Asymbiotic N_2 Fixation in Paddy Soils

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1. INTRODUCTION

It is well known that, even in the absence of any fertilizer or manure, flooded rice (*Oriza sativa*) can grow in the same plot for centuries without any depletion of the yield. The maintenance of soil fertility has been assumed to result mainly from asymbiotic N_2 fixation by soil or water microorganisms.[1-3] Three categories of microorganisms may be involved: (1) *free photosynthetic microorganisms*, blue-green algae and photosynthetic bacteria;[4-6] (2) *symbiotic blue-green algae*, such as *Anabaena* associated with various *Azolla* species;[7-9] and (3) *heterotrophic bacteria* associated with algae or with the rice root system. Since excellent reviews have already been presented of the recent work on ecological aspects of N_2 fixation by the two former groups of microorganisms, this note concentrates on N_2 fixation by the free-living heterotrophic bacteria of the rice rhizosphere. Our aim is to provide a survey of the latest findings on: (1) the application of the C_2H_2-C_2H_4 assay to complex rice-soil systems; (2) the use of gnotobiotic systems (model systems); (3) factors affecting asymbiotic N_2 fixation in the rice rhizosphere; (4) field estimations of N_2[C_2H_2] fixation; and (5) the populations of diazotrophs in the rice rhizosphere.

2. APPLICATION OF THE C_2H_2-C_2H_4 ASSAY TO COMPLEX RICE-SOIL SYSTEMS

The various uses of the C_2H_2-C_2H_4 assay have been comprehensively reviewed.[10] However, some specific difficulties may arise due to sampling and incubation chambers, diffusion of C_2H_2 and C_2H_4 through the plant-soil system, duration of incubation and illumination during incubation, and extrapolation of N_2 fixation data in time and in space.

2.1. Sampling and Incubation Chambers

Three types of sampling and incubating procedures (for any plant-soil system) have been developed for field C_2H_2-C_2H_4 assays: (1) root excision or

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rhizosphere soil collection, followed by incubation in different kinds of closed chambers;[11,12] (2) extraction of soil cores, including the plants to be studied, which are incubated in air-tight containers;[13] and (3) field techniques employing internal standards.[14]

When plants are grown in jars or tubes under controlled conditions *in the laboratory*, the entire plant-soil system is usually incubated with C_2H_2 in different kinds of chambers (Fig. 1). Whatever type of plant-soil system is studied, the sampling procedure should disturb the system as little as possible. The use of fairly large undisturbed cores[13] or the field technique employing internal standards[14] is, therefore recommended whenever possible.

2.2. Diffusion of C_2H_2 and C_2H_4 through the Plant-Soil System of Rice

The rice plant transports O_2 from the atmosphere to the roots and the rhizosphere soil *via* tissue gas spaces.[15,16] Thus, when other gases, e.g. N_2 (ref. 17) and C_2H_2 (ref. 18), are present in the gas phase of a rice-soil system, they would be expected to diffuse readily to the rhizosphere through the plant. This assumption was only partly supported by the results of a preliminary experiment with C_2H_2 (Fig. 2). Rice (IR8)-soil (Boundoum) systems developed in the device D[19] (Fig. 1) were submitted to two different kinds of incubation. C_2H_2 was injected into either upper compartment U (6 replicates) or lower compartment L (5 replicates). Fig. 2 shows that slightly less C_2H_4 (14.8 ± 2 nanomoles/seedling. hr) was produced in the lower compartment when C_2H_2 was injected into the upper compartment than when it was injected into the lower one (19.9 ± 4.0 nanomoles/seedling. hr). Moreover, C_2H_4 accumulated in the upper compartment was much smaller than that in the lower compartment, indicating a poor diffusion of C_2H_4 from roots to shoots (the latter are made of tubes into which gases diffuse mostly through nce), one cannot entirely rely upon this pathway to ensure a satisfactory diffusion of C_2H_2 or C_2H_4 from the gas phase to rhizosphere and *vice versa*, especially when large cores or field devices[14] are operated. The lag, which occurs during C_2H_2-C_2H_4 assays (reported for the rice-soil system[20] as well as other complex systems[11]), might reflect to some degree this hindrance in gas diffusion.

