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Factors affecting N_2 fixation in the rice rhizosphere

Y. R. DOMMERGUES and G. RINAUDO

A rice rhizosphere is colonized by many strains of N_2-fixing bacteria with different physiological features. It also harbors, in addition to N_2 fixers, other microorganisms that behave as synergists or antagonists.

N_2 fixation in the rhizosphere depends primarily on the plant and the N_2-fixing bacteria. Because the rhizosphere is a component of the whole soil-plant-atmosphere system, the factors affecting soil and atmosphere should affect the growth and activity of the rhizospheric N_2-fixers. This paper reviews studies of the influence of the following edaphic and climatic factors: N_2 content in soil, carbon and energy supply, light, temperature and air humidity, soil oxygen and water regime, inorganic soil N, pH and soil content in elements other than N, and biological factors. The sensitivity of the rice N_2-fixing system to environmental factors explains the occurrence of large variations with time and space. Different possibilities of increasing N_2 fixation in the rice rhizosphere are discussed.

HETEROTROPHIC N_2-FIXING BACTERIA DEVELOP in association with the roots of higher plants. This association, termed *associative symbiosis* by Burns and Hardy (1975), is thought to be a possible source of new N for the ecosystem, especially in tropical conditions. This review deals with associative symbiosis in rice.

Although blue-green algae (either free-living or associated symbiotically with azolla) are thought to be the main agents of N_2-fixation in paddy fields, heterotrophic N_2-fixing bacteria in the rice rhizosphere could also contribute to the N input when no limiting factor impedes their activity.

We attempt to describe the rice N_2-fixing system, and analyze the intrinsic and extrinsic factors that affect the functioning of the rice associative symbiosis. We also explore possibilities for increasing N_2-fixation.

THE RICE NITROGEN-FIXING SYSTEM

The nitrogen-fixing bacteria of the rice rhizosphere

Miscellaneous heterotrophic bacteria are known to fix N_2 (Postgate 1974, Knowles 1977). Some of them, especially *Azotobacter* sp. (Rouquerol 1964, Rinaudo 1974), *Beijerinckia* sp., *Clostridium* sp. (Bhattacharya 1958, Ishizawa

Office de la Recherche Scientifique et Technique Outre-Mer, Boite Postale No. 1386, Dakar, Senegal.

et al 1970, Sulaiman 1971, Matsuguchi et al 1975, Ishizawa et al 1975, IRRI 1976), *Desulfovibrio*, *Desulfotomaculum* (V. Jacq, pers. comm.), and methane oxidizing bacteria (Bont et al 1978) have been frequently found in paddy soils. *Derxia* was reported to be selectively stimulated by flooding (Döbereiner and Campello 1971).

Until now, however, little is known about the composition of N_2-fixing micropopulations in the rice rhizosphere. In the tropics, rice roots are colonized chiefly by *Spirillum* sp. (Laksmi Kumari et al 1976, Nayak and Rajaramamohan Rao 1977, Silva and Döbereiner 1978, Rinaudo, unpubl. results), *Clostridium* sp., and *Enterobacter* sp. (IRRI 1976; Hamad-Fares 1976, Thesis, Université of Nancy, France; Watanabe et al 1977; Rinaudo, unpubl.). *Azotobacter* and *Beijerinckia* occur only sporadically. Many undetermined species that would be worth studying taxonomically have also been isolated from rice roots.

The five main physiological features that are assumed to be essential for rhizosphere N_2 fixation are:

1. protection against oxygen,
2. competitiveness, or ability to colonize rice roots and use the substrates that are available in the different regions of the rhizosphere,
3. N_2-fixing efficiency,
4. ability to fix N_2 in the presence of combined N, and
5. ability to excrete N_2 fixed as ammonium that can be directly absorbed by the plant.

Preliminary studies of the physiological features of N_2-fixers from the rice rhizosphere indicate that large differences exist among the N_2-fixing strains that have been isolated from the rice rhizosphere. The rice rhizosphere appears to be inhabited mainly by microaerophilic strains; obligate or facultative anaerobes are present but are usually less abundant (Trolldenier 1977, Balandreau and Knowles 1978). This high proportion of microaerophilic strains is probably the result of the oxidative character of the rhizosphere of rice, especially at the seedling stage (Yoshida 1975). Nutritional requirements are also diversified but strains with some specific requirements seem more favored than other strains. Thus, Watanabe and Cholitkul (1979) found that glucose-utilizing N_2-fixers were up to 100 times more numerous than malate-utilizing N_2-fixers in the rhizosphere of IR26 and IR36 rices growing in the Philippine soil. But such results should be generalized with caution. Consequences of the diversity in the qualitative and quantitative composition of N_2-fixing population in the rhizosphere of rice are discussed later.

Colonization of rice rhizosphere by nitrogen-fixing bacteria

The rhizosphere is a peculiar microhabitat made up of the soil-root interface. Typically it can be divided into three areas.

1. The rhizosphere *sensu stricto* (or outer rhizosphere) comprising the region of the soil immediately surrounding the plant roots and the microbial populations inhabiting therein.

2. The rhizoplane (or root surface) formed by the root surface and the microorganisms living on it.
3. The endorhizosphere (or inner rhizosphere or histosphere) formed by the root cortical tissue invaded and colonized by heterotrophic microorganisms.

"To some degree these three areas should be regarded as a single microbial milieu with no sharp demarcations between them" (Old and Nicolson 1975).

The location of N_2-fixers in different areas of the rhizosphere has been indirectly explored through washing techniques similar to that proposed by Harley and Waid (1955).

