3. Heterotrophic N_2 fixation in paddy soils

T. YOSHIDA and G. RINAUDO

1. Introduction

1.1. Maintenance of N fertility of paddy soils

The yield of rice crops in many South Asian countries affects the food supply of almost half the world's population. Nitrogen is the element that often becomes a limiting factor for crop production. In many parts of Asian rice-growing countries, chemical fertilizers are not yet commonly used and rice yields greatly depend on the natural fertility of paddy soils. Ancient farmers practiced the burnt-land type of agriculture to produce their food, mainly upland rice. They had to move from land to land every 2 to 3 years because the soil fertility did not last long. However, the farmers growing rice in flooded soil have been growing it for more than a thousand years in the same rice paddies. The continuous supply of elements, particularly nitrogen, despite its removal over the years by rice crops, is considered caused by the fixation of atmospheric N_2 by microorganisms in paddy soils [41]. But it is not yet certain which microorganisms contribute to the fixation of atmospheric nitrogen.

Long-term NPK experiments at the various prefectures in Japan have demonstrated the continuous natural supply of nitrogen in paddy soils. Konishi and Seino [57] showed that at least about 20 kg N ha^{-1} is annually fixed from the atmosphere in paddy fields that received no fertilizer (Treatment None, Table 1). The addition of phosphorus and potassium increases nitrogen enrichment in the paddy field (Treatment PK, Table 1).

Table 1. Nitrogen balance in rice growing paddy field for 22 years (1929–1950) Ishikawa Agricultural Experiment Station, Japan [57]

Treatment	Gains or losses of nitrogen[a] (kg ha^{-1})	
	Without Ca	With Ca
None	+ 420	+ 651
PK (–N)	+ 741	+ 838
NK (–P)	–507	–372
NP (–K)	– 57	+ 82
NPK	+ 39	+ 95

[a] Positive values were considered as gains through atmospheric N_2 fixation. The calculation was done as follows: (N taken up by plants) – (N added as fertilizers + lost soil N)

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Matsuo and Takahashi [75], in a long-term experiment in Japan, calculated the nitrogen balance in paddy fields without nitrogen application and estimated the average natural supply of nitrogen in such fields at 49 to 91 kg ha^{-1} $year^{-1}$ and 70 kg ha^{-1} $year^{-1}$ for all the districts in Japan.

In Hokkaido, Japan, a 41-year rice experiment without nitrogen fertilizer showed average 2.8 t ha^{-1} grain yield with no decline in soil fertility [100]. The amount of nitrogen removed from the paddy was 53 kg ha^{-1} $year^{-1}$. Koyama and App [58] reported an input of 35–50 kg N $crop^{-1}$ into flooded rice soils in temperate and tropical regions.

In a Maahas clay area at the experimental field of the International Rice Research Institute (IRRI), Philippines, net amount of soil nitrogen released was estimated at 57 kg ha^{-1} in fields submerged for 110 days during the dry season and 77 kg ha^{-1} in fields submerged for 94–98 days during the wet season [82, 101]. The input and output of natural nitrogen in the rice-growing soil seem well balanced. Input of natural nitrogen such as nitrogen in rain or irrigation water did not account for the balance. However, if the soil mineralized 57 kg ha^{-1} every cropping season, its total nitrogen (2,800 kg ha^{-1}, on the assumption of 0.14% soil N) would be compensated for in about 50 croppings unless the soils gain nitrogen by some mechanisms. Nevertheless without nitrogen fertilizer the rice yield in different paddies located in the Philippines has remained at several tons ha^{-1} over the past many years [48] (Table 2).

1.2. Measurement of N_2 fixation in the field

There are three major methods of studying the biological N_2 fixation in rice soils: the Kjeldahl technique, the N-15 isotope technique, and the acetylene reduction (C_2H_2-C_2H_4) technique. A direct estimate of N_2 fixation is obtained by determining the increase in total fixed N in samples. Total nitrogen is determined by the Kjeldahl method, which is adequate for a system that is capable of vigourous N_2 fixation, but is considered unreliable for a system that shows a small percentage of increase in fixed nitrogen. However, the method is satisfactory if length of exposure to N_2 results in a reasonably high percentage change in total nitrogen in the system used.

An experiment by App *et al.* [4] using this technique explains the natural nitrogen fertility of flooded rice fields and indicates that the rice plants significantly improve the nitrogen economy of such soils (Table 3).

More specifically, a direct and probably more accurate method for measuring N_2 fixation would be the N-15 isotope technique. But this tracer technique requires the use of expensive $^{15}N_2$, particularly if it is used on a large scale, as in a growth chamber or a paddy field. It also needs analysis with a mass spectrometer, a rather expensive piece of equipment and one requiring some laborious procedures in sample preparation. Therefore, most of the research on N_2 fixation in rice soils using the N-15 isotope technique has been restricted to experiments in the

Table 2. Yields of a traditional variety and two modern varieties grown without nitrogen fertilizer [48]

Wet season	Yield (t ha^{-1})														
	IRRI			Maligaya station			Bicol station			Visayas station			All stations		
	Peta	IR8	IR20	Peta	IR8	IR20	Peta	IR8	IR20	Peta	IR8	IR20	Peta	IR8	IR20
1968	3.9	4.1	3.7	4.1	3.7	4.0	3.8	5.1	4.5	2.4	3.0	3.1	3.6	4.0	3.8
1969	2.9	5.6	5.2	3.8	5.2	4.4	3.4	4.6	4.0	4.1	4.0	5.2	3.6	4.8	4.7
1970	2.8	4.9	4.6	2.8	4.0	4.3	0.0	2.8	2.4	3.5	2.2	3.5	2.3	3.5	3.7
1971	1.3	3.6	3.8	2.9	2.5	3.2	3.1	3.2	2.9	4.6	3.9	3.8	3.0	3.3	3.4
1972	2.9	3.3	4.0	3.2	3.6	3.6	3.8	4.2	3.8	3.4	3.2	3.5	3.3	3.6	3.7
Average	2.8	4.3	4.3	3.4	3.8	3.9	2.8	4.0	3.5	3.6	3.3	3.8	3.2	3.8	3.9

Table 3. Nitrogen balance sheet for flooded soil planted to rice determined by the Kjeldahl method [4]

Planted to rice	Exposed to light	Supplemental treatment	Mg N: Crop ± S.E. (X)	Mg N: Change in soil ± S.E. (Y)	Mg N: Misc. Inputs (Z)	Mg N: N balance (X + Y − Z)	N gain as % of crop N
			Experiment A (4 crops)				
+	−	None	997 ± 15 (a)	−795 ± 39 (a)	21	181^{**} (a)	18
+	−	Stubble removed	1042 ± 22 (a)	−875 ± 66 (a)	21	148^{*} (a)	14
−	−	None	0 (b)	−243 ± 40 (b)	0	-243^{**} (b)	−
			Experiment B (6 crops)				
+	+	None	1175 ± 11 (a)	−604 ± 70 (a)	27	544^{**} (a)	46
+	−	None	1148 ± 16 (a)	−961 ± 50 (b)	27	160^{*} (b)	14
−	+	None	0 (b)	193 ± 82 (c)	24	$169^{n.s}$ (b)	−
			Experiment C (6 crops)				
+	+	None	1203 ± 12 (b)	−789 ± 61 (a)	27	387^{**} (a)	32
+	+	P, Fe	1211 ± 43 (b)	−895 ± 77 (b)	92	723^{**} (b)	60
+	+	P, Fe, algae	1273 ± 33 (b)	−261 ± 80 (b)	98	914^{**} (b,c)	72
+	+	P, Fe azolla	1681 ± 21 (a)	−421 ± 67 (b)	106	1153^{**} (c)	69

Note: Significantly different from zero at the 5% and 1% level represented by * and **. Means followed by the same letter not significantly different at the 5% level by Duncan Multiple Range Test. S.E. means standard error of mean laboratory. The isotope technique is direct and, perhaps, much less subject to sampling errors than the Kjeldahl technique, however. Recently, the N_2-fixing activities of the rice root zone were measured by ^{15}N-labeled dinitrogen in water culture conditions [52]. A similar experiment using the tracer technique in flooded soil conditions [37, 133] indicated that the N-15 isotope technique is sensitive enough to detect within 1 or 2 weeks the N_2-fixing activities in the rice soil-plant system under *in situ* conditions.