2.3. Duration of Incubation

Estimations of N_2[C_2H_2] fixation rates based on long-term incubations are higher than those based on short-term incubations (Fig. 3). Thus fixation rates, estimated at the beginning of the light period, were only 0.5 micromoles C_2H_4/g dry root.h when based on 1 hr incubations, but were 3-4 micromoles C_2H_4/g dry root.h when based on 8 hr incubations. Corresponding rates, estimated at the beginning of the dark period, were 0.2 and 1.0 respectively.

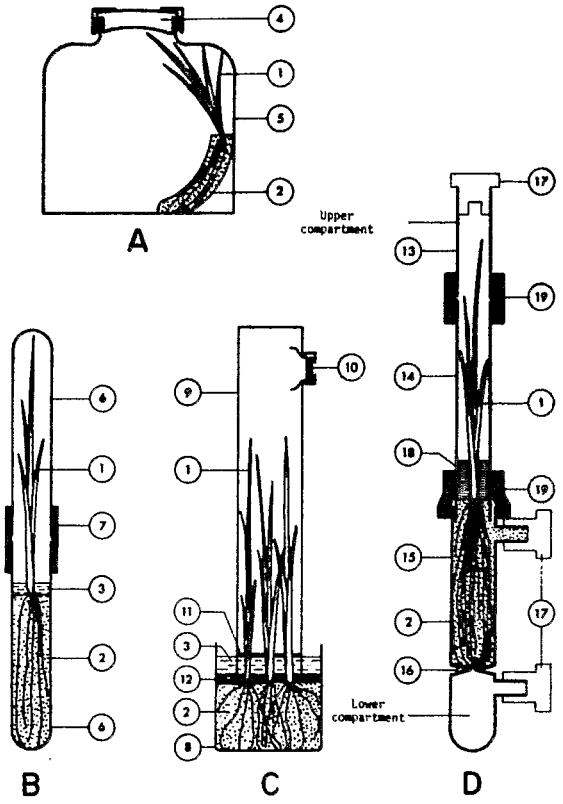

Figure 1.

Incubation devices for measuring $N_2[C_2H_2]$ fixation in rice-soil systems: A. classical serum bottle; B. double test-tube device; C. beaker with incubation canopy made of polyethylene bag; D. device allowing C_2H_2 separate injection into upper (shoot) compartment and lower (root) compartment.[19] 1. Rice seedling; 2. Soil; 3. Water; 4. Rubber cap; 5. Serum bottle (500 ml); 6. Pyrex test tube (14 x 140 mm); 7. Rubber attachment; 8. Beaker (150 ml); 9. Polyethylene bag (50 x 300 mm); 10. Sampling port; 11. Paraffin oil; 12. Red sand (for isolating algae from light); 13. Pyrex tube with one side arm (14 x 100 mm); 14. Pyrex tube (14 x 200 mm); 15. Pyrex tube with two side arms (18 x 180 mm); 16. Vigreux points; 17. Vacutainer stoppers; 18. Paraffin oil seal (for isolating algae from C_2H_2); 19. Vacuum tubing.

High $N_2[C_2H_2]$ fixation rates induced by long-term incubations may be attributed to either a direct effect of C_2H_2 on rhizosphere bacteria[21] or an indirect effect through a stimulation of exudation. Such stimulation could possibly result from pathologic reactions of the plant to C_2H_2 or C_2H_4, or,

