ATMOSPHERIC NITROGEN FIXATION BY PHOTOSYNTHETIC MICROORGANISMS IN A SUBMERGED PHILIPPINE SOIL

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ABSTRACT

Photosynthetic nitrogen-fixing microorganisms help maintain the nitrogen level of soil in rice paddies when environmental factors favors the growth of the 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 the soil have been submerged under light. The longer the submergence of the soil, the less nitrogen microorganisms in the soil was fixed. In a greenhouse experiment, the fixed nitrogen appeared to be not immediately available to rice plant. The amount of nitrogen that can be fixed in the field by nitrogen-fixing microorganisms in paddy water was estimated with the acetylene reduction method during the rice-growing period. The amount of nitrogen fixation by these microorganisms is not adequate to account for the amount of nitrogen uptake by rice during the rice-growing period.

In many parts of Asia, fertilizers are not used. Rice production depends on the natural fertility of the soil. The continuing supply of nitrogen over the years despite removal of the element by the rice crop 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 was 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 could be explained only by microbial nitrogen fixation in the soil, since the possible sources of the nitrogen, such as rain or irrigation water and rice plant residues, are not significant enough to bring about the nitrogen balance.

Various kinds of microorganisms fix atmospheric nitrogen, but it is not yet known which of these actually take part in nitrogen fixation in paddy soils. This paper reports on the 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 minutes, washed with tap water several times, and placed on a nylon mesh in a nutrient solution. After 15 days the seedlings 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 the 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 the greenhouse. Each piece of cloth had a hole in the center to allow seedling to grow through.

In the dark treatment no algal growth was observed, while in the light treatment prominent algal growth was observed. Soil samples were taken during four growth stages of the rice plant: transplanting, 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 the pots seeded with IR8 were examined for the presence of microflora at different stages of plant growth. The dilution frequency method was used to estimate algal content. Kratz and Mayers 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 for estimating the number of nitrogen-fixing bacteria grown on the Azotobacter medium (4) and Clostridium medium (5). Parker's method (6) was used for attaining anaerobiosis in the jars where the clostridium plates were incubated. All the plates and test tubes containing the inoculum were incubated at 30\(^\circ\)C.
The microorganisms grown on each medium were isolated and purified. The cultures of blue-green algae were purified by Wieringa's method (7), and their genus was tentatively identified.

The test for nitrogen-fixing capacity in the soils were carried out by using the isotope $^{15}\text{N}_2$. The surface-soil was removed from the pots containing the different treatments about 2 months after transplanting. Ten grams of the wet soils were placed in glass tubes (1.1 cm x 20 cm) to give a soil column of approximately 9 cm. Water was added to give 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 $^{15}\text{N}$, 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 greenhouse. After the incubation period the total nitrogen content of the soil in the different tubes was determined by the Kjeldahl method to include nitrite and nitrate as recommended by Bremner (8). The distillates were used for the $^{15}\text{N}$ analysis.

The total nitrogen in the soil was determined at the start and at the end of the experiment using 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 tiller number, was measured and transplanting, 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. The submerged soil were kept in an illuminated (3000 lux) incubation room at $30^\circ$ C for 2, 4, and 6 weeks. To examine the effect of nitrogen fertilizer on the nitrogen fixation the 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 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 the 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 the activities of non-photosynthetic nitrogen-fixing bacteria. All the samples were incubated for a month at 30°C in the illuminated incubation room. After incubation, the total nitrogen of the soil sample was analyzed by the Kjeldahl method. The distillates were used for the \(^{15}\text{N}\) analysis. The isotope \(^{15}\text{N}\) 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 the \(N_2\)-fixing microorganisms in paddy water. The water samples were taken from rice fields of the International Rice Research Institute without application of nitrogen fertilizer every 2 weeks. The paddy water was collected in glass containers from several sites from paddies that were planted with rice and unplanted. Ten milliliters of the samples after being homogenized in a Waring Blender were placed in 50-ml Erlenmeyer flasks fitted with rubber needle-puncture stoppers. The atmosphere in each flask was replaced with He-O\(_2\) (8:2) gas mixture and 0.05 atm of pure acetylene. After incubation of samples at 30°C under light for 5 hours 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 the light treatment than in the dark treatment 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 the pots without nitrogen fertilizer. There were less nitrogen-fixing blue-green algae compared to the total algae in the soils to which ammonium fertilizer had been added. It has been
known that nitrogen-fixation is immediately inhibited when ammonium nitrogen is added to cultures of nitrogen-fixing organisms and that the ammonium nitrogen is preferentially assimilated (10). The number of nitrogen-fixing bacteria did not change significantly during plant growth and among treatments. Most of the bacterial counts on the Azotobacter medium varied from 1 to $9 \times 10^5$ per g dried soil in the 0-2 cm layer, and from 0.5 to $4 \times 10^5$ in the 2-7 cm layer. The number of bacteria in the Clostridium medium was 2 to $8 \times 10^5$ per g dried soil in both the 0-2 cm and 2-7 cm layers. The bacterial count in the samples heated at $80^\circ$C for 20 min. did not show any significant difference from these values, suggesting that a large number of the 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 cultured further for purification and identification. Of 308 isolates, 267 belonged to the Nostoc, 38 to the Anabaena, 1 to the Stigonema, 1 to the Tolypothrix and 1 to the Scytomena 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 transplanting. The data suggest that a significantly higher rate of nitrogen was fixed with the light treatment than with the dark treatment (Table 1). The microorganisms involved in the nitrogen fixation were most likely photosynthetic microorganisms since not much nitrogen was observed in the samples incubated in the dark. But, the values may not indicate true $N_2$-fixing activities of the soils in pots since the soil samples have 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 the Maahas clay soil exposed to the sunlight on rice plant growth and yield was investigated. Both light and dark treatments showed no significant difference as measured by weight of straw, filled grains, and nitrogen uptake by the
Table 1. Nitrogen fixation of Maahas clay soil under various soil treatment.

