ATMOSPHERIC NITROGEN FIXATION BY PHOTOSYNTHETIC MICROORGANISMS IN A SUBMERGED PHILIPPINE SOIL

Tomio YOSHIDA, Rosabel A. RONCAL, and Ellen M. BAUTISTA

The International Rice Research Institute,
Los Baños, Laguna, Philippines

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Photosynthetic nitrogen-fixing microorganisms help maintain the nitrogen level of soil in rice paddies when environmental factors favor the growth of microorganisms. Our studies showed that blue-green algae in particular have a significant role in nitrogen-fixation in light. The most active nitrogen-fixation by microorganisms occurred in the soil shortly after it had been submerged under light. The longer the submergence, the less nitrogen microorganisms were fixed. In a greenhouse experiment, the fixed nitrogen appeared not to be immediately available to the rice plant. The amount of nitrogen that can be fixed in the field by nitrogen-fixing microorganisms in paddy water was estimated using the acetylene reduction method during the rice-growing period. The amount of nitrogen fixation by these microorganisms is not sufficient to account for the amount of nitrogen uptake by rice during the rice-growing period.

In many parts of Asia, fertilizers are not used, and rice production depends on the natural fertility of the soil. A continuing supply of nitrogen over the years, despite removal of this element by rice crops, is claimed to result from the fixation of atmospheric nitrogen by microorganisms in paddy fields (1, 2). If the source of nitrogen for rice crops were only soil organic matter, it is estimated that the total nitrogen present in the soil would not last for many years. Thus, the maintenance of soil fertility in rice paddies for years can be explained only by microbial nitrogen fixation in the soil, since its possible sources, i.e. rain or irrigation water and rice plant residues, are not significant enough to bring about a nitrogen balance.

Various kinds of microorganisms fix atmospheric nitrogen, but it is not known which of these actually take part in nitrogen fixation in paddy soils. This paper reports a possible effect of photosynthetic microorganisms on the fertility of a Philippine soil.

MATERIALS AND METHODS

A pot experiment was conducted in the greenhouse. Seeds of IR8 and Peta were sterilized in 2% formalin solution for 15 min, then they were washed with tap water several times, and placed on nylon mesh in a nutrient solution. After 15 days seed-
lings were transplanted to porcelain pots, each containing 10 kg of soil. The soil was Maahas clay (Organic matter, 2.0%; Total-N, 0.098%; pH 6.4) taken from a coconut field adjacent to the IRRI experimental farm.

Half the pots were treated with 8 g of N as ammonium sulfate while the other half were not given additional nitrogen. All pots were treated with 4 g P$_2$O$_5$ as superphosphate and 4 g K$_2$O as potassium chloride. Three replicate pots were used per treatment.

To test the effect of sunlight on nitrogen fixation, a set of pots was covered with black cloth to prevent light from penetrating. Another set was covered with white cloth so that the light intensity was almost the same as that ordinarily present inside a greenhouse. Each piece of cloth had a hole in the center for seedling growth.

In the dark treatment no algal growth was observed, while in the light treatment prominent algal growth was observed. Soil samples were taken during the four growth stages of the rice plant; transplantation, maximum tillering, panicle initiation, and maturity, from two regions of the soil profile: 0–2 cm (upper layer) and 2–7 cm (lower layer).

The Maahas clay samples obtained from pots seeded with IR8 were examined for the presence of microflora at different growth stages. The dilution frequency method was used to estimate the algal content. KRATZ and MEYERS' medium (3) and Bristol's solution (4) were used to determine the amounts of total algae and nitrogen-fixing blue-green algae, respectively. The plate count method was used to estimate the number of nitrogen-fixing bacteria grown on the Azotobacter medium (4) and Clostridium medium (5). PARKER's method (6) was used to obtain anaerobiosis in jars in which the clostridium plates were incubated. All plates and test tubes containing the inoculum were incubated at 30°C.

Microorganisms, which grew on each medium were isolated and purified. Cultures of blue-green algae were purified with WIERINGA's method (7), and the genus was tentatively identified.