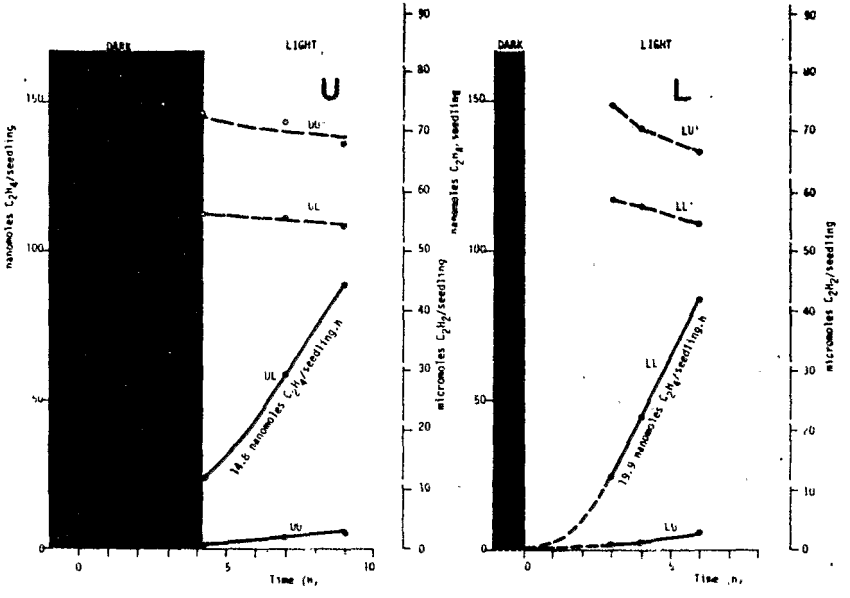

Figure 2.

Time course of C_2H_2-C_2H_4 reduction in a rice(IR8)-soil system, using incubation device D, Figure 1. U, *C_2H_2 injected into upper compartment*: UU, C_2H_4 produced in upper compartment; UL, C_2H_4 produced in lower compartment; UU', C_2H_2 remaining in upper compartment; UL', C_2H_2 remaining in lower compartment. L, *C_2H_2 injected into lower compartment*: LU, C_2H_4 produced in upper compartment; LL, C_2H_4 produced in lower compartment; LU', C_2H_2 remaining in upper compartment; LL', C_2H_2 remaining in lower compartment.

more likely, from an increase of photosynthesis due to the accumulation of CO_2 in the incubation chamber through soil respiration. Thus long-term incubations should not be used, except when there is no other way to overcome inadequate gas diffusion into the soil.

2.4. Illumination during Incubation

During incubation, plant-soil systems should be illuminated in conditions closely simulating those in the field because the activity of N_2-fixing bacteria in the rhizosphere is influenced by photosynthesis.[13,22] Experimental results summarized in Fig. 2 show that illuminating a rice-soil system previously placed in the dark promoted $N_2[C_2H_2]$-fixing activity. The problem of diurnal variations of $N_2[C_2H_2]$-fixing activity induced by day-night sequence will be discussed later.