The acetylene reduction method which is simple, sensitive and inexpensive, greatly enhanced N_2 fixation studies [22, 45]. Because it indirectly measures potential biological N_2 fixation, it has been considered unreliable for critical experiments aimed at estimating N_2 fixation. But N_2 fixation has been studied by the C_2H_2-C_2H_4 technique under *in situ* conditions in paddy fields [3, 60, 62, 63, 119].

Alimagno and Yoshida [3] used a device for *in situ* acetylene ethylene assay of submerged rice soils. Similarly, the N_2-fixing activities in the rice rhizosphere were measured with a metal cylinder attached to a plastic bag for C_2H_2-C_2H_4 assay in *in situ* field conditions [62]. The N_2-fixing activity was markedly higher in planted areas of the field than in non-planted areas between plant rows. Lee and Watanabe [60] found that stirring the soil-water system within the assay device maximized the recovery of the evolved ethylene.

1.3. Contributions of heterotrophic and photosynthetic organisms

It appears from the foregoing review that the continuous supply of nitrogen despite its removal by rice crops, is caused by microorganisms in paddy fields. It is interesting now to know which microorganisms actually take important part in the N_2 fixation in paddy fields. The blue-green algae have been receiving more attention than the bacteria in relation to the N_2 fixation in rice soils. Much work has been done on N_2 fixation by blue-green algae in rice soils particularly, in Japan and India (refer to Chapter 5 of this book). Little work regarding the study on the photosynthetic bacteria in rice soils has been reported [56, 77, 123].

The photosynthetic microorganisms may have a great potential in the N_2 fixation in paddy fields if the various ecological factors are suitable for the growth and maintenance of the N_2-fixing inhabitants. A greenhouse experiment aimed at determining the role of the photosynthetic microorganisms in rice soils showed that N_2-fixing activity is much higher in flooded conditions under light than under dark [130]. But there were no significant differences, as measured by the weights of straw and filled grain and by nitrogen uptake in rice between the light and dark treatments during two continuous croppings.

In tests using the *in situ* device for assaying photosynthetic N_2 fixation in paddy fields, the estimated amounts of N_2 fixation ranged from 2.3 to 5.7 kg N ha^{-1} in one location and 18.5 to 33.3 kg N ha^{-1} in another, in the Philippines [3]. Inoculating paddy fields with N_2-fixing blue-green algae such as *Anabaena* sp., *Nostoc* sp., *Aulosira fertilissima*, or *Tolypothrix tenuis* benefited rice growth and yield [113] in India, although it did not significantly affect growth and yield of rice plants in both field experiments at Los Baños, Philippines [2].

Rouquerol [98] reported that *Azotobacter* and *Clostridium* are responsible for the N_2 fixation in a rice field in southern France. Okuda and Yamaguchi [77] studied the distribution of photosynthetic N_2-fixing bacteria in paddy soils and reported that the occurrence of the bacteria appears important in the N_2 fixation in paddy soils in Japan. Becking [15] reported that *Beijerinckia* is distributed widely in tropical regions, mainly in lateritic soil, and more abundantly than *Azotobacter* in soil with low pH. However, no published paper gives a quantitative estimation of N_2 fixation by these heterotropic N_2-fixing bacteria. Jensen [53] or Alexander [1] consider the frequently estimated annual gains of 20–50 kg N ha^{-1} by heterotrophic N_2-fixing bacteria too high because these bacteria must consume at least 1–2.5 tons of organic materials of the same nutritive value as glucose.

Many papers report that the addition of organic materials to flooded soil stimulates the N_2-fixing activities of the soils [14, 67, 68, 72, 85, 89, 90]. But none of these studies reported quantitative estimates of N_2 fixation in the paddy field.

Atmospheric N_2 was recently found to be fixed by heterotrophic N_2-fixing bacteria in the rhizosphere of the rice plant [17, 34, 40, 47, 62, 63, 66, 111, 128, 129]. The rice rhizosphere has some unique characteristics, which include the air-transporting root tissues and the aerobic-anaerobic interface between the oxidative root tissue and the anaerobic soil [126]. The N_2-fixing activity increases as the plant ages, reaching a maximum at mid-reproductive stage. Rice plants rapidly translocate into plant parts the N_2 fixed in the rhizosphere [52, 133]. The amount of N_2 fixed in the rice rhizosphere by heterotrophic bacteria has been considered significant for rice production without fertilizers [4, 128, 133] Wada *et al.* [114] reported that the most important site of N_2 fixation was the reduced horizon of the paddy field, and the role of the rhizosphere in the estimation of N_2 fixation in paddy fields could not be neglected (Fig. 1.). Matsuguchi [72] reported that heterotrophic N_2 fixation shared more than 60 percent of the total N_2 fixed in the field. Direct measurement of N_2 fixation by the Kjeldahl technique gave an additional evidence of simultaneous N_2 fixation by heterotrophic and phototrophic organisms as shown in Fig. 2 [4, 49].

1.4. Conclusion

Scientists have long recognized the maintenance of nitrogen fertility in rice paddies. The mechanism involved in this phenomenon is believed to be the biological N_2 fixation. The evidence supporting the theory has increased in the last several decades. Major biological agents contributing to nitrogen enrichment are, apparently, photosynthetic microorganisms, including the N_2-fixing blue-green algae and heterotrophic bacteria in rice paddies. The magnitude of the contribution of each N_2 fixer to the maintenance of nitrogen fertility of rice paddies may depend on environmental conditions and has not yet been clearly determined. How-

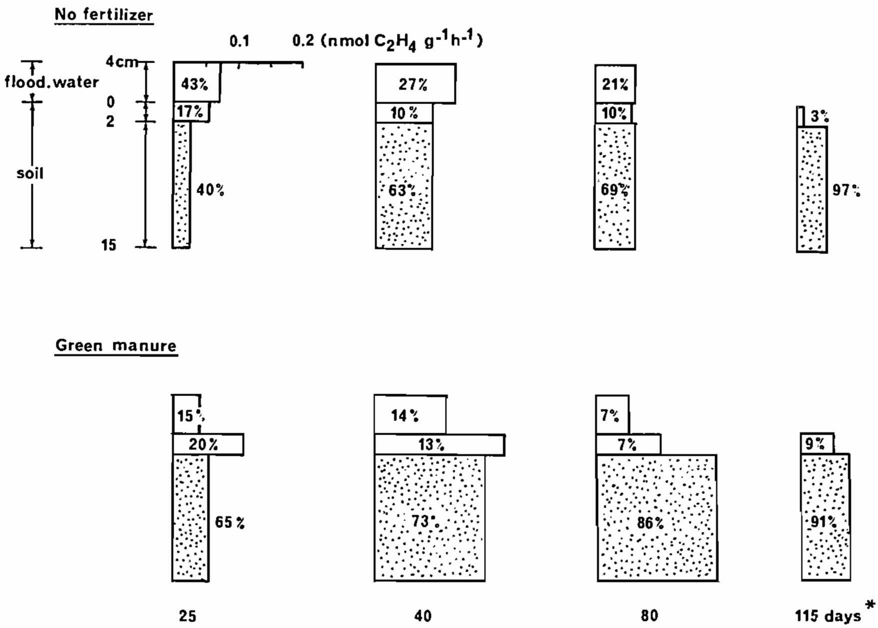

Fig. 1. Distribution of N_2-fixing activity in soil at different sites in Konosu [114]. * days after transplanting.