The test for nitrogen-fixing capacity in soils was carried out with isotope $^{15}$N$_2$. Surface-soil was removed from pots containing the different treatments about 2 months after transplanting. Ten grams of wet soil was placed in glass tubes (1.1 cm × 20 cm) to make a soil column of approximately 9 cm. Water was added to produce a 5-cm column above the soil surface. The tubes were placed in Mason jars which were then sealed, evacuated, and flushed with helium ten times. A gas phase containing oxygen (0.1 atm), nitrogen (0.2 atm of 37 atom percent excess $^{15}$N), and helium (0.7 atm) was introduced into the jars. The effect of light was determined by incubating one set in light and another in the dark for 30 days in a greenhouse. After the incubation period the total nitrogen content of the soil in the different tubes was determined by the Kjeldahl method, including nitrite and nitrate as recommended by BREMNER (8). Distillates were used for $^{15}$N analysis.

The total nitrogen in the soil was determined at the start and at the end of the experiment with the Kjeldahl method. The total nitrogen in the straw and in the grains was determined by the Kjeldahl method. Plant growth, i.e. the height of plants and the tiller number, was measured at transplantation, maximum tillering, panicle initiation, and maturity. The weights of straw and filled grains were determined after the rice plants had been harvested and oven-dried.
To determine the effect of soil submergence on nitrogen fixation, the moisture content of some soil samples was kept at field capacity; other samples were flooded with distilled water to a depth of 5 cm. Submerged soils were kept in an illuminated (3,000 lux) incubation room at 30°C for 2, 4, and 6 weeks. To examine the effect of nitrogen fertilizer on nitrogen fixation, soil samples in the glass tubes were mixed with ammonium sulfate, ammonium chloride, and urea at levels of 200 kg/ha N (80 ppm N) and 400 kg/ha N (160 ppm) before flooding. All treatments were replicated three times.

The soil columns were placed in Mason jars and sealed with a silastic sealant (Silastic RTV 731, Dow Corning Corp., Midland, Michigan). The jars were evacuated and flushed with helium 10 times and a gas mixture as previously described. For anaerobic nitrogen fixation, oxygen was not introduced. Instead, helium (0.8 atm) was added. Half of the samples in the Mason jars were covered with black cloth to test activities of the non-photosynthetic nitrogen-fixing bacteria. All samples were incubated for a month at 30°C in an illuminated incubation room. After incubation, the total nitrogen of the soil samples was analyzed by the Kjeldahl method. The distillates were used for 15N analysis. The isotope 15N was analyzed at the Institute of Physical and Chemical Research in Tokyo.

The acetylene-reduction method was used to estimate the amount of nitrogen fixation by N2-fixing microorganisms in paddy water. Water samples were taken from rice fields at the International Rice Research Institute without the application of nitrogen fertilizer every 2 weeks. Paddy water was collected in glass containers from several sites from paddies that had been planted with rice or were unplanted. After homogenization in a Waring Blendor, ten milliliters of the samples was placed in 50-ml Erlenmeyer flasks fitted with rubber needle-puncture stoppers. The atmosphere in each flask was replaced with an He-O2 (8 : 2) gas mixture and 0.05 atm of pure acetylene. After incubation at 30°C under light for 5 hr the amount of ethylene in the flask was analyzed by gas chromatography (9).

RESULTS AND DISCUSSION

More algal flora grew in the 0–2 cm surface layer of the soil than in the 2–7 cm layer. The algal population was higher in light treated samples than in dark treated ones during the rice-growing period. In the surface soil samples without nitrogen fertilizer in the light, the major algal flora were nitrogen-fixing blue-green algae. Nitrogen fertilizer seemed to enhance algal growth, but more blue-green algae generally were found in pots without nitrogen fertilizer. There were less nitrogen-fixing blue-green algae as compared to the total algae in soils to which ammonium fertilizer had been added. Nitrogen-fixation is known to be immediately inhibited when ammonium nitrogen is added to cultures of nitrogen-fixing organisms and ammonium nitrogen is known to be preferentially assimilated (10). The number of nitrogen-fixing bacteria neither changed significantly during plant growth nor among treatments. Most of the bacterial counts on the Azotobacter medium varied from 1 to 9×10⁴ per g of dried soil in the 0–2 cm layer, and from 0.5 to 4×10⁴ in the 2–7 cm layer. The number of bacteria in the Clostridium medium was 2 to 8×10⁵ per g of dried soil in both the 0–2 cm and 2–7 cm layers. The bacterial count in samples heated at 80°C for 20 min.
showed no significant difference from these values, suggesting that a large number of bacteria were in the resting stages, probably as spores. We also found, however, that many bacteria in each medium were probably not *Clostridium* or *Azotobacter*. These bacteria were counted and included in the bacterial count on each medium.