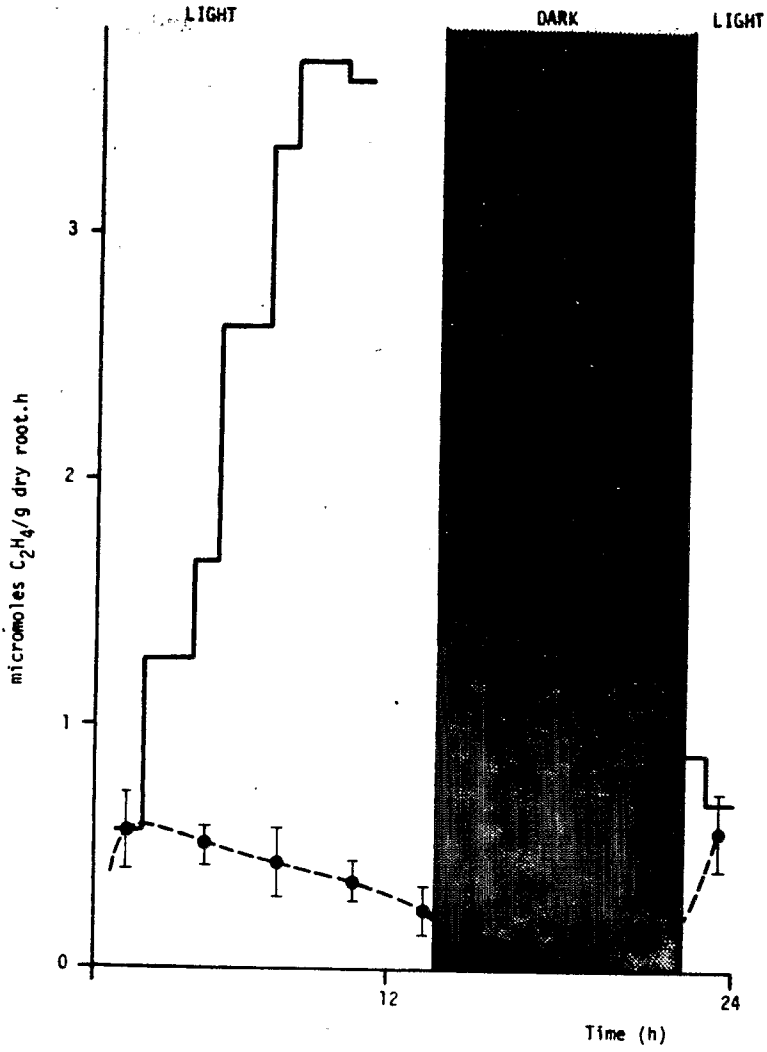

Figure 3.
Estimations of $N_2[C_2H_2]$. fixation of a rice(IR8)-soil system based on short-term or long-term incubations. ●---● diurnal variations of $N_2[C_2H_2]$-fixing activity based on short-term incubations, limits show standard errors of means; —— cumulated curve of C_2H_2-C_2H_4 reduction over long-term (up to 12 hr) incubations. For long term incubations, there were only 2 assays, thus no diurnal variation curve could be drawn.

2.5. Extrapolation of N_2 Fixation Data in Time and in Space

The conversion of N_2 fixation data obtained from necessarily limited sampling units (excised roots, cores, field incubation devices) to fairly large areas involves sampling on a distribution pattern taking into account variations in soil characteristics.[23] However, such spatial variations are assumed not to be of much consequence within most paddy fields. More important are diurnal and seasonal variations in N_2 fixation activity. These changes require that sampling be performed at regular intervals throughout 24-hr periods[22] and at many times throughout the year. Only integrated values based on data obtained in such a way can be considered valid.

3. USE OF GNOTOBIOTIC SYSTEMS

Ecological studies on N_2 fixation in the rice rhizosphere require the use of gnotobiotic systems comprised of sterile rice seedlings and a known population of diazotrophs. A rice (IR8)-*Beijerinckia* model system was found to fix N_2 as actively as did complex rice-soil systems studied in the laboratory (Table 1), the activity averaging 1,000 nanomoles/g dry root.hr. In the field, the extremely high activity observed (30,000 nanomoles/g dry root.hr) was attributed mostly to blue green algae. Gnotobiotic systems such as ours, which exclude algae, confirm the postulated high potentiality for N_2 fixation of rice-diazotrophic bacteria associations.

4. FACTORS AFFECTING ASYMBIOTIC N_2 FIXATION IN THE RICE RHIZOSPHERE

As the rhizosphere is by no means an isolated compartment of the plant-soil system, microbial activity in general and asymbiotic N_2-fixing activity in the rice rhizosphere in particular is dependent not only upon soil factors but also upon atmospheric parameters and, moreover, upon the plant itself. Table 2 summarizes the different factors that have been studied to date in the rice-soil system.

4.1. Climatic Factors

The effects of illumination have been investigated in growth chambers and in the field. In growth chambers, $N_2[C_2H_2]$ fixation of rice seedlings (20-27 days)-soil systems was 10 times lower at 3,000 lux (200 nanomoles/g dry root.hr) than at 30,000 lux (2,000 nanomoles/g dry root.hr).[24] Fig. 2 shows that, 1-2 hr after illuminating a rice-soil system, nitrogenase activity was significantly stimulated. In the field (Fig. 4), diurnal variations induced by the day-night sequence were noted;[22] in growth chambers, diurnal variation curves were similar, except for the absolute maximum activity, which was 1/10 that

Table 1.

$N_2[C_2H_2]$-fixing activity of various rice-soil systems and of a gnotobiotic system.