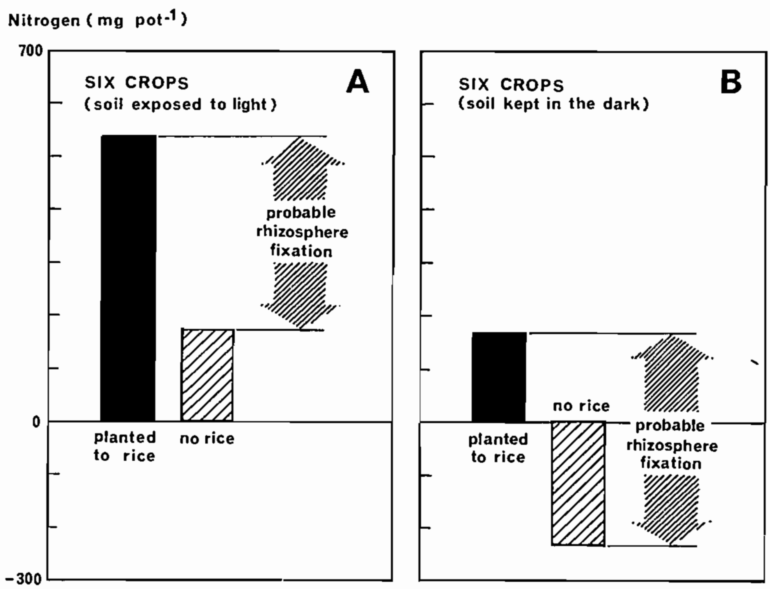

Fig. 2. Direct measurement of nitrogen balance suggests significant N_2 fixation in the root zone of paddy rice as a complement to that by blue-green algae (soil exposed to light: A) or in the absence of the blue-green algae (soil kept in the dark: B) [49].

ever, the role in the nitrogen enrichment of both phototrophic and heterotrophic N_2-fixing microorganisms appears important; probably that of the former is more important in the earlier stage of the rice-growing season and that of the latter is more important in the reproductive stage of the rice plant.

2. Microbiology of heterotrophic N_2 fixation

2.1. Microorganisms involved

2.1.1. Qualitative studies. A wide range of N_2-fixing bacteria occurs in rice soils: *Pseudomonas, Azotomonas, Azotobacter, Beijerinckia, Flavobacterium, Arthrobacter, Bacillus, Clostridium* [10]. *Desulfovibrio, Desulfotomaculum* and methane-oxidizing bacteria [19] have also been frequently found in paddy soils. *Derxia* was found particularly prevalent in waterlogged soils [27]. Recently, different strains of *Azospirillum* [39, 59, 76, 102], *Enterobacter* sp. [44, 118], *Pseudomonas, Arthrobacter, Spirillum, Vibrio* (Rinaudo, unpublished), and possibly *Achromobacter* [116, 121] have been isolated from washed or surface-sterilized rice roots. Such a large range of N_2-fixing heterotrophs suggests that heterotrophic N_2 fixation adapts to widely diverse soil environments.

2.1.2. Quantitative estimations. According to Ishizawa *et al.* [50] and Balandreau *et al.* [9], aerobic N_2 fixers are more abundant than anaerobic N_2 fixers in rice soils and in the rice rhizosphere. Counts of N_2 fixers on washed rice roots [111] revealed a similar trend. Ishizawa and Toyota [51] pointed out that *Azotobacter* was distributed more widely in wetland rice soils than in dryland ones, and also more widely in non-volcanic soils than in volcanic ones, although the bacterial population in most soils was as sparse as 10^3 g $(soil)^{-1}$ or less. In alkaline rice soils such as those in Egypt [69] the number of *Azotobacter* can be considerable 10^3 – 10^7 g $(soil)^{-1}$, while in neutral to acid soils it is much lower [50]. *Beijerinckia* occurs abundantly in rice soils [16]. In over 40 paddy fields surveyed in Thailand, Matsuguchi *et al.* [74] found that the populations of *Azotobacter*, and *Clostridium* which were 10^0–10^4 g $(soil)^{-1}$ and 10^3–10^6 g $(soil)^{-1}$, respectively, tended to be high in soils with high pH and large amounts of organic matter and phosphorus. *Azospirillum* has often been isolated from surface sterilized rice roots. Unfortunately, most work on these bacteria are qualitative and quantitative estimates are lacking.

2.2. Distribution of N_2-fixing microorganisms

2.2.1. Location of N_2-fixing bacteria in non-rhizosphere soil. Little attention has been paid to heterotrophic N_2 fixers in non-rhizosphere soil. However, a considerable amount of organic matter (stuble and root litter from crop residues) is left in the field after harvest. Recent studies in Japan suggest that: (1) organic debris are active microsites for non-symbiotic N_2 fixation [115], (2) rice straw decomposition and subsequent heterotrophic N_2 fixation proceed not only in the surface layer, where aerobic or microaerophilic conditions facilitate both processes but also in the lower plow layer [72].

2.2.2. Location of N_2-fixing bacteria in the rhizosphere. After the rediscovery of *Spirillum lipoferum* [28], one of the most interesting problems was the location of N_2 fixers in or on root tissues and its establishment in association with the plants.

This point was recently discussed by Diem and Dommergues [23]. Intracellular colonization may occur but is generally discrete: Lakshmi *et al.* [59] observed only some *Azospirillum* cells within rice root hairs. In contrast, intercellular colonization of root cells, often reported, seems mainly restricted to older parts of roots (nodal zone) where the cells are generally damaged [6, 25, 78]. Umali-Garcia *et al.* [112] reported that *Azospirillum* cells were observable within the middle lamella of the walls of living root cells. That observation may be correlated with the ability of most *Azospirillum* strains to use the pectin as sole carbon source [24, 108]. Asanuma *et al.* [6] found that rhizoplane microorganisms of rice seedlings were distributed in every region except the root tip zone, and mainly at ruptured sites of epidermal cells and cell junctions. They were also dispersed and they adhered to mucigel, the bare epidermal surface, and root hairs. The ratio of bacterial coverage on the root surface was surprisingly small: 1–9% of the total root surface.

2.2.3. Distribution of N_2-fixing microorganisms in different sectors of the rhizosphere. The rhizosphere can be divided into three sectors: (1) the rhizosphere soil comprising the region of soil immediately surrounding the plant roots and the organisms living therein, (2) the rhizoplane formed by the root surface and the microorganisms living on it, and (3) the endorhizosphere formed by the root cortical tissue involved and colonized by heterotrophic microorganisms [32]. Brown [21] considered many N_2-fixing bacteria (e.g.) *Azotobacter* as not true rhizosphere bacteria. Colonization by *Azotobacter*, when it occurred, was limited to the rhizosphere soil, and very few cells were found on the root surface. In contrast, Döbereiner and Ruschel [30] reported that *Beijerinckia* grew better on the root surface of waterlogged rice than in the surrounding soil. The results obtained by Döbereiner's group [29] suggested that plants with the C_4 photosynthetic pathway are preferentially infected by *Azospirillum lipoferum* while C_3 plants are infected by *A. brasilense.* In flooded rice, all *Azospirillum* isolates from surface-sterilized roots (15 min in 1% chloramine T) belonged to the species *A. brasilense,* 96% of them being nir^- (nitrite reductase$^-$), while in non-planted soil only 50% of the isolates belonged to *A. brasilense* nir^-.