Watanabe and Cholitkul (1979) defined outer rhizosphere bacteria as those that live in the soil attached to the roots, rhizoplane bacteria as those that are detached from the roots by vigorous shaking, and endorhizosphere bacteria as those counted on root samples from which rhizoplane bacteria have been detached by shaking. Through the procedure similar to that of Okon et al (1977), Fetiarison and Diem (unpubl.) considered as endorhizosphere bacteria those that remained alive after the root had been surface-sterilized by chloramine T (Gautheret 1959). For these last authors rhizoplane bacteria were those that remained attached to the roots after 20 washings.

Differences in the procedures used for separating the microbial populations in the different areas of the rhizosphere explain the large differences in the numbers that have been reported. Enumeration methods can also be responsible for the discrepancies. The best method appears to be the MPN method proposed by the IRRI researchers. It uses simultaneously glucose and malate (Döbereiner's) semisolid media supplemented with yeast extract, and positive tubes are detected by the conventional acetylene assay. Results are usually expressed in number per gram of fresh root. In the endorhizosphere of two rice varieties (IR26 and IR36), Watanabe and Cholitkul (1979) detected about 10^7 to 10^8 N_2-fixers (internal bacteria) with glucose and 10^5 to 10^6 N_2-fixers with malate. According to Fetiarison and Diem (unpubl.) rhizoplane N_2-fixers ranged from 10^4 to 10^5 and endorhizosphere bacteria were not more than 10^3. Low numbers of rhizosphere N_2-fixers could partially be explained by the effect of the antiseptic used for sterilizing root surfaces.

As with rhizosphere N_2-fixers associated with maize (Okon et al 1977), rhizosphere N_2-fixers associated with rice are more abundant in the rhizoplane than in the endorhizosphere but that is not always the case (I. Watanabe, IRRI, pers. comm.). Endorhizosphere bacteria can be easily detected after the roots are vigorously shaken and washed, the technique used by Watanabe and Cholitkul (1979), or even surface-sterilized, a technique used by Fetiarison and Diem (unpubl.), thus indicating a rather close association between rice roots and N_2-fixing bacteria.

We note that Watanabe and Cholitkul (1979) at this symposium report populations as high as 10^7 to 10^8 N_2-fixers/g (fresh weight) root. But these figures do not imply that the root surface is covered by a sheath of bacteria.

Recently, Rovira et al (1974) who studied the rhizosphere of four plants from permanent grasslands, indicated that the bacterial cover of the root surface did not exceed 4–10%. Since such a pattern of colonization may exist in flooded rice, it should be studied more extensively in the future.

Other rhizosphere microorganisms

N_2-fixers are not the sole inhabitants of the rice rhizosphere; they thrive in the presence of other microorganisms, which behave as antagonists (especially by competing for the energy provided by the root system) or as synergists. Until now, this topic is poorly documented. According to data reported by Watanabe and Cholitkul (1979), N_2-fixers make up 80% of the total heterotrophic bacterial population in the histosphere of IR26 rice. Similar results were reported by Trolldenier (1977), who found that N_2-fixers represented 50% and 23% of the total heterotrophic bacterial population in the rhizospheres of IR20 and IR8, respectively. Those data suggest that other bacteria compete for available energy sources in the rhizosphere less intensely than expected. But other microorganisms such as actinomycetes as suggested by Diem et al (1977), or protozoa as demonstrated by Danso et al (1975) for *Rhizobium*, can be involved in antagonistic processes. Conversely, rhizosphere N_2-fixers may be associated with other N_2-fixers or nonfixing microorganisms, which could increase their efficiency. Such associations have been reported (Rubenchik 1963, Dommergues and Mutaftschiev 1965, Kobayashi et al 1965, Florenzano et al 1968, Line and Loutit 1973), but their significance in the rice rhizosphere is still a subject for speculation.

INTRINSIC FACTORS AFFECTING NITROGEN FIXATION IN THE RICE RHIZOSPHERE

Nitrogen fixation in the rice rhizosphere depends primarily upon the plant and the rhizospheric N_2-fixing bacteria.

Influence of the plant

Because the response of legumes to association with *Rhizobium* is variable, the response of Graminaceae, especially rice, to their association with rhizospheric N_2-fixing bacteria could be expected to be similarly variable. Actually several reports confirm that the plant determines the N_2-fixing potential of the association.

Intravarietal variability, besides intervarietal variability, occurred. By screening 17-day-old seedlings from a collection of 30 induced mutants of the variety Cigalon (obtained by gamma irradiation) growing in Camargue alluvial soil, Marie and Rinaudo (Orstom, unpubl.) found that rhizospheric acetylene reducing activity (ARA) ranged from 861 $\pm$ 456 to 7368 $\pm$ 1971 nmol C_2H_4/g root (dry weight) per hour; the same ARA expressed in nmol of C_2H_4 per seedling per hour ranged from 27.6 $\pm$ 11.4 to 369 $\pm$ 80.4. Another collection

of 19 rice mutants (Césariot) grown in the same conditions exhibited ARA ranging from 500 ± 310 to 5320 ± 5010 nmol C_2H_4/g root (dry weight) per hour. Such data cannot be extrapolated to the field without utmost caution, but they clearly indicate that N_2 fixation in the rice rhizosphere could be increased by plant breeding. The large variations in ARA that occurred in these screenings were attributed mainly to variations in root colonization by N_2-fixers (Rinaudo, unpubl.).