	Incubation		Presence of algae	$N_2[C_2H_2]$-fixing activity (nanomoles C_2H_4/g dry root.hr)	
	Device	Time(hr)		Day	Night
Field experiment					
Lamto soil, Ivory Coast (Taichung native 1 rice)	(a)	3	+	30,000	2,000
Laboratory experiments					
(i). Rice (IR8)-soil systems					
Djibelor soil, Senegal	A(b)	24	+		3,800
Djibelor soil, Senegal	B(b)	3	+	1,500	200
Camargue soil, France	C(b)	3	(c)	600	100
Boundoum soil, Senegal	D(b)	3	+		600
(ii). Gnotobiotic system					
Rice(IR8)-*Beijerinckia* sp.	(d)	3	0	1,000	

(a) Field device described previously.[14]

(b) Cf. Fig. 1.

(c) Algae were present, but their activity was stopped by application of sand and paraffin oil at the soil surface.

(d) Not described here.

Table 2.
Factors known to affect N_2 asymbiotic fixation in the rice rhizosphere.

Ecosystem compartments	Atmosphere	: light; CO_2; air humidity.
	Plant	: N_2 and O_2 diffusion through the plant; exudates.
	Rhizosphere soil	: water content; soil type; dissolved gases (N_2, O_2); inorganic and organic N; potassium.

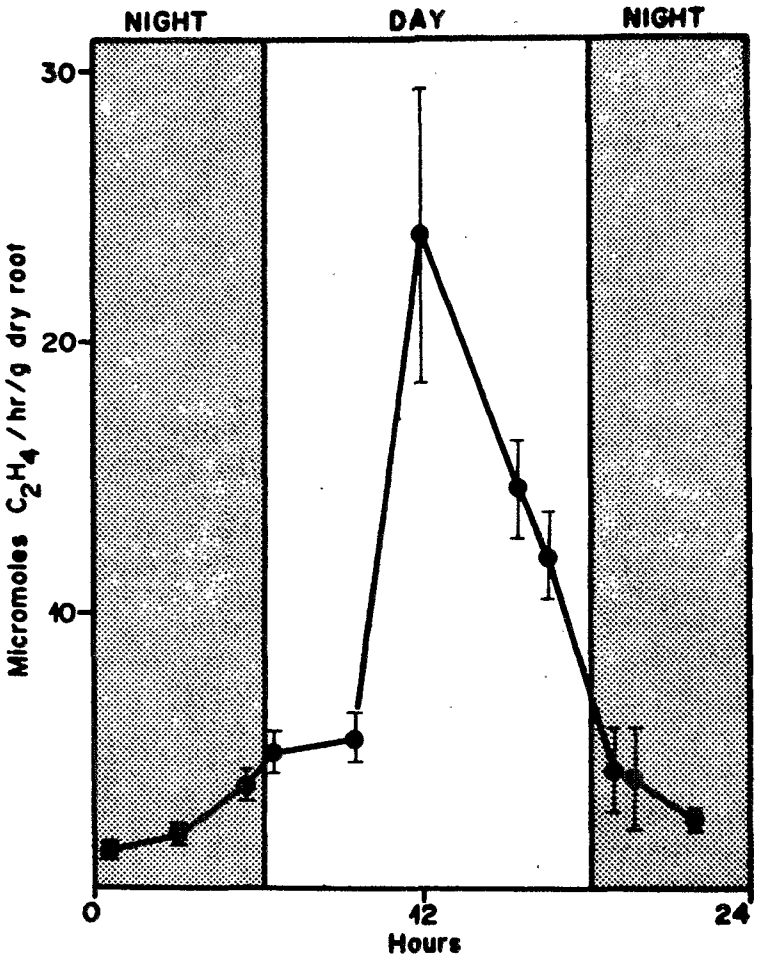

Figure 4.
Diurnal variations of $N_2[C_2H_2]$-fixing activity measured in a paddy field at Lamto (Ivory Coast). Limits show standard errors of means.

of the *in situ* (Fig. 5). $N_2[C_2H_2]$ fixation in some plant-soil systems was shown to be very sensitive to the water stress induced by a decrease in air humidity.[22] This effect is supposedly attributable to stomatal closure; in the case of flooded rice fields, however, this climatic parameter should not be a major limiting factor.