Watanabe *et al.* [121] studied the distribution of aerobic heterotrophic bacteria among the three sectors of the rhizosphere of rice cultivar IR26. Bacterial isolates were taken from the plates used for these counts, and checked for nitrogenase activity. Similar studies including actinomycetes counts have been done in Senegal with rice cultivar Moroberekan [97]. The results are summarized in Table 4. The percentage of bacterial isolates that were nitrogenase positive increased from rhizosphere soil to endorhizosphere in both the 'rice cultivar IR26-Philippine soil' and 'rice cultivar Moroberekan-Senegalese *sol-Dior*' systems. The percentages of endorhizosphere isolates showing nitrogenase activity (81% and 50%, respectively) were very high. Surprisingly, when Moroberekan was grown on Senegalese *sol gris*, the percentage of nitrogenase-positive isolates was lower in the endorhizosphere than in the rhizosphere soil (16% and 53%, respectively), whereas the proportion of actinomycetes decreased from endorhizosphere to rhizosphere soil (7.2% and 1.7% respectively). On the basis of these data Roussos *et al.* [97] suggested that actinomycetes might affect the rhizosphere colonization by N_2-fixing microorganisms.

A comparison of the results obtained in a Philippine soil and a Senegalese *sol gris* revealed other important differences: (1) all isolates from the endorhizosphere of the Philippine soil were presumably *Achromobacter*, whereas the most important N_2-fixing group in Senegalese *sol gris* was constituted by a pink pigmented strain belonging probably to the genus *Arthrobacter*; (2) Watanabe and Barraquio [116] found that glucose-utilizing N_2 fixers were more numerous than malate-using N_2 fixers, whereas Roussos *et al.* [97] observed that malate was more widely used by isolates from all three samples; (3) nutritional requirements (vitamins or amino acids) of N_2-fixing isolates from the Philippine soil were more marked than those of the Senegalese *sol gris*.

2.3. Conclusion

Miscellaneous bacteria other than *Azotobacter, Clostridium,* or *Azospirillum* thrive in the rice rhizosphere. Many are microaerophilic. Obligate or facultative anaerobes are present but usually less abundant. According to the different studies reported here, the rhizosphere population of N_2 fixers varies widely both qualitatively and quantitatively.

The high proportion of N_2-fixing bacteria among other rhizosphere microorganisms suggests that the rice rhizosphere could be a potential site of active N_2 fixation. However we shall see hereafter that the expression of this potential is limited by environmental factors or agents, such as actinomycetes, that impede this activity. Besides, one should be aware that the occurrence of N_2-fixing populations as high as 10^7 to 10^8 microorganisms g (dry root)$^{-1}$ does not imply that the root surface is covered by a sheath of bacteria since the bacteria coverage on the root surface never exceeds 10%.

Table 4. Colonization of rhizosphere soil, rhizoplane and endorhizosphere of three rice-soil systems

Rice-soil system	Total microflora	% of total microflora	
		N_2-fixing bacteria	Actinomycetes
Rice cv IR26-Philippine Soil [116]			
Rhizosphere soil	2.3×10^7	2.4	n.d.
Rhizoplane	1.5×10^7	76	n.d.
Endorhizosphere	1.1×10^8	81	n.d.
Rice cv Moroberekan-Senegalese *sol gris* [97]			
Rhizosphere soil	2.0×10^8	53	1.7
Rhizoplane	1.8×10^8	25	4.1
Endorhizosphere	1.2×10^8	16	7.2
Rice cv Moroberekan-Senegalese sol Dior[a]			
Rhizosphere soil	2.4×10^9	17	0.5
Rhizoplane	1.6×10^9	28	0.1
Endorhizosphere	2.4×10^7	50	0.1

Number of microorganisms expressed per g dry soil rhizosphere soil or dry root (rhizoplane or endorhizosphere); n.d. = not determined
[a] From Rinaudo (unpublished data)

3. Factors affecting heterotrophic N_2 fixation in paddy soils

The ecological aspects of N_2 fixation by rhizosphere microorganisms have been recently reviewed [8, 12, 23, 35, 55]. The review of Dommergues and Rinaudo [35] particularly concerns the rice rhizosphere. N_2 fixation in the rice rhizosphere depends primarily upon the plant and the rhizospheric N_2-fixing bacteria. Climatic and soil factors also directly or indirectly affect the rhizospheric microorganisms through the plant.

3.1. Effect of the plant

3.1.1. Plant genotype. Several reports confirm that the plant's genotype influences its association with N_2-fixing bacteria in its rhizosphere [42, 47, 61, 63, 91]. According to Lee *et al.* [63] the levels of N_2 fixation measured by the acetylene reduction activity (ARA) are highly correlated with the rice dry root weight at heading stage, the amount of organic material supplied by roots to the rhizosphere probably being one of the main factors responsible for such correlation.

In a first experiment, Dommergues and Rinaudo [35] found that various mutants of two rice varieties (Cesariot and Cigalon) obtained by gamma irradiation exhibited rhizospheric ARAs ranging from 500 to 5,320 (Cesariot) and 860 to 7,370 (Cigalon) nmol C_2H_4 g (dry root)$^{-1}$ h^{-1} when grown in a non-sterile Camargue soil (Table 5). A second experiment was set up with part of the same plant material, the original Cigalon variety, and two rice mutants L, which exhibited the lowest ARA, and H, which exhibited the highest ARA in the first experiment, but growing in a non-sterile Senegalese *sol gris*. Unlike in the first experiment, the ARA of the three rice genotypes did not differ significantly (Table 5). The discrepancies in results between the two experiments were attributed to differences in the soil chemical and biological properties. These data lead to the conclusion that the results of screening of rice genotypes for their ARA should be interpreted with utmost caution and their generalization to undefined environmental conditions avoided.

Table 5. Rhizospheric ARA of rice cv Cigalon and two mutants grown in alluvial soil from Camargue and a Senegalese *sol gris* (3-week old rice seedlings)

Rice 'Cigalon'	ARA (nmol C_2H_4 g (dry root)$^{-1}$ h^{-1} ± S.E.)	
	Camargue soil (first experiment)	Senegalese *sol gris* (second experiment)
Original cv	4079 ± 1383	2589 ± 466
Mutant L	861 ± 456	3682 ± 1254
Mutant H	7368 ± 1971	2330 ± 595

S.E. = standard error of mean

3.1.2. Variations with time. Since climatic conditions vary with time, variations in the rate of some plant physiological processes and, consequently, in N_2- fixing activity associated with the plants, can be expected. Actually diurnal variations were reported by Balandreau *et al.* [11], Trolldenier [111], Rinaudo *et al.* [94] and Boddey and Ahmad [18]. Early reports on the variation in ARA throughout the rice-growing season indicated maximal activity at heading stage [63, 128]. *In situ* assays [18, 94, 120] confirmed this finding.

3.2. Climatic factors

3.2.1. Light intensity. When the plants are still young, the energy-yielding substrates available to N_2 fixers are mainly made up by root exudates. Since root exudation depends upon light intensity, shading 10-day old rice seedlings dramatically decreased rhizospheric ARA [34]. This decrease was attributed to a reduction of the carbon supply to the rhizosphere as ARA was similarly drastically reduced when 14- or 21-day old plants were decapitated. The diurnal fluctuations in rhizopheric ARA of rice seedlings reported by Rinaudo *et al.* [94] were thought to result from the close relationship of ARA to the photosynthetic activity of the plant. However, such relationship was not observed in more mature plants. Lee *et al.* [62] reported that decapitation of the shoot had little effect on *in situ* ARA within 24 hours. When a plant is aging, root lysates and root litter possibly provide N_2 fixers with an extra supply of energy-yielding compounds that greatly enlarge the carbon pool.