The tubes used in estimating the number of blue-green algae present in the soil were further cultured for purification and identification. Of 308 isolates, 267 belonged to the *Nostoc*, 38 to the *Anabaena*, 1 to the *Sligonema*, 1 to the *Tolyphorix* and 1 to the *Scytonea* genus. Some typical isolates were cultured and purified. *Azotobacter* and *Clostridium* isolates were also obtained.

The capacity for nitrogen fixation in each pot was examined two months after transplantation. Data suggest that a significantly higher rate of nitrogen was fixed during the light treatment as opposed to during dark treatment (Table 1). Microorganisms involved in nitrogen fixation were most likely photosynthetic microorganisms, since not much nitrogen was observed in samples incubated in the dark. But, values may not indicate the true N\(_2\)-fixing activities of soils in the pots, since the soil samples had been incubated for \(^{15}\)N\(_2\)-fixation for a month. It would not be unexpected that during that period of the assay under the light some N\(_2\)-fixing blue-green algae grew in the tubes. The addition of ammonium fertilizer greatly depressed the amount of molecular nitrogen fixed in the soils.

The effects of prominent algal growth on Maahas clay soil exposed to the sunlight for rice plant growth and yield was investigated. Both light and dark treatments

<table>
<thead>
<tr>
<th>Soil treatment</th>
<th>Rice varieties</th>
<th>In light</th>
<th>In the dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKL</td>
<td>No plant</td>
<td>0.395</td>
<td>0.038</td>
</tr>
<tr>
<td>PKD</td>
<td>No plant</td>
<td>0.493</td>
<td>0.020</td>
</tr>
<tr>
<td>PKL</td>
<td>IR8</td>
<td>0.459</td>
<td>0.026</td>
</tr>
<tr>
<td>PKD</td>
<td>IR8</td>
<td>0.514</td>
<td>0.006</td>
</tr>
<tr>
<td>PKL</td>
<td>Peta</td>
<td>0.517</td>
<td>0.047</td>
</tr>
<tr>
<td>PKD</td>
<td>Peta</td>
<td>0.410</td>
<td>0.027</td>
</tr>
<tr>
<td>NPKL</td>
<td>No plant</td>
<td>0.043</td>
<td>0.048</td>
</tr>
<tr>
<td>NPKD</td>
<td>No plant</td>
<td>0.020</td>
<td>0.000</td>
</tr>
<tr>
<td>NPKL</td>
<td>IR8</td>
<td>0.075</td>
<td>0.017</td>
</tr>
<tr>
<td>NPKD</td>
<td>IR8</td>
<td>0.070</td>
<td>0.024</td>
</tr>
<tr>
<td>NPKL</td>
<td>Peta</td>
<td>0.151</td>
<td>0.031</td>
</tr>
<tr>
<td>NPKD</td>
<td>Peta</td>
<td>0.089</td>
<td>0.000</td>
</tr>
</tbody>
</table>

\(^{15}\)N P K: Nitrogen, phosphorus, and potassium fertilizer. L: Light, D: Dark.
showed no significant difference as measured by the weights of straw, filled grains, and by nitrogen uptake in the rice during the two continuous croppings. Measurement of the total nitrogen content of the soil after the second crop showed that significantly more nitrogen occurred in surface soils kept in the light than in soils kept in the dark.

The non-significant effect of nitrogen-fixing microorganisms on the growth of the rice plant in spite of their large populations and high activity in the light suggests that fixed nitrogen was immobilized in the soil, perhaps as microbial bodies, in the organic fraction of the soil.

In the experiments on the effect of submerging soil, we found that nitrogen fixation occurred to a greater extent in submerged soils than it did in soils kept under upland conditions; especially when soils were incubated in the light under aerobic conditions (Table 2). There was no significant nitrogen fixation in the dark, regardless of soil pretreatment. Under submerged conditions, maximum nitrogen fixation was obtained shortly after the soil had been submerged under the light. Some nitrogen fixation could have been due to algae which grew on the soil surface and on flood water during incubation in the test for nitrogen fixation. A fairly large amount of nitrogen was also fixed even under anaerobic conditions in the submerged soil. This indicates that the role of photosynthetic bacteria is that of the major microflora responsible for nitrogen fixation under anaerobic conditions. Interestingly KOBAYASHI et al. (11) reported that a large number of anaerobic photosynthetic bacteria are found in Philippine soils. Although some nitrogen-fixing blue-green algae can grow and fix nitrogen even in the dark to a limited extent (12-14) their ecological significance in nitrogen fixation in the soil is doubtful.