Influence of qualitative and quantitative composition of rhizosphere nitrogen-fixing populations

It can reasonably be assumed that the qualitative and quantitative compositions of the N_2-fixing populations of the rhizosphere govern, at least partially, the level of N_2-fixing activity. To confirm this assumption would require experiments based on the manipulation of gnotobiotic systems composed of sterile rice plants inoculated with one or several defined N_2-fixing strains. Unfortunately, until now, attempts to create operative gnotobiotic systems have been only partially successful and the results inconsistent for at least two reasons:

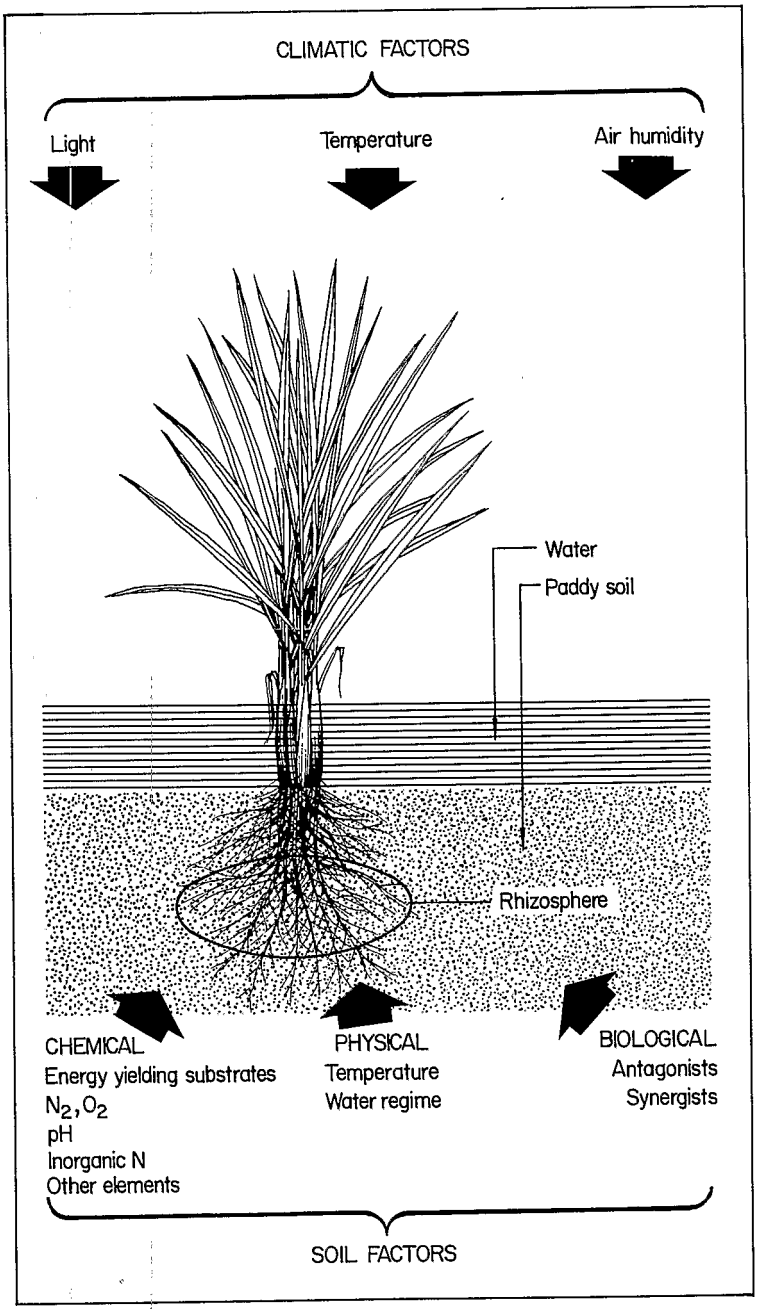
1. use of excessive oxygen in the experimental setups; and
2. inability of N_2-fixers to use directly the substances provided by the roots.

The last reason is strengthened by the finding of Diem and Rinaudo (unpubl.) that rice roots inoculated by a mixture of four N_2-fixing bacteria and two pectinolytic bacteria exhibited an ARA three times that which occurred when inoculation was performed with N_2-fixing bacteria alone. Rinaudo (unpubl.) found that inoculating nonsterile soils with root segments from an active N_2-fixing rice rhizosphere, generally resulted in a significant increase in ARA, and a decrease in the variability of the results. Whether such increase in ARA is caused by the improved root colonization or by the establishment of more efficient N_2-fixing strains, or by both, is not known.

EXTRINSIC FACTORS AFFECTING RHIZOSPHERIC NITROGEN FIXATION

Ecological aspects of N_2 fixation by rhizospheric microorganisms have been reviewed (Knowles 1977, Diem and Dommergues 1979). This paper concentrates on the rice rhizosphere. Microbial populations associated with plant roots must be viewed as a component of the whole soil-plant-atmosphere system (Dommergues 1978). A corollary of this concept is that factors affecting the different components of the system, namely plant and soil, should affect the growth and activity of rhizospheric microorganisms. Actually, climatic factors (especially light intensity and temperature) and soil factors (especially oxygen tension and water regime, chemical characteristics, and biological characteristics), act directly or indirectly through the plant, upon rhizospheric microorganisms, which include N_2-fixing bacteria (Fig. 1).

The use of acetylene assay as a sensitive, rapid, and simple means to measure N_2 fixation has contributed greatly to the progress of ecological aspects of



1. Extrinsic factors affecting N_2 fixation in the rhizosphere of rice.

N_2 fixation in the rhizosphere in recent years. However, one should be aware that using the C_2H_2 assay to evaluate ARA in complex systems, such as the rice rhizosphere, raises some difficult problems in the field and even in laboratory studies. Even relatively short periods of experimental incubation with C_2H_2, such as those adopted by Rinaudo et al (1978), which are about 4–6 hours, are subject to criticism since they may lead to a multifold enhancement of ARA, as demonstrated by David and Fay (1977). Therefore, most of the results presented here should be interpreted as qualitative and relative rather than as quantitative and absolute. Another difficulty that is more arduous to overcome in the field than in the laboratory, results from the interference of blue-green algae, which in nature fix N_2 at the same time as rhizospheric N_2-fixing bacteria. Fortunately, the IRRI researchers recently found that blue-green algae could be eliminated during the ARA measurement by removing the floodwater and a small layer of the surface soil and by subsequently adding distilled water (Watanabe et al 1978). Another procedure was proposed by Boddey et al (1978) who inhibited ARA caused by algae by using a herbicide, propanil.