Light intensity may directly or indirectly affect the heterotrophic N_2 fixation in the non-rhizosphere soil. The direct effect is mainly related to the growth and activities of photosynthetic N_2-fixing bacteria. The indirect effect is the stimulation or inhibition of N_2-fixing heterotrophs in soil by the growth of photosynthetic N_2 fixers. The photosynthetic bacteria have been considered as important N_2 fixers in paddy soils [123]. The photosynthetic bacteria would have greater advantage than the blue-green algae in fixing N_2 under low light intensity and anaerobic environment.

The indirect effect of photosynthetic microorganisms on N_2 fixation can be associated with other heterotrophic N_2 fixers in paddy soils, but little information on their behaviour in the field is available [123].

3.2.2. Temperature. Relatively low temperatures limit: (1) photosynthesis, translocation, and exudation, thereby reducing the supply of energy substrate required by N_2 fixers [10] and (2) the activity of N_2 fixers. So, it is logical to assume that increased temperatures up to 30–35 °C would enhance rhizosphere ARA, although few data prove this assumption.

The effect of temperature on the non-rhizosphere soil is more related to the geographical factor than to the photosynthetic activity of plant and subsequent energy supply from roots to which it is greatly related on rhizospheric soil. The biological N_2-fixing activities are generally considered much higher in the tropical regions than in the temperate regions because of the temperature differences. A heterotrophic N_2-fixing bacterium, *Beijerinckia*, is reportedly distributed mainly in tropical regions [15]. However, its occurrence may not be due to the temperature differences but to some other soil factors such as pH or competition between microbes as reported by Becking [16].

3.3. Soil factors

3.3.1 Variation with soil type. Rinaudo *et al.* [93, 94, 95, 96] and Garcia *et al.* [38] showed that the same rice variety grown in different non-sterile soils exhibited different N_2-fixing abilities. For example, the ARA of 17-day old seedlings (Sefa 319 G) was 32 times higher when grown in Camargue soil (France), than when grown in Boundoum soil (Senegal). These differences in ARA could obviously be attributed to soil-related factors, indicating that soil is an essential part of the environment, which has often been overlooked. Of course further studies would be necessary to elucidate the roles of chemical and biological soil characteristics responsible for the variations in ARA such as those mentioned above.

3.3.2. Gaseous nitrogen. Flooded rice soil seems to have disadvantages for the biological N_2 fixation because of the limited supply of N_2 gas into the soil.

Takai *et al.* [106] reported that the amount of N_2 gas dissolved in flooded soils was generally constant for 2 weeks after flooding and the amount of methane gas increased considerably thereafter. If the flooded soils are planted to rice, N_2 gas would be supplied into soils through the plant's air-transporting system [131, 132]. In the absence of the rice plant, however, the partial pressure of N_2 gas in flooded soil may be inadequate to promote the N_2 fixation by heterotrophic N_2 fixers. The atmospheric N_2 gas diffuses into paddy water and further into the surface soil of the rice field. According to Magdoff and Bouldin [68], the concentration of N_2 diffused from the atmosphere approaches theoretically zero at a depth of about 6.6 cm below the surface of flooded soil.

Alternate flooding and drying provides favorable conditions for a high rate of N_2 fixation mainly because of the increase in the partial pressure of N_2 gas [68].

3.3.3. Inorganic nitrogen. Repression of nitrogenase synthesis by various forms of combined nitrogen in all types of N_2-fixing organisms (except derepressed mutants) is well established. Yoshida *et al.* [130] noted that 160 ppm of added combined nitrogen completely inhibited ARA in a rice soil system. Applied nitrogen, either as ammonium or nitrate, significantly inhibits the N_2-fixing activites of rhizosphere soil at 50 ppm N [66]. Trolldenier [111] found that the ARA of excised rice roots was significantly affected by an even lower application of combined nitrogen (10 ppm urea-N). However, the effect of combined nitrogen is more complex than expected. According to Balandreau *et al.* [9], the application of up to 40 ppm

NH_4-N (120 kg N ha^{-1}) caused no inhibition of ARA in the rice rhizosphere; on the contrary, ARA slightly increased probably because of the increase of exudate input into the soil. Similarly, Trolldenier [111] found that the application of up to 140 kg N ha^{-1} eventually caused an increase in rhizospheric ARA. Since rice plants absorb combined nitrogen all the more rapidly as they develop, nitrogenase repression occurs only at the early stages of growth. Starter doses of nitrogen fertilizers can therefore benefit plant growth without hindering biological N_2 fixation.

3.3.4. pH and inorganic elements other than nitrogen. Laboratory experiments indicate that the growth of pure culture of N_2 fixers is pH dependent. Most field surveys confirm these data: *Azotobacter* is generally restricted to soils of neutral and alkaline pH whereas *Beijerinckia* is more tolerant to acidic conditions [15]. *Azospirillum* species occur mainly in soils of pH 5.6–7.2 [31]. In contrast, the effects of pH on the overall N_2-fixing activity in the rhizosphere are unknown. A field survey [38] suggested that pH effect is less marked than expected.

The status of other elements besides mineral nitrogen may also affect N_2 fixation through alteration of plant exudation, about which little is known, however. Phosphorus content must be an important limiting factor, especially in poor soils, as reported for legumes. In long-term fertility assays in Thailand, Watanabe and Cholitkul [117] observed that when rice plants responded to phosphorus addition, N_2-fixing activity associated with rice was enhanced.

Trolldenier [110] found that potassium deficiency caused a decline in oxygen content and in redox potential, which might provide better conditions for N_2 fixation, but have detrimental effects on plant growth.

3.3.5. Soil oxygen and water regime. The soil water content does not directly affect N_2 fixation but controls it by affecting the rates of gas exchanges. Therefore, the effect of oxygen and that of the water regime on N_2 fixation cannot be dissociated.

The N_2-fixing enzymes are principally anaerobic and any system including them has an oxygen-scavenge system. Oxygen in air transported by the air-transporting system of flooded rice would be mostly consumed by respiration of root tissue which would provide favorable conditions for N_2-fixing bacteria in the rhizosphere.

Rice *et al.* [90] showed that the occurrence of an aerobic-anaerobic interface was of great importance for N_2 fixation in soil-straw mixtures: the products of the degradation of hemicellulose and cellulose in the 2 mm thick aerobic zone diffused across the interface to support N_2 fixation by *Clostridia* in the anaerobic zone. There are some similarities between such a model system and the rhizosphere of flooded rice, which is the site of an aerobic-anaerobic interface resulting from air diffusion from the leaves to the roots. Rice plants growing in anaerobic water-saturated soil can supply oxygen to the root, thus creating a gradient in oxygen concentration around its roots [5, 65, 83]. It is likely that in this gradient, a micro-zone with the oxygen concentration optimum for the development and activity of each of the various N_2-fixing strains that are diversely protected against oxygen, will exist (Rinaudo, unpublished data). Since this protection is generally not well developed in most free-living N_2-fixing bacteria, except *Azotobacter*, it is not surprising that rhizosphere ARA was constantly reported to be more pronounced when the plants were grown in waterlogged than in upland conditions [95, 128]. The addition of organic materials into paddy soils stimulates the respiration of aerobic microorganisms and enhances it to create an anaerobic environment in the paddy soil by expelling molecular oxygen and lowering the oxidation-reduction process. The quantity of oxygen supplied by diffusion through the floodwater into the soil is small and enough only in the few millimeters of surface soil. Magdoff and Bouldin [68] showed evidence supporting the hypothesis that N_2 fixation is enhanced when the products of anaerobic decomposition of cellulose are subjected to aerobic condition by such processes as diffusion, mixing, and drying.

3.3.6. Energy supply. Fixation of significant amounts of N_2 is dependent upon a suitable supply of carbon and energy. When plants are still young, root exudates and root lysates, mainly epidermal and cortical cells [70], are the major sources of energy substrates for rhizospheric microorganisms. With plant aging, root litter possibly provides N_2 fixers with an extra supply of energy-yielding compounds [35]. There is good evidence that the amounts of organic material released into the soil by the roots of actively growing plants are increased by root damage [7] and the presence of soil microorganisms [13].