For the period of presubmergence, prominent algal growth was observed on the surface of the flood water as well as on the soil surface. Less nitrogen fixation occurred when the soil was presubmerged for 2 to 4 weeks. Soil incubated for 6 weeks showed the least nitrogen increase under aerobic conditions. Apparently the younger the algal bodies, the more active the nitrogen fixation. STEWART (2), in referring to the results obtained by DUGDALE (15), indicated that the total nitrogen fixed in a

Table 2. The effect of soil submergence on nitrogen fixation in Maahas clay soil.

<table>
<thead>
<tr>
<th>Soil treatment</th>
<th>Conditions for the nitrogen fixation assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light-aerobic</td>
</tr>
<tr>
<td>Field capacity</td>
<td>0.126</td>
</tr>
<tr>
<td>Submergence just before assay</td>
<td>1.379</td>
</tr>
<tr>
<td>2-week submergence</td>
<td>0.617</td>
</tr>
<tr>
<td>4-week submergence</td>
<td>0.717</td>
</tr>
<tr>
<td>6-week submergence</td>
<td>0.349</td>
</tr>
</tbody>
</table>
lake was at the maximum when _Anabaena_ were increasing their population and that little nitrogen fixation occurred when algae were most abundant. **Calder (16)** showed that a considerable amount of fixed nitrogen resulted only when soil preparations were alternately flooded with 2 cm of water then allowed to dry out. He also observed that a luxuriant growth of blue-green algae occurred either as a gelatinous sheet on the soil surface or as lobed floating masses on the water layer of the preparations. These observations suggest that nitrogen fixation in the intact algal cell is closely associated with the growth of algae and is probably introduced by such practices as the repetition of soil flooding and drying.

We found no significant amount of nitrogen fixation in any sample incubated in the dark. Under our experimental conditions, non-photosynthetic nitrogen-fixing bacteria, such as _Azotobacter_ and _Clostridium_, were not active nitrogen-fixers in the soil. This does not necessarily mean that heterotrophic nitrogen-fixing microflora are unimportant in nitrogen fixation in paddy soils. They can be active agents in fixing nitrogen gas when organic materials are incorporated into the soil under waterlogged conditions (17). The rhizosphere of the rice plant is also the site where the plant excretes organic matter continuously.

The amount of fixed nitrogen obtained in our study may not be completely accounted for by photosynthetic nitrogen fixation. **Bellve (18)** indicated that more nitrogen fixation occurs in sand with organic matter than in sand alone in the light, suggesting the possibility of some kind of association between photosynthetic microorganisms and non-photosynthetic heterotrophic bacteria.

We also examined the effect of nitrogen fertilizers on nitrogen fixation. The presence of ammonium nitrogen fertilizer affected the fixation of molecular nitrogen by soil microorganisms both in the dark and in the light. At 80 ppm N the atom percent excess of $^{15}$N fixed under light for 30 days was 0.042, 0.003, or 0.024 in soils treated with ammonium sulfate, ammonium chloride, or urea, respectively, while the value of the control soil was 0.150 atom percent excess of $^{15}$N. Nitrogen fixation was almost completely inhibited when nitrogen was applied to the soil at 160 ppm N which is equivalent to 400 kg/ha N.

Nitrogen-fixing microorganisms, with one exception, use ammonium and nitrate nitrogen. Fixation is partially inhibited by low concentrations of ammonium-nitrogen but high concentrations are necessary for complete inhibition (2). It is not known whether the function of enzymes in nitrogen fixation or formation of the enzymes themselves is inhibited by a high concentration of ammonium nitrogen.

One disadvantage of the isotope $^{15}$N method, as pointed out by Dawson (19), is that the experiments can be conducted only on a very small scale and, therefore, is hardly representative of a rice paddy. In addition, because of its low sensitivity, samples must be maintained under experimental conditions for many days before the level of $^{15}$N enrichment becomes high enough to be measured by a mass spectrometer. Changes occur during the incubation period and the samples are not the same as they were at the start. It should be emphasized that the experimental results obtained would not necessarily be applicable to field conditions. The acetylene reduction method would be appropriate for studying nitrogen fixation in paddy fields (9).

The estimated amounts of nitrogen fixation in paddy water during a rice-growing period, using the acetylene reduction method, were 3.2 kg N/ha in fields with growing
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rice crops and 10.9 kg N/ha in an unplanted flooded field. The amount of nitrogen fixation by microorganisms in paddy water did not account for the amount of nitrogen uptake by rice.

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