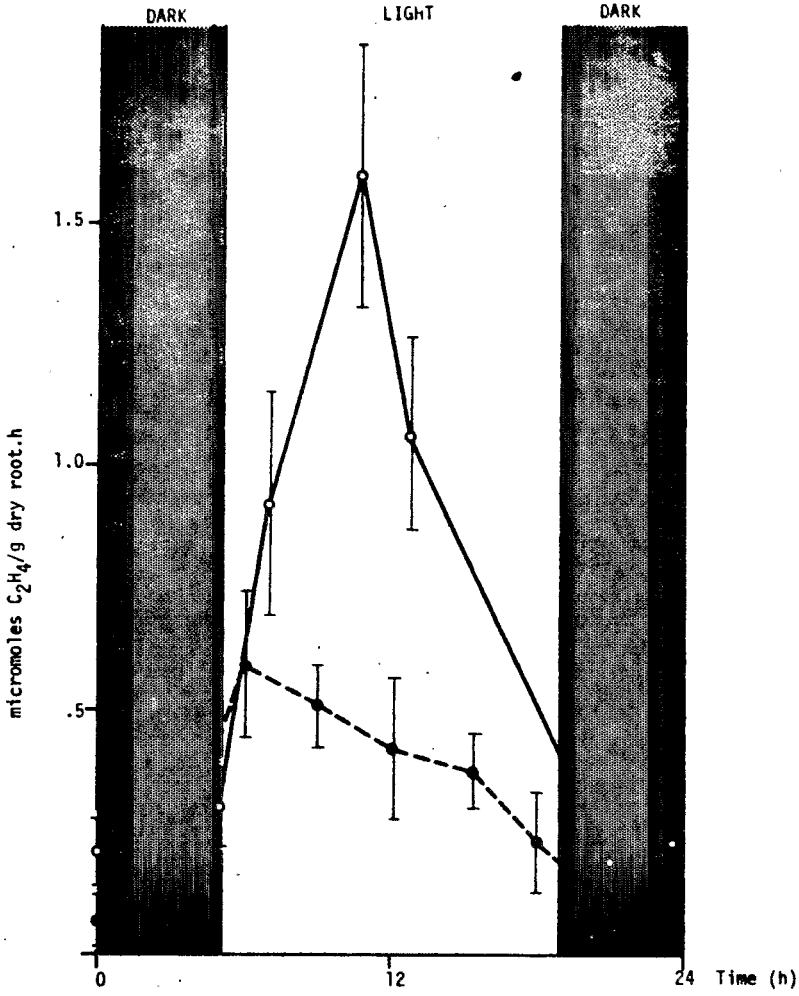

Figure 5.

Diurnal variations of $N_2[C_2H_2]$-fixing activity of two rice(IR8)-soil systems placed in growth chambers: ●—● with light duration of 14 hr, incubation device as shown in Figure 1.B, Boundoum soil; ●- -● with light duration of 15 hr, incubation device as shown in Figure 1.C, Camargue soil.

Atmospheric CO_2 concentration significantly affects N_2 fixation in symbiotic systems[26] and is assumed to play a similar role in rice-soil systems. Its significance in the field is, as yet, unknown, but preliminary laboratory investigations show that either CO_2 depletion or CO_2 accumulation (*vide supra*) during C_2H_2 incubation may explain some anomalous results. Thus, $N_2[C_2H_2]$-fixing activity of a gnotobiotic rice (IR8)-*Beijerinckia* system was only 300 nanomoles/g dry root.hr when growing in a chamber where the gas phase was not replenished, but exceeded 1,500 nanomoles/g dry root.hr four days after a CO_2 injection raising the CO_2 content of the gas phase up to 5,000 ppm (I. Fares-Hamad, personal communication).

4.2. Edaphic Factors

Waterlogging is known to favor asymbiotic N_2 fixation in any system.[27] The rice-soil system is no exception.[28] Yoshida's investigations[25,29] illustrate this fact and, in addition, stress the major role of the rhizosphere as $N_2[C_2H_2]$ fixation is consistently much higher in planted than in unplanted plots (Fig. 6).