The amounts of available substrates in the rhizosphere of rice grown under non-sterile conditions is poorly documented. At IRRI, the amount of exudates from IR8 and lR22 was evaluated by the isotope technique: the proportions of the total assimilated ^{14}C released into the soil were 1.9% and 2.2% at flower initiation, and 3.2% and 6.7% at harvest. But this amount of carbon indicated only carbon excreted from roots and remained in the rhizosphere soil, and did not include carbon mineralized by rhizosphere bacteria (IRRI Ann. Rep. 1973).

Data obtained by Martin [70] show a transfer of photosynthetate to the root system of field-grown wheat between early tillering and flowering that is equivalent to about 1,800 kg C ha^{-1}. 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^{-1} 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^{-1} N_2 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^{-1}. The actual amount of N_2 fixed per 1,000 kg available C ha^{-1} would be much less because no allowance has been made for root respiration, and that will induce competition for carbon among the different microbial populations, many of which may not be capable of N_2 fixation.' (K. Martin's comments on the communication presented by Dommergues and Rinaudo [35] at the Symposium 'Nitrogen and Rice' 1979, p. 259.)

In rice, N_2 fixers make up a rather large percentage of the total microflora so that one can assume that a significant part of the energy flowing out of the rhizosphere can be used for N_2 fixation if other limiting factors do not impede the process.

Most heterotrophic N_2-fixing bacteria in non-rhizosphere soil depend on available carbon materials for their field energy source. Organic materials in soil generally are not readily decomposable in nature as humic acid. Thus, organic materials incorporated freshly into paddies as dead plant tissues or root tissues left in soil would be a good source of energy in N_2 fixation by the heterotrophic bacteria in natural paddy soil environment. Assuming that the total dry weight of rice plants is 10 t ha^{-1} (25% as roots), organic matter residues that remain in a paddy field would be about 2.5 t ha^{-1}. Rice straw much more favorably stimulates N_2 fixation by heterotrophic bacteria in paddy fields. Fixation measured by the N-15 technique was equivalent to 42–45 kg N_2 ha^{-1} in a soil at field capacity and 13–150 kg N_2 ha^{-1} in flooded soil when the soil was amended with less than 1% straw [90]. Rates of fixation were as high as 500–1,000 kg N_2 ha^{-1} in the soils amended with 5–20% straw and incubated under flooded conditions in this study. However, that is not the usual case in the paddy field since 1% straw would be equivalent in amount to 20 t ha^{-1} assuming that 1 ha contains 2 million kilograms soil.

3.3.7. Interaction of N_2-fixing bacteria with other soil microorganisms. N_2-fixing bacteria thrive in the presence of other microorganisms that behave as antagonists or as synergists. Most of the studies related to such interactions have been performed *in vitro* [33, 54, 56, 64]. However some recent observations or experiments concern processes occurring in the rhizosphere of plants growing in non-sterile soils.

Some data suggested the antagonistic effect of actinomycetes on *Azotobacter* [21, 79, 105] or *Beijerinckia* [26]. Roussos *et al.* [97] pointed out that the low percentage of nitrogenase-positive isolates in the endorhizosphere of rice cultivar Moroberekan grown in Senegalese *sol gris* might be attributed to antagonistic actinomycetes.

Actually the effect of actinomycetes on root colonization by N_2-fixing bacteria appears to be more complex than expected. Thus by inoculating rice growing in a non-sterile soil with two actinomycete strains, Rinaudo *et al.* [96] observed a 25% decrease in the rhizosphere ARA (Experiment 1, in Table 6). But when a mixed inoculum comprising the same actinomycete strains plus an N_2-fixing bacterium was used, the rhizosphere ARA was 75% higher than when inoculation was made with the N_2-fixing strain alone (Experiment 2, in Table 6). The decrease in ARA observed in Experiment 1 was explained by possible antagonism between the introduced actinomycete strains and by the native N_2-fixing microflora. In Experiment 2, the increase in ARA resulting from the inoculation with the mixed culture was attributed to a synergistic interaction between the introduced N_2-fixing strain and the actinomycetes.

According to Remacle and Rouatt [86], in the early stages of barley, pectinolytic bacteria contribute to the decomposition of roots and seed reserves, and liberate available carbohydrates, thus stimulating the multiplication of *Azotobacter*.

Table 6. Inoculation of the rhizosphere of rice cv Moroberekan grown in a non-sterile soil (*sol gris*) by an N_2-fixing bacterium (F4) and two actinomycetes (Al and A15) [96]

Inoculum	ARA (nmol C_2H_4 h^{-1} ± S.E.)	
	Plant^{-1}	g (dry root)$^{-1}$
Experiment 1		
Control	173± 23	4640± 900
Al + A15	130± 83	3100±1950
Experiment 2		
F4	132± 34	3100± 950
F4 + Al + A15	234±176	5690±3850

S.E. = standard error of mean

Such a stimulatory effect was observed in the rhizosphere of rice grown in sterilized soil inoculated with a mixture of N_2-fixing bacteria and pectinolytic strains [24]. Interestingly, in a survey dealing with 34 *Azospirillum* strains isolated from the rice rhizosphere in Senegal, Diem *et al.* [24] found that 53% of the strains studied could use pectine as a substrate to fix N_2.

Other soil organisms may be involved in antagonistic processes. The results obtained by Rinaudo and Germani [92] show that nematodes of the genus *Hirschmanniella* (specific parasites of flooded rice) could be held responsible for the limitation of non-symbiotic N_2 fixation in submerged rice soils (Fig. 3).

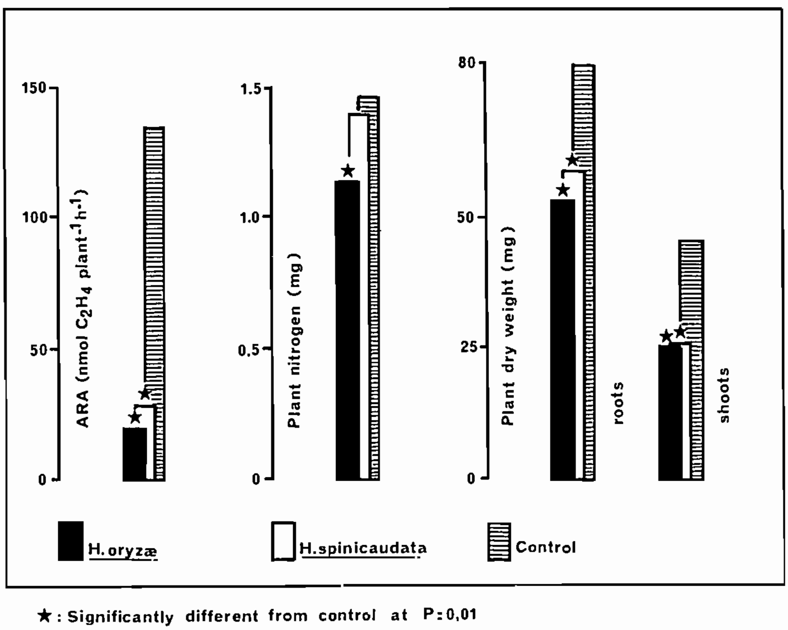

Fig. 3. Effect of the infestation of the soil with *Hirschmanniella oryzae* and *Hirschmanniella spinicaudata* on the ARA and the plant growth of 3-week old rice seedlings (cv Moroberekan) [92].