Presence of nitrogen in the rice rhizosphere

Nitrogen fixation in the rice rhizosphere is contingent on the presence of N_2 in that microhabitat. Because the rice plant and other aquatic vascular plants have an air-transporting system through which air diffuses to the root, one can assume that N_2 diffuses from the atmosphere through the rice plant into the rhizosphere. Yoshida et al (1975) calculated the amount of N_2 in planted soil at the tillering stage to be equivalent to about 160 kg/ha, and that in the field at transplanting about 18.5 kg/ha. These studies indicate that the N_2 content of the soil rhizosphere should not limit N_2 fixation.

Carbon and energy substrates; light

Heterotrophic N_2 fixation is frequently limited by shortages of carbon and energy (Knowles 1977). In the rhizosphere of rice, as in any other rhizosphere, energy-yielding compounds come from three sources:

1. *Root exudates*, i.e. materials released from healthy, intact roots, which have been grouped according to their mobility through the soil as diffusable-volatile, diffusable-water soluble, and nondiffusable compounds. The latter category comprises the mucigel (Rovira and Davey 1971).
2. *Root lysates*, i.e. compounds resulting from the autolysis of root hairs and sloughed root cap cells as well as epidermal and cortical cells of still functional roots (Martin 1977).
3. *Root litter*, i.e. moribund or dead, decaying roots deposited in the soil (process termed rhizo-deposition by Waid 1974) as the roots and rootlets are renewed (process termed root turnover), without noticeable variation

in the total biomass in natural prairie formations (Warembourg and Morrale 1978).

When plants are still young, root exudates and root lysates are presumably the major sources of carbon and energy substrates for rhizospheric microorganisms. Since root exudation depends in part upon light intensity, it is not surprising that shading rice seedlings dramatically decreases rhizospheric ARA (Dommergues et al 1972). When a plant is aging, root litter possibly provides N_2-fixers with an extra supply of energy-yielding compounds. Root residues are rich in carbohydrates with high molecular weight, such as cellulose, which cannot be used directly by the N_2-fixers. These residues must be transformed to simpler intermediates by associated rhizospheric microorganisms to serve as substrates for N_2-fixers.

Knowles (1977) discussed the problem of the efficiency of N_2 fixation, which can be expressed as mg N_2 fixed per gram of carbohydrate consumed or as moles of N_2 fixed per mole of carbohydrate consumed. This efficiency is generally considered low. To fix 1 mol of N_2 anaerobic heterotrophic N_2-fixers require 8–10 mol of glucose, aerobic heterotrophic N_2-fixers 3–4 mol, and *Rhizobium* about 1 mol. Therefore, fixation by heterotrophs could not be great. However, there is evidence that efficiencies in the rhizosphere, as in other natural habitats, may be greater than expected. Thus, Knowles (1977) indicated that growth factors, soil extract, colloidal clay, anaerobiosis, and low concentrations of energy-yielding substrates, may increase the efficiency of N_2 fixation by 2 to 3 times, compared with values obtained on the basis of pure culture studies.

Whatever the respective contributions of root exudates, root lysates, and root litter may be, the resulting pool of energy-yielding substrates is shared both by rhizosphere N_2-fixers and the bulk of other rhizospheric microorganisms. But because N_2-fixers make up a rather large percentage of the total heterotrophic bacterial population in the rice rhizosphere, a significant part of the energy flowing out of the rhizosphere *sensu lato*, can be assumed to be used for N_2 fixation.

Actual N_2 fixation can be predicted by considering the amount of energy-yielding substrates available for N_2-fixers in the rhizosphere, and the efficiency of N_2 fixation, as defined above. Unfortunately the amount of available substrates in the rhizosphere, except that of exudates (which are only a part of the total energy supply for rhizosphere N_2-fixers) is poorly documented. At IRRI, the amount of exudates from IR8 and IR22 was evaluated by the isotope technique (application of $^{14}CO_2$ for 4 hours to rice placed in a growth chamber). At harvest stage, 3.2 and 6.7% of the total ^{14}C assimilated was exuded from IR22 and IR8 roots and remained in the soil and water (IRRI 1973). These data indicate that exudation by itself is not an important source of energy for rhizospheric N_2-fixers.

Air and soil temperatures and air-water deficit

Relatively low temperatures limit:

- photosynthesis, translocation, and exudation, thereby reducing the supply

of energy-yielding compounds required by N_2-fixers, and
- the activity of N_2-fixers, some of which, such as *Spirillum* sp., are sensitive to low temperatures.

According to Day and Döbereiner (1976), the ARA of *Azospirillum lipoferum* is maximal between 32 and 40°C with a pronounced drop above 40°C. The influence of air-water deficit[1] upon rhizospheric ARA of 18-day-old rice seedlings grown in a phytotron was investigated by Hamad-Fares (1976, thesis, University of Nancy, France) who found a marked optimum for a low water deficit (about 5 mm Hg). In paddy fields high water deficit is unlikely as long as soil is flooded, but it may be important during drought.

Soil oxygen and water regime

Nitrogenase is susceptible to inhibition and damage by oxygen. But mechanisms to control access of oxygen and protect the N_2ase have evolved in N_2-fixers. Studies on such mechanisms have been reviewed recently by Postgate (1974) and Yates (1977).