Dissolved or adsorbed gases in paddy soils are apparently at least partially provided by the rice itself (*vide infra*).

Combined forms of N affect N_2 fixation in complex systems and pure cultures similarly. Yoshida *et al.*[30] noted that 160 ppm of added combined N completely inhibited $N_2[C_2H_2]$ fixation in a rice-soil system. Similar results were found by Balandreau *et al.*,[18] when 100 ppm of inorganic N inhibited 90 % of the $N_2[C_2H_2]$-fixing activity. If the absorption rate of inorganic N by rice is higher than the inorganic N influx in the rhizosphere, the rice plant could indirectly contribute to the derepression of N_2ase in the system. Soil organic N did not appear to restrict $N_2[C_2H_2]$ fixation, as three rice-soil systems with increasing organic N content (0.151 ; 0.257 ; 0.300 %) exhibited the increasing $N_2[C_2H_2]$-fixing activity of 10, 20, 38 nanmoles C_2H_4/g dry soil.hr respectively.[20]

Beside mineral N, the status of other nutrients, namely P, Mo and K, may also affect N_2 fixation by altering plant exudation. Trolldenier[31] found that insufficient K nutrition gave rise to larger excretion of organic compounds by rice roots, causing higher bacterial activity. This result suggests that N_2 fixation could be indirectly stimulated by K deficiency.

4.3. The Plant Factor

A most prominent, though poorly understood, factor controlling N_2ase activity in a plant-soil system is the plant variety.[11,32] Yoshida and Ancajas[3] reported that $N_2[C_2H_2]$ fixation of rice systems involving a *Peta* variety was four times higher than that of a system with an IR8 variety. The effect may

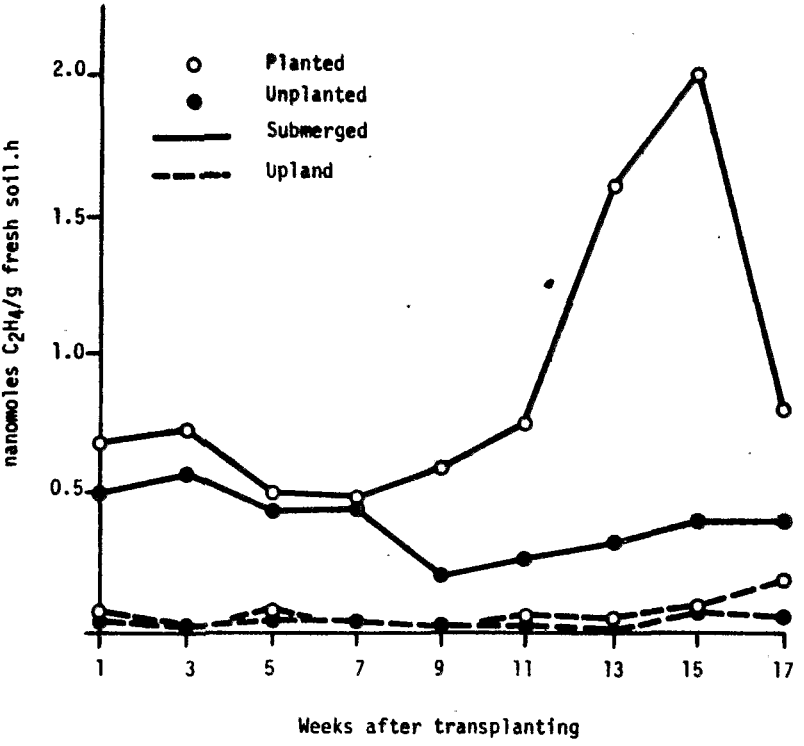

Figure 6.
Variations of $N_2[C_2H_2]$-fixing activity in planted (rice IR20) and unplanted upland and submerged soils during 1971 dry season at Los Baños (Philippines).[3,25]

be attributed to differences in: (1) exudate composition;[33] (2) amount of exudation; and (3) diffusion of gases (N_2, O_2) from the plant shoots to the rhizosphere.[29]