3.4. Conclusion

Comparing the symbiotic N_2-fixing system with the rice rhizosphere N_2-fixing system shows differences which are summarized in Table 7. In the rice rhizosphere, N_2 fixers appear to find a favorable niche, but there is no tight relationship between plant growth and activity of the N_2-fixing microorganisms probably because they remain in competition with the other components of the microflora. The looseness of the relationship between the plant and the N_2 fixers explains that this system is very sensitive to the effect of environmental factors and, thus is most unstable. Therefore a recent international symposium on N_2 fixation aptly recommended that this relationship, often termed as an 'associative symbiosis', should be referred to as 'biocoenosis' (National Academy of Sciences, 1979).

4. Managing N_2 fixation in paddy soils

The yield potential of a rice crop cannot fully be realized without the application of fertilizer nitrogen. However, in many rice-growing countries the use of fertilizers is very much limited, and yield production virtually depends on the natural fertility of paddy soil. Two major aspects concern the nitrogen nutrition to promote rice growth: (1) the enrichment of nitrogen element in paddy fields, and (2) the efficiency of nitrogen in rice production.

Rice growing countries with sufficient supply of chemical fertilizers, on the other hand, are currently very much interested in exploring a possible use of biological source of nitrogen for agriculture because of their environmental problems and shortage of energy sources.

To enrich the nitrogen element we should consider improving the environmental conditions for increasing N_2 fixation. The various factors related to the free-living or associative N_2 fixation already discussed and to the introduction of a symbiotic N_2-fixing system should be considered. The efficient use of fixed nitrogen in the field management by preventing nitrogen losses through leaching or denitrification, and the promotion of associative N_2 fixation in the rice rhizosphere by seed inoculation of N_2-fixing bacteria or screening of rice varieties for higher N_2 fixation are to be investigated in the years to come.

4.1. Organic matter application

The production of straw in Japan is 19 million and its direct application to paddy fields was recently widely adapted by Japanese farmers [107]. An investigation in 1972 indicated that the average production in Japanese paddy was 4.93 t ha^{-1} for grain and 4.89 t ha^{-1} for straw. Straw materials added to rice paddies should be sources of a good energy for N_2 fixers. Carbon dioxide released into air from the decaying straw material could be used by the crop for photosynthesis [125], and subsequently may affect the N_2 fixation in the rice rhizosphere.

Table 7. Comparison of legume and rhizosphere N_2-fixing systems

Nitrogen-fixing system	Bacterial strains	Specificity	Bacterial infection	Amount of energy (expressed in moles of glucose) required to fix 1 mole of nitrogen	Protection against oxygen	Fate of fixed N_2
Legume-*Rhizobium* symbiosis	*Rhizobium*	Certain	Intracellular	1	By plant cell and pigments such as leghae-moglobin	Directly used by the legume as NH_4^+-N
Rhizosphere N_2-fixing system	Many belonging to different genera, often associated with other microorganisms	Probably no 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	Protection by O_2 consumption of root respiration Partially protection by water-logging	Indirectly and partly directly used by the plant

However, in many Southeast Asian countries, the rice straw are traditionally burned to prevent rice disease problems. Williams *et al.* [122] reported no measurable difference in 5-year rice yield averages between burning and incorporation at any level of nitrogen fertilizers even when the rate of straw incorporation was increased to 30 t ha^{-1}.

The application of organic materials such as straw or cellulose stimulates biological N_2 fixation in flooded soils [14, 20, 67, 68, 71, 72, 73, 84, 89, 124]. However, there are a few reports on the actual measurement of N_2-fixing activity in rice field into which organic materials were incorporated during a growth period of rice plant [72, 114]. A field experiment by Matsuguchi [72] clearly indicated that the application of rice straw stimulated the N_2-fixing activity of paddy soils even with the addition of nitrogen fertilizer at 50 kg N ha^{-1} (Fig. 4).

Ponnamperuma [81] reported that straw incorporation, increased the total N content in paddy soil to 39–57 kg N ha^{-1} per season.

4.2. *Promotion of associative N_2 fixation*

The nitrogen nutrition of the rice plant is very much related to its photosynthetic activity and carbon metabolism. Carbon supply to the rice rhizosphere during the reproductive stages should be a very important factor in promoting the N_2 fixation by the associative N_2-fixing bacteria. It has been suggested that appreciable energy for heterotrophic diazotrophs may be supplied in a number of aquatic systems [55] and cereal crops [70]. Any method to stimulate rice plants to provide energy should

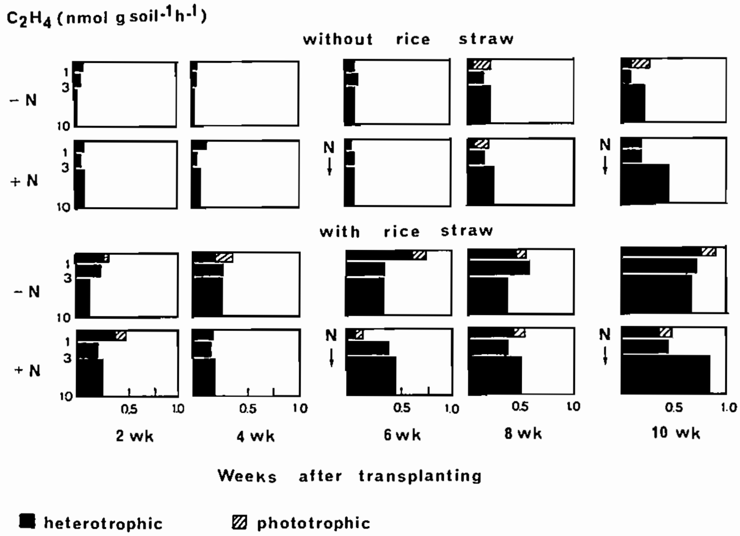

Fig. 4. Effect of N application on heterotrophic and phototrophic N_2-fixing activities in paddy plow layers (Tochigi Ando soil) with and without rice straw [72].

be considered significant and investigated. Hale and Moore [43] reviewed extensively the works on the various factors affecting root exudation such as plant, environment, and chemicals.

The associative N_2-fixation in the rhizosphere can also be promoted by the application of organic materials to the paddy field in addition to the N_2 fixation of free-living heterotrophs.

The screening of available rice cultivars for higher N_2-fixing activities in the rice rhizosphere should be worthwhile. Varieties seem to differ in N_2-fixing activity and the correlation between root weight and nitrogen fixing activity is high (Fig. 5). Further systematic screening for rice varieties in accordance with photosynthetic activity, color intensity of leaves, or root-oxidizing activity should be explored.

At the reproductive stage, the N_2-fixing bacteria are the majority of microflora

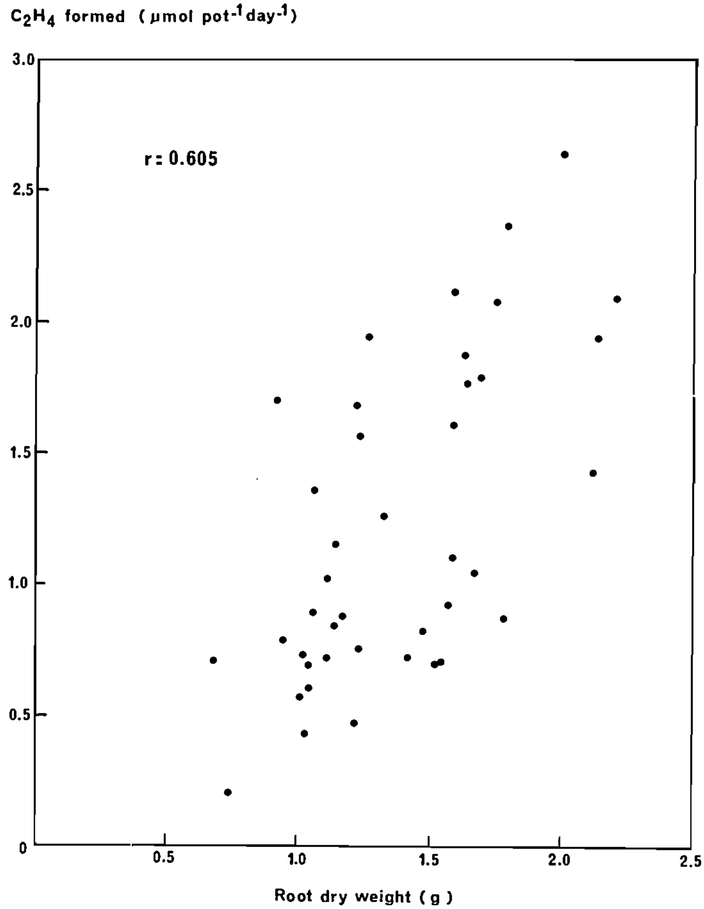

Fig. 5. Acetylene-reducing activity and root weight of 41 rice varieties (each value is the mean of 5 replications) [61].

