mainly in the oxidized rhizosphere as first stated by Yoshida and Ancajas[23] who found nitrogenase activity much lower in unplanted soil than in planted soil. The presence of a large number of aerobic nitrogen-fixing bacteria in the rhizosphere, making up a considerable percentage of the total number of saprophytic bacteria, indicates their high competitive ability in this particular habitat. Most likely the lower partial pressure of oxygen compared with that in the rhizosphere of upland plants seems to be especially advantageous for aerobic nitrogen fixers, as shown by their faster growth and the higher nitrogenase activity of root samples at subatmospheric oxygen tension. Unlike aerobic nitrogen fixers anaerobic nitrogen-fixing bacteria make up a rather small portion of the rhizosphere[4]. Anaerobic nitrogen fixers are less important, as reflected by the lower nitrogenase activity of root samples incubated under anaerobic conditions.

Factors that influence the oxidative status therefore control nitrogen fixation. Additional evidence was obtained with rice grown in solution culture. Since the nutrient solution is in contact with air, its redox potential remains at a higher level than that of a flooded soil. A decrease in redox potential of the solution favors nitrogen fixation to some extent. But a further decrease, causes nitrogen fixation to drop sharply.

Nutrient deficiency, especially deficiency of potassium causes increased growth of saprophytic bacteria and decline in oxygen content[19] and in redox potential[20] as well. In solution culture the diminution of the unfavorably high redox potential caused by potassium deficiency may provide better conditions for nitrogen fixation. A submerged soil's deeper redox potential[13], however, acts as a strong sink for oxygen coming from the root. Weakening of the oxidizing power caused by potassium deficiency and the consumption by rhizosphere bacteria of oxygen excreted from the roots may have rather opposite effects. The relationship between oxidizing power and nitrogen fixation must be studied further.

In a study of the effect of combined nitrogen on nitrogen fixation

in the rhizosphere of rice, Balandreau *et al.*[4] applied several levels of ammonium sulfate at seeding time and assayed nitrogenase activity 16 days later. At the assay all ammonia was consumed or immobilized. Up to 40 ppm added ammonia slightly stimulated nitrogenase activity while higher concentrations had a depressing effect. The increase of nitrogenase activity at lower concentrations of ammonia was attributed to increase in exudation. In our experiment in which at assay time the roots were kept in nitrogen containing solutions the inhibiting threshold was lower, as can be expected. Ten ppm N applied as urea inhibited nitrogenase activity.

With symbiotic nitrogen fixation of soybeans foliar application of urea inhibited nitrogen fixation in nodules, indicating[11], a negative sink effect of shoot N on the development of the N_2-fixation system. The experiment, using the root split technique, signifies a similar inhibition for a nonsymbiotic system, namely nitrogen fixation on rice roots.

Nitrogen fixation after nitrogen dressing seemed to be inhibited only temporarely, however, as assays showed after ample application of nitrogen fertilizer (140 kg/ha N). Studies[16 18] indicate that rice plants rapidly absorb ammonium nitrogen. The critical threshold is exceeded only in the first stages of growth and immediately after dressing. As suggested by Balandreau and Fares-Hamad[2] in the long run, nitrogen fertilization may enlarge the capacity for nitrogen fixation by promoting plant growth. A survey of nitrogenase activity in a fertilizer trial at the IRRI farm confirmed the higher activity in the rhizosphere of nitrogen fertilized plants.

Symbiotic systems show diurnal variations in nitrogenase activity, indicating a dependence on the supply of photosynthates[11]. In a non-symbiotic system, such as the rhizosphere of *Paspalum notatum*[8] no diurnal fluctuation was observed. Data from Balandreau *et al.*[3] and the result of our own study clearly show diurnal variation of nitrogenase activity in the rhizosphere of rice. The effect of photosynthesis is additionally supported by the nitrogenase activity observed after a day of low solar radiation. Similarily Dommergues *et al.*[10] found in laboratory experiments that the rhizosphere of rice responded to higher light intensity with higher nitrogenase activity.

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