Some heterotrophic bacteria, such as *Azotobacter*, benefit from sophisticated protection mechanisms (such as respiratory or conformational protection). In nature, however, N_2 fixation by heterotrophic N_2-fixers is most often affected by oxygen, indicating that few actively fixing strains are efficiently protected against inhibition and damage by oxygen. In soil, water content does not directly affect N_2 fixation, but controls it by affecting the rates of gas exchanges, especially the oxygen content of the soil atmosphere and soil solution. Therefore, the effect of oxygen and that of the water regime on N_2 fixation cannot be dissociated in the field.

In natural environments, the occurrence of an aerobic- anaerobic interface favors high N_2-fixing activities (Rice et al 1967, Magdoff and Bouldin 1970). Therefore, the rhizosphere of flooded rice, which is the site of an aerobic-anaerobic interface resulting from air diffusion from the leaves to the roots, should be a privileged microhabitat for N_2 fixation. It is likely that in this interface, a microzone will exist in which pO_2 is optimum for the activity of each N_2-fixing bacterium. ARA studies clearly show that N_2 (C_2H_2) fixation in the rhizosphere of rice growing in a waterlogged soil is much higher than that occurring in nonwaterlogged conditions (Yoshida and Ancajas 1973). In principle, moisture deficiency is excluded in paddy fields; however, periods of drought may occur. There is some indication that resulting water stress drastically stops N_2 fixation in the rhizosphere.

Inorganic nitrogen

Repression of N_2ase synthesis by NH_4^+ in all types of N_2-fixing organisms (except certain derepressed mutants) is well established. In view of the results

[1] Air water deficit is calculated according to the formula:

$$D = V\frac{(100-H)}{100}$$

where V is the vapor pressure of water expressed as mm of Hg at a given temperature and H is the air relative humidity expressed as a percentage.

of in vitro studies, it is not surprising that N_2-fixing activity in a complex system, such as rice rhizosphere, is affected by NH_4^+. Actually the addition of NH_4^+-N to soil reduced rhizosphere N_2 fixation, but only when the average NH_4^+-N concentration in the bulk rhizosphere soil was at least 40 ppm (Balandreau et al 1975a).

Although no experimental data are available, it is presumed that, in such a situation, NH_4^+-N concentration in the rhizoplane was much lower because the plant root itself acted as a NH_4^+ sink, depleting NH_4^+-N in the rhizoplane (Lee et al 1977). MacRae (1975) also found that the application of NH_4^+-N at 50 ppm and higher inhibited $N_2(C_2H_2)$ fixation in the rice rhizosphere. A similar inhibition occurred when inorganic N was applied as NO_3^--N. In a fertility trial with wetland rice (Trolldenier 1977), N dressing (140 kg N/ha) inhibited N_2 fixation only temporarily. Since rice plants rapidly absorbed NH_4^+-N, repression seemed to be restricted to the first stages of growth. Considering these data, one could predict that *starter doses* of N fertilizers could benefit the plant growth without hindering biological N_2 fixation.

pH and elements other than nitrogen

Laboratory experiments indicate that the growth of pure cultures of N_2-fixers, such as *Azospirillum lipoferum* (Day and Döbereiner 1976) is pH-dependent. By contrast, a field survey (Garcia et al 1974) strongly suggests that pH effect would be less marked than expected. But further studies are required to elucidate the role of soil pH in situ. Effects of applying such elements as phosphorus and molybdenum have been studied in the field, but no attempt has been made to separate the effect on rhizospheric heterotrophic N_2 fixation from that on photosynthetic N_2 fixation.

Biological factors

Gibson (1977), in a review of the ecology of the legume-*Rhizobium* symbiosis, stressed that any consideration of the environment would be incomplete without reference to the biological factors. These factors include plant pathogens, which affect the plant growth; the development of the root system and processes such as photosynthesis, translocation of photosynthates, and exudation; and microorganisms, other than N_2-fixers that thrive in the rhizosphere. No reports about the effect of plant pathogens upon rhizosphere fixation are available. The interactions between N_2-fixers and other rhizosphere microorganisms are already discussed.

Variations in rhizospheric nitrogen fixation

Variations with time. The levels of environmental factors (e.g. light, temperature...) vary with time. That implies variations in the rate of some plant physiological processes (especially photosynthesis, translocation of photosynthates, and exudation). Consistent variations in the activity of N_2-fixers, which are responsive to environmental factors and to plant exudation, can be

foreseen. In fact, ARA variations have been studied by many authors, using young rice seedlings grown in a phytotron in the absence of blue-green algae. Diurnal variations were reported by Balandreau et al (1975b) and Trolldenier (1977).

A pattern of ARA variations was reported by Lee et al (1977) who found that ARA in IR26 rice rhizosphere began to increase 4 weeks after transplanting and attained a maximum at heading stage, then decreased rapidly.

Variations with soil type. Rinaudo et al (1978) compared the rhizospheric ARA of 21-day-old rice seedlings of the same rice (Sefa 319G) grown in three different soil types and found that ARA in Senegalese *sol gris* was 3.8 and 2.8 times higher than that in Maahas clay and Maligaya clay, respectively (Table 1). A second experiment conducted in similar conditions showed that the rhizospheric ARA of 17-day-old (Sefa 319G) seedlings was 32 times higher when grown in Camargue soil (France) than when grown in Boundoum Senegalese soil (Table 1). Even if the method used for ARA evaluation is liable to be criticized since incubation time of the whole undisturbed plant-soil system was 5–6 hours, it must be emphasized that increases in ARA up to 32 times can be attributed to soil alone, indicating that soil is an essential part of the environment that has been often overlooked. Of course, one should be aware that further studies are necessary to elucidate the roles of chemical and biological soil characteristics responsible for the reported variations in ARA.