5. FIELD MEASUREMENTS OF $N_2[C_2H_2]$ FIXATION

Estimations based both on N balance and C_2H_2-C_2H_4 assays demonstrated the high potential of flooded paddy fields for N_2 fixation. A N balance of a paddy field in the IRRI experimental station suggested that N_2 input from diazotrophic organisms approximated 70 kg N_2/ha.yr as the N level of the soil remained constant (Table 3). C_2H_2-C_2H_4 assays performed in Japan, Philippines and the Ivory Coast gave figures of the same order of magnitude (Table 4). Other graminae-soil systems located in temperate (*Brachypodium ramosum, Dactylis glomerata, Lolium perenne, Zea mays*) or in tropical (*Loudetia simplex, Hypparrhenia* sp., *Panicum maximum*) conditions appeared

Table 3.
Nitrogen balance in IRRI experimental farm at Los Baños, Philippines.[25]

N-Source	Average N-output kg N/ha	Average N-input kg N/ha
Rice grain	50.0	—
Rice straw	25.0	—
Rain water	—	2.3
Irrigation water	—	3.8

to be much less efficient (Table 4). Paddy fields often deserve the reputation of highly effective N_2-fixing ecosystems; however, some poor N_2-fixing paddy fields also exist. Such paddy fields were recently studied in Morocco, where $N_2[C_2H_2]$-fixing activity in the soil was low, even though the activity was associated with planktonic *Gloeotrichia* species.[36] These data suggest that bacterial N_2 fixation in the rice rhizosphere was of limited importance there.

6. DIAZOTROPHIC BACTERIA OF THE RICE RHIZOSPHERE

Preliminary investigations showed that aerobic diazotrophs may predominate in the rice rhizosphere (Table 5), where a relative high pO_2 exists.[15,37] Diazotrophic non-photosynthetic bacteria isolated from rice rhizospheres belong to the following genera: *Pseudomonas, Azotomonas, Azotobacter, Beijerinckia, Flavobacterium, Arthrobacter, Bacillus, Clostridium.*[18,38-44] In many soils, even neutral ones, *Azotobacter* was recently found to play only a trivial role compared with other diazotrophs. Although the infrequent occurrence of *Beijerinckia* in temperate soils has been reported, it may have been overlooked elsewhere because of the lack of the sensitive C_2H_2-C_2H_4 procedure now in use in many laboratories.[18] Cultures of diazotroph associations grown in Feodorov-Kalininskaya medium* were found to reduce C_2H_2 more actively than did monospecific cultures (Fig. 7). However, the stability and efficiency of such associations in the rice rhizosphere have not yet been tested.

A strain of *Beijerinckia* sp. isolated from a rice rhizosphere in Camargue (France) profusely colonized rice roots in gnotobiotic systems (Figs. 8 and 9). The observed pattern of colonization was not very different from that of *Rhizobium* on *Trifolium* roots.[45] The colonizing ability of *Beijerinckia* strains therefore confirms previous results concerning the establishment of *Beijerinckia* in the rice rhizosphere.[38]

*Feodorov-Kalininskaya culture medium:[46] K_2HPO_4, 1.74 g; KH_2PO_4, 0.91 g; $MgSO_4.7H_2O$, 0.3 g; $CaCl_2.6H_2O$, 0.1 g; NaCl, 0.5 g; $FeCl_3.6H_2O$, 0.01 g; trace elements solution, 1 ml; yeast extract, 10 mg (expressed as N content); carbon source, 10 g; distilled water, 1,000 ml.

Table 4.

Field measurements of $N_2[C_2H_2]$ fixation in paddy fields and some gramineous ecosystems.

Ecosystems	Location	$N_2[C_2H_2]$ fixed		References
Paddy fields (*Oriza sativa*)				
—	—	10-200	kg N/kg dry soil.day	34
unplanted	Los Baños, Philippines	42.5	kg N/ha/dry season	25
planted	Los Baños, Philippines	79.8	kg N/ha/dry season	25
planted	Lamto, Ivory Coast	72	kg N/ha.yr	22
Gramineous ecosystems				
Brachypodium ramosum sod	Montpellier, France	2	kg N/ha.yr	this paper
Dactylis glomerata sod (cv. Floreal