<table>
<thead>
<tr>
<th>Soil treatment *</th>
<th>Rice varieties</th>
<th>Conditions for assay of nitrogen fixation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>In light</td>
</tr>
<tr>
<td>PK L</td>
<td>No plant</td>
<td>0.395</td>
</tr>
<tr>
<td>PK D</td>
<td>No plant</td>
<td>0.493</td>
</tr>
<tr>
<td>PK L</td>
<td>IR8</td>
<td>0.459</td>
</tr>
<tr>
<td>PK D</td>
<td>IR8</td>
<td>0.514</td>
</tr>
<tr>
<td>PK L</td>
<td>Peta</td>
<td>0.517</td>
</tr>
<tr>
<td>PK D</td>
<td>Peta</td>
<td>0.410</td>
</tr>
<tr>
<td>NP K L</td>
<td>No plant</td>
<td>0.043</td>
</tr>
<tr>
<td>NP K D</td>
<td>No plant</td>
<td>0.020</td>
</tr>
<tr>
<td>NP K L</td>
<td>IR8</td>
<td>0.075</td>
</tr>
<tr>
<td>NP K D</td>
<td>IR8</td>
<td>0.070</td>
</tr>
<tr>
<td>NP K L</td>
<td>Peta</td>
<td>0.151</td>
</tr>
<tr>
<td>NP K D</td>
<td>Peta</td>
<td>0.089</td>
</tr>
</tbody>
</table>

* NPK: Nitrogen, phosphorus and potassium fertilizer. L: Light, D: Dark

rice during the two continuous croppings. Measurement of the total nitrogen content of the soil after the second crop showed that a significantly more nitrogen occurred in the surface soils kept in the light than in the 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 population and high activity in the light suggests that the 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 the nitrogen fixation occurred to a greater extent in the submerged
soils than in the soils kept under upland conditions, especially when the soils were incubated in the light under aerobic conditions (Table 2). There was no significant nitrogen fixation in the dark regardless of the soil pretreatment. Under submerged conditions, maximum nitrogen fixation was obtained in the soil shortly after the soil had been submerged under light. Some of the nitrogen fixation could have been due to the algae which grow on the soil surface and on flood water during the incubation in the test for nitrogen fixation. A fairly large amount of nitrogen was also fixed even under the anaerobic conditions of the submerged soil. This points out the role of the photosynthetic bacteria as the major microflora responsible for nitrogen fixation under anaerobic conditions. It is interesting to note that Kobayashi et al. (11) reported that a large number of anaerobic photosynthetic bacteria are found in the Philippine soils. Although some nitrogen-fixing blue-green algae can grow and fix nitrogen even in the dark to a limited extent (12, 13, 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. The 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), referring to the results obtained by Dugdale (15), showed that the total nitrogen fixed in a lake was at the maximum when Anabaena were increasing their population and little nitrogen fixation occurred when algae were most abundant. Calder (16) showed that a considerable amount of fixed nitrogen resulted only when the soil preparations were alternately flooded with 2 cm of water and 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 intact algal cell is closely associated with the growth of algae probably introduced by such a practice as 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 the 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 all accounted for by photosynthetic nitrogen fixation. Bjalve (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 50 ppm N the atom percent excess $^{15}$N fixed under light for 30 days were 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 $^{15}$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.

All nitrogen-fixing microorganisms, with one exception, use ammonium and nitrate nitrogen. The fixation is partially inhibited by low concentrations of ammonium-nitrogen but high concentrations are necessary for complete inhibition (2). It is known whether the function of the enzymes in nitrogen fixation or in the formation of the enzymes themselves is inhibited by the high concentration of ammonium nitrogen.

One of the disadvantages 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 are hardly representative of a rice paddy.
Table 2. The effect of soil submergence on nitrogen fixation of Maahas clay soil.

<table>
<thead>
<tr>
<th>Soil treatment</th>
<th>Conditions for assay of nitrogen fixation</th>
<th>Atom % excess $^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light-aerobic</td>
<td>Light-anaerobic</td>
</tr>
<tr>
<td>Field capacity</td>
<td>0.126</td>
<td>0.124</td>
</tr>
<tr>
<td>Submergence just</td>
<td>1.379</td>
<td>0.194</td>
</tr>
<tr>
<td>before assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-week submergence</td>
<td>0.617</td>
<td>0.419</td>
</tr>
<tr>
<td>4-week submergence</td>
<td>0.717</td>
<td>0.346</td>
</tr>
<tr>
<td>6-week submergence</td>
<td>0.349</td>
<td>0.063</td>
</tr>
</tbody>
</table>

In addition, because of its low sensitivity, the 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 in 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 in the experiment would not necessarily be applicable to field conditions. The acetylene reduction method would be appropriate for the study of nitrogen fixation in paddy field (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 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.
REFERENCES