INCREASING NITROGEN FIXATION IN THE RICE RHIZOSPHERE

Nitrogen-fixing bacteria can be associated with the roots of flooded rice and thus make up a N_2-fixing association. But until now the actual amount of N_2 fixed by rice growing in different climatic and edaphic conditions is poorly documented. One result is undisputable: the large variations with space and time indicate that the rice N_2-fixing system is more susceptible to environmental parameters than the legume-*Rhizobium* system. This higher suscepti-

Table 1. ARA of the rhizosphere of rice (Sefa 319G) grown in different soils.

	ARA nmol C_2H_4/h ($\pm$ CI)		Root dry wt (mg/seedling)
	Per seedling	Per g (dry root)	
First experiment (21-day-old seedlings)			
Maahas clay, Philippines	61	352	174
Maligaya clay, Philippines	126	484	266
Sol gris, Senegal	201	1357	149
Second experiment (17-day-old seedlings)[a]			
Boundoum soil, Senegal	8 $\pm$ 2	168 $\pm$ 48	49
Camargue soil, France	174 $\pm$ 99	5420 $\pm$ 3637	34

[a] Confidence interval CI was calculated only in the second experiment (5 replicates), but not in the first (3 replicates).

bility results from the differences between the two systems, which are summarized in Table 2. Thus, the rice N_2-fixing system appears to be a loose association. Nevertheless there are some indications that its N_2-fixing activity can be increased by eliminating or at least reducing the impact of the major limiting environmental factors and by manipulating separately or simultaneously the components of the system (the rice plant itself and the associated N_2-fixing bacteria).

Soil management and fertilization

Practically the only extrinsic factors affecting N_2 fixation that can be easily tackled are the edaphic factors. As already indicated, the most important limiting factor is the excess inorganic N. Large applications of N fertilizers are recommended for high rice yields. But they inhibit rhizospheric N_2 fixation. To prevent this inhibition in legumes, Hardy et al (1973) suggested the use of other forms of N fertilizers that do not inhibit N_2 fixation, while providing the plant with the complementary N required for their growth. Such new forms of chemical fertilizers, which they designated as *compatible fertilizers*, could also be recommended for use in rhizosphere N_2 fixation. The possibility, although promising, has not yet been seriously explored. The influence of population and placement of conventional N fertilizers on rhizosphere N_2 fixation should also be studied.

Because acidic tropical soils are most often deficient in some elements, especially phosphorus, calcium, and molybdenum, application of related fertilizers can be expected to stimulate the activity of rhizosphere N_2-fixers either directly or by promoting the development of the root system, thereby increasing the amount of energy-yielding substrates in the soil.

Plant breeding and selection

Preliminary results indicate that by screening collections of rice germplasm, one could select the most desirable genotypes according to their ability to associate themselves with rhizospheric N_2-fixing bacteria. Bulow (1978) stressed that new variability may also be produced by mutation breeding, hybridization, and genetic manipulation.

However, one should be aware of the limitations of such an approach, which results from:

- the imperfect methodology for estimating rhizospheric N_2-fixing capacity, and
- the great variability of the rhizospheric ARA and its dependence on environmental parameters, which cannot always be controlled in situ, and on the plant behavior during its growth cycle.

Manipulation of the rhizosphere microflora

Recent developments in knowledge of N_2 fixation in the rice rhizosphere suggest three groups of approaches based on the manipulation of the rhizosphere

Table 2. Comparison of legume and rice N_2-fixing systems.

Nitrogen-fixing system	Bacterial strains	Specificity	Bacterial infection	Amount of energy (expressed in moles of glucose) required to fix one mole of nitrogen	Protection against oxygen excess	Fate of fixed nitrogen
Legume-*Rhizobium* symbiosis	*Rhizobium*	Certain	Intracellular	1	By plant cell and pigments such as leghaemoglobin	Directly used by the legume as NH_4^+—N
Rice rhizosphere nitrogen-fixing system	Many belonging to different genera; often associated with other microorganisms	Possibly some broad specificity	Apparently limited to rhizoplane, moribund or dead root cortical cells, and to root residues	3–4 (aerobic fixers) 8–10 (anaerobic fixers). Some environmental factors could presumably increase efficiency two or three fold).	No protection by host-plant. Bacterial protection often inadequate. Partial protection by waterlogging.	Not yet ascertained

microflora: selection and improvement of rhizosphere N_2-fixers, introduction of microbial associates, and improvement of root colonization by selected strains or microbial associations.

Selection and improvement of rhizosphere N_2-fixers. The five main features that are essential for rhizosphere N_2 fixation have already been indicated: protection against oxygen, competitiveness and ability to colonize rice roots and use the substrates that are available in the rhizosphere, nitrogen-fixing efficiency, ability to fix N_2 in the presence of combined nitrogen, and ability to excrete fixed N_2 as ammonium. Screening wild strains for such features is obviously desirable. Another possibility is to use the conventional techniques of microbial genetics to obtain new strains that have some of these desirable properties. Encouraging results have already been reported in that field by Gauthier and Elmerich (1977) who obtained mutants of *Spirillum lipoferum* that had a higher N_2ase activity than the wild strain. Microbial geneticists are now able to construct N_2-fixing bacteria that can fix N_2 in the presence of ammonia, by manipulating the genes determining glutamine synthetase (*gln* genes) so that they become constitutive (Postgate 1977). It is also possible to obtain N_2-fixers that cannot incorporate the N_2 they fix, by blocking glutamate synthetase, a constitutive enzyme that incorporates ammonia resulting from fixation into amino acid glutamate. Thus Shanmugam and Valentine (1975) obtained mutants of *Klebsiella pneumoniae* that fixed N_2 and excreted ammonia when grown with little glutamate.