inhabiting the rice rhizosphere [121]. The N_2-fixing bacteria inhabiting both the inside root tissue and surface root are closely associated with the rice plant. However, there is no report on specific N_2-fixing bacteria associated closely with rice roots. Artificial inoculation of some grasses with bacteria known to fix N_2 in the rhizosphere such as *Azospirillum lipoferum* was reported to increase the yields of grasses [104]. Successful inoculations of maize or wheat with *Azospirillum brasilense* have also been recently reported [46, 87, 88]. For rice, few studies have been carried out except by Gauthier and Rinaudo [39]. Using 32 different strains of *Azospirillum*, these authors showed that the rhizosphere ARA of 3-week old rice seedlings grown in test tubes could be significantly altered by inoculation: rhizosphere ARA could be either increased (+ 60%) or decreased (– 80%) in a non-sterile Senegalese *sol gris*. Such responses varied widely with the soil type. Another experiment carried out using similar experimental devices showed that inoculation with *Azospirillum brasilense* Sp7, significantly increased the plant growth, but this increase was not related to the ARA (Table 8). Thus it was hypothesized that growth-stimulating substances produced by *Azospirillum* Sp7 were involved. Up to now, this beneficial effect of inoculation observed in the case of young seedlings, has not been observed in larger-scale experiments. The lack of response in the latter experiments was attributed to the fact that introduced *Azospirillum* strains had been eliminated by competition, a process which did not occur in small devices, where the inoculum was much larger compared to the native microflora.

4.3. Field management

Maintaining soil in flooded conditions seems to be the key to the nitrogen enrichment in paddy soils [80]. Flood fallowing increased total N content by an average of 50 kg ha^{-1} per season in Maahas clay soil [81]. Rice plants readily translocated the nitrogen fixed in the rhizosphere into plant parts [52, 133]. The rhizosphere of flooded rice at the reproductive stage would have played a role in the nitrogen nutrition of rice by N_2 fixation, thereby contributing to rice yield.

The grain yield of rice correlates with the amount of nitrogen at the reproductive stages of rice plants, which determines the number of spikelets per unit area and percentage of ripened grains. In Japan rice plants showing high grain yield generally absorb 30–40% of the total plant N during the reproductive stages after panicle initiation. Japanese farmers now topdress nitrogen fertilizers to paddy rice at the reproductive stage. The nitrogen application between panicle initiation to heading stage is called 'Hogoe' and that between heading to maturity is called 'Migoe' in Japan.

However, application of fertilizer nitrogen appears to suppress the activities of N_2-fixing *Azospirillum* in the rice rhizosphere [76]. Trolldenier [109] reported that in a long-term fertility trial, the application of N, P, or K to the paddy field significantly stimulated the nitrogenase activity in the rice rhizosphere. Inoculation of N_2-fixing bacteria known as nitrogenase-depressed mutant strains, which are able to

Table 8. Influence of soil inoculation with *Azospirillum brasilense* Sp7, upon ARA and growth of 3-week old rice seedlings cv Moroberekan (Gauthier and Rinaudo, unpublished)

Soil	Inoculum	ARA ($nmol\ C_2H_2\ h^{-1}\ plant^{-1} \pm$ S.E.)	Plant growth: Aerial parts ($mg\ plant^{-1} \pm$ S.E.)	Plant growth: Roots ($mg\ plant^{-1} \pm$ S.E.)
Sol gris	0	60 ± 56	48 ± 18	68 ± 11
	+	50 ± 21	136 ± 13	85 ± 12
Bel Air	0	200 ± 33	75 ± 14	45 ± 5
	+	322 ± 146	106 ± 17	47 ± 3
Boundoum	0	61 ± 16	62 ± 15	48 ± 7
	+	47 ± 31	125 ± 18	84 ± 9

S.E. = standard error of mean

produce nitrogenase even in the presence of ammonium and to export large quantities of fixed nitrogen [99], may be significant in promoting N_2 fixation in paddy fields that received nitrogen fertilizers.

The chemical fertilizers in rice production should be applied without prohibiting the biological N_2-fixing activities. Similarly, some pesticides have a depressive effect on the heterotrophic N_2-fixing bacteria in soils [103]. Caution should be observed in their application in rice paddies. Symbiotic N_2 fixation by legume is still considered the most important biological process in agricultural industry since it greatly affects plant production of legumes and soil nitrogen fertility. Intercropping or mix-cropping of legumes with the rice crop may promote nitrogen enrichment in rice paddies. Legumes that are able to grow in flooded soil conditions and enrich soil with nitrogen should be worth studying [36].

4.4. Conclusion

Recent advancements in biological N_2-fixation studies are revealing the mechanisms involved in the natural environment of the nitrogen element in rice fields. However, they only answer the question why conventional rice farmers were able to produce rice for the last several hundred years without chemical fertilizers.

To produce more rice to feed the increasing populations under the circumstances of energy shortage we, in the rice-growing countries, should be aware of field management problems in promoting N_2 fixation in paddy soils. Unfortunately, there is yet no feasible N_2-fixation technique and it is hoped that a revolutional technology would soon solve this problem. At the same time it would be a very important consideration to manage field practice such as the application of fertilizers and other agriculture chemicals or water controls in a way to avoid inhibiting N_2-fixing activities by soil bacteria.

5. Epilogue

Only recently scientists have realized why paddy fields can maintain their nitrogen fertility for a long time. The rapid progress of N_2-fixation studies in this chapter, proves the importance of heterotrophic N_2 fixation in maintaining the nitrogen fertility in paddy soils under natural environments. However, the exact contribution of phototrophic N_2 fixers and heterotrophic N_2 fixers to the nitrogen nutrition of the rice crop is still unclear. It appears to depend on the environmental conditions of the paddy, plant age, or some other factors.

The major limiting factors in heterotrophic N_2 fixation in flooded rice paddies are the supply of organic materials as energy source and perhaps the supply of N_2 in paddy. The presence of the rice plant is apparently the most important factor. The maximum N_2-fixing activity of rice rhizosphere is reached at the plant's flowering stage, as in the case of nodule activity. At this stage, the rice plant has developed its root system and attained maximum values in dry weight, surface area, and air-transporting system. Flooding soil creates a unique ecological site, a so-called aerobic-anaerobic interface in both the soil surface and the rhizosphere of the rice plant. The ecological site seems important in the heterotrophic N_2 fixation in paddy.

Microbial studies of N_2 fixation in the rice rhizosphere should be promoted to identify the N_2-fixing bacteria that contribute most to the fixing of N_2. It is unlikely that a specific bacterial species is associated with rice roots as in the rhizobium–legume relationship.

In the coming years, varietal screening for higher N_2 fixation, inoculation of seed with an associative N_2-fixing bacterium, or management of paddy fields to make conditions favorable for heterotrophic N_2 fixation, would be further explored to promote biological N_2 fixation in rice paddy.

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