Schubert and Evans (1977) discovered that in symbiotic N_2-fixing systems, a variable portion of the available energy was lost as hydrogen originating from a side reaction of N_2ase. Moreover, evidence was obtained that symbiotic N_2-fixing microorganisms possessed a hydrogenase that could recycle the hydrogen produced through N_2ase; the more active the hydrogen-recycling apparatus, the more efficient the N_2-fixing system was. Postgate (1977) recalls that free-living N_2-fixing bacteria, such as those thriving in the rice rhizosphere, have a H_2ase that can recycle the hydrogen formed through N_2ase and assumes that a genetic approach to tightening up such systems may well be possible.

Nitrogen fixing microbial associations. We have already indicated that N_2 fixation was significantly enhanced when N_2-fixers were grown together with beneficient microbial associates (synergists). Preliminary experiments already mentioned suggest that association of N_2-fixers with pectinolytic (and possibly cellulolytic) microorganisms is a prerequisite for N_2 fixation when the main source of energy is root debris.

Promoting root colonization by desirable strains or associations. It would be useless to select or obtain new efficient N_2-fixing strains or associations, if they could not be established in the rice rhizosphere. To date, inoculation techniques based on the application of liquid microbial or peat-base inoculants to the rice rhizosphere have led to inconsistent results. A new type of inoculant was recently devised. It is obtained by distributing bacterial cells in a matrix of acrylamide, which is then polymerized and crushed before it is mixed with

the soil (Dommergues et al 1979). The main advantage of this technique is that bacterial cells protected by the polyacrylamide gel are kept alive in the soil much longer than bacterial cells from a liquid culture. Inoculating 21-day-old rice seedlings with cowpea *Rhizobium* strain CB 756 (which behaved as a free N_2-fixing bacteria) significantly promoted ARA in the rice rhizosphere only when polyacrylamide-entrapped *Rhizobium* was used as inoculant (Table 3), indicating that new techniques that might promote root colonization by desirable N_2-fixing strains or microbial associations could be developed.

Table 3. Comparison of two inoculants (liquid culture and polyacrylamide-entrapped bacteria) upon ARA of 21-day-old seedlings of rice (Sefa 319G) grown in Boundoum alluvial soil (Senegal) (Rinaudo, unpubl. data).

Inoculant	ARA (nmol C_2H_4/h)[a]	
	Per seedling	Per g root (dry wt)
Control (autoclaved bacteria)	70 a	240 a
Liquid culture	90 ac	310 a
Polyacrylamide-entrapped bacteria	160 bc	660 b

[a] In a row any two numbers that are followed by the same letter are not significantly different at the 5% level (Snedecor and Cochran 1967). The bacterium used for inoculation was *Rhizobium* cowpea strain CB 756.

CONCLUSION

The rhizosphere of flooded rice, with associated N_2-fixers, can fix N_2.

According to intrinsic and extrinsic factors, N_2-fixing rates range from zero to indefinite values that are probably much lower than the N_2 fixation rates of nodulated legumes. Two questions arise:

1. Is it possible to increase the potential for N_2 fixation by the rice N_2-fixing system as described here?
2. If it is, how large could the expected increase be?

The answer to the first question is yes, because tools than can act upon rhizosphere N_2-fixing systems are now available. In practice, the most promising approaches aimed at medium-term improvements are:

- the use of proper fertilizer stimulating N_2 fixation and especially discovery of compatible N fertilizers,
- screening of material stored in germplasm banks for the ability to associate with N_2-fixing bacteria and to exhibit the higher N_2-fixing activity,
- obtaining, by screening or genetic manipulation, strains with the desirable features, and
- improvement of root colonization by devising new carriers for preparing inoculants, extending the survival and the competitiveness of the strains or associations of strains to be introduced in the rhizosphere.

With our present knowledge, it is not yet possible to answer the second question. Whatever the increase in N_2 fixation rate may be, differences varying in degree according to soil and climate are to be expected. These differences imply that experiments should be made at a large number of sites. Even in the best conditions, the rate of rhizospheric N_2 fixation predictably will not be high enough to meet by itself the rice N requirements for high yields. But even a relatively small contribution of rhizospheric N_2-fixing bacteria to the N input in flooded paddy fields (for instance, 20 kg/ha per crop) may significantly reduce the need for N fertilizers and possibly add to the N input through blue-green algae and azolla.

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DISCUSSION

FREDERICK: Do the N_2-fixing bacteria require certain types of carbon compounds? If so, which ones and how many kg are required per kg of N_2 fixed?

Dommergues: Carbon nutrition of some N_2-fixing strains is already known; thus, some *Azospirillum* strains are known to use glucose, others malate, and others both sources of carbon. To the best of our knowledge no N_2-fixing strain is able directly (by itself) to use the high-molecule-weight compounds that make up a large part of root residues; in other words they lack the enzymatic equipment (cellulase, pectinase, for instance) that would be required. I ask Dr. Martin to comment on your second question.

Martin: My information on amount of carbon available to heterotrophic N_2-fixing organisms in the rhizosphere is limited to dryland wheat. There are such obvious differences between wheat and paddy rice that I am not sure that the values for wheat are applicable to rice, but they give an indication of the magnitude of the possible losses of carbon from cereal roots. Data obtained from the Waite Institute in Adelaide show a transfer of photosynthate to the root system of field-grown wheat between early tillering and flowering that is equivalent to about 1,800 kg C/ha. This is in contrast to a carbon content at the root system, which can be recovered at flowering by washing away the soil, of some 400 kg C/ha. Thus, there has been a wastage of root tissue during the active growth period equal to more than 70% of the root mass produced.

Allowing for 30% of the root carbon occurring in liquid and other compounds, which is not readily available for microbial decomposition, there would be some 1,000 kg C/ha available to the microflora and microfauna.

There is some doubt about the amount of carbon required for the heterotrophic fixation of N_2 but a common estimate is 30–40 kg C/kg N fixed. If the amount of carbon and N_2 located in the root system of flooded rice is of the same order of magnitude as for wheat, one can obtain an upper limit of heterotrophic N_2 fixation equivalent to 25–30 kg N/ha. The actual amount of N_2 fixed per 1,000 kg of available C/ha would be much less because no allowance has been made for root respiration, and that will induce competition for the carbon among the different microbial populations, many of which may not be capable of N_2 fixation.

EUNUS: In your presentation you have indicated that N_2-fixation varies due to varietal differences. Can you comment on what plant characteristics should be taken into account for breeding rices for adequate N fixation?

Dommergues: Two characteristics appear to be highly desirable—ability to produce large amount of root tissue, and susceptibility of roots to colonization and infection by N_2-fixers and associated microorganisms.

BECKING: With regard to substrate addition for energy and the mentioned calculation, I would like to add that such a substrate will not be used solely by N_2-fixing microorganisms, but also by a large number of N-heterotrophs in the soil. Secondly, the presence of combined N in soil will inhibit the N_2-fixing activity of N_2-fixing bacteria present.

Dommergues: We are well aware that the energy yielding substrates available in the rhizosphere are not solely used by N_2-fixers, but also by other microorganisms. Thus, the figures given by Dr. Martin should be considered as the upper limit. The effect of combined N in soil was discussed in some detail in the report, which will be published.

HONG: In the final part of your conclusion you stated that even a relatively small contribution of rhizospheric N_2-fixing bacteria to the N input in a flooded paddy (for instance, 20 kg/ha per crop) may significantly reduce the need for N fertilizers. In my opinion, it may not be always true. As a matter of fact, it is not clear yet whether the N_2 fixed by certain organisms is directly available to the present crop or the following crop.

Dommergues: We do not yet know whether N_2 fixed in the rhizosphere is used by the standing crop or by the following ones. However, there is some indication that only a fraction of fixed N_2 is available and absorbed by the standing crop (see Boyce Thompson Institute experiment using ^{15}N). The fraction of fixed N_2 that is immediately available could probably be increased if N_2-fixing bacteria excreting ammonium could be established in the rhizosphere.

DURBIN: You said that soil type affected ARA of seedlings and explained this by a difference in N_2-fixing bacteria populations. Do you mean a difference in numbers, species types, or numbers of bacteria found on or in the rice roots?

Dommergues: Reported variations in ARA can probably be attributed to differences both in the quantitative and the qualitative characteristics of the N_2-fixing populations and associated microorganisms living in the rhizosphere.

ALEXANDER: How do you envision inoculating the rice root to allow for an extensive enough colonization that the inoculum exploits an adequate amount of the root exudates?

Dommergues: Because bacteria are not very mobile in the soil, the inoculum, applied as a powder, should be mixed thoroughly with the soil so that most of the root system is infected.

CRASWELL: If energy is the factor most limiting rhizosphere N_2 fixation, a high-yielding plant fertilized with N is needed to provide the necessary carbon in the rhizosphere. This could be achieved without N_2ase being inhibited by too much mineral N in the soil, if a slow-release fertilizer is used. Have you studied the effect of slow-release fertilizer on rhizosphere N_2 fixation?

Dommergues: In a recent experiment in our laboratory, application of a slow-release fertilizer (urea formaldehyde) did not inhibit nodulation or N_2 fixation on soybean. This type of experiment has not yet been performed in the case of a nonsymbiotic system (such as the rhizosphere N_2-fixing system of rice), but we can reasonably presume that results should be similar. I do think that studying the effect of slow-release fertilizers (and also formulation and placement of N fertilizers) upon N_2 fixation in the rhizosphere would be really worthwhile.

ROVIRA: The mobility of bacteria in the rhizosphere is slight, hence maybe we should use an *enrichment* technique by growing successive crops in which the bacteria on the root fragments act as inoculum.

Dommergues: I agree with Dr. Rovira. The experiment he proposes is really worth being carried out.

ROVIRA: There will be a great competition by the general soil and rhizosphere microflora with the N_2-fixing bacteria for energy in the rhizosphere.

Dommergues: Your point raises the general problem of competition, which obviously is one of the most arduous that we encounter in soil microbiology.

VENKATARAMAN: You suggest the possibility of breeding rice varieties for rhizosphere N_2 fixation. In view of the absence of any specific varieties, and the different response of the same genotype in different soil, I wonder whether breeding will be a profitable approach.

Dommergues: Our latest data show that breeding alone cannot generally lead to an increase of rhizospheric N_2 fixation, because the qualitative and quantitative composition of the rhizosphere microflora plays a major role in governing N_2 fixation levels. Therefore, screening for the most desirable rice varieties should not be dissociated from microbiological manipulation of the rhizosphere micropopulations.

DOBEREINER: You mentioned pronounced effects of rhizobium inoculation on N_2ase activity in the rhizosphere of rice seedlings. Could you further comment on this rather unexpected result?

Dommergues: Rhizobium strain CB 756 was established in the rice rhizosphere using our new technique of inoculation (bacteria embedded in a polymer). Because that strain is known to fix N_2 in vitro (outside the nodule) and thus can behave as a nonsymbiotic N_2-fixer, it is not surprising that it exhibits some ARA when living in the rice rhizosphere.

ASPIRAS: What would be the effect of systemic pesticides on ARA in the rhizosphere of the rice plant?

Dommergues: We have not yet studied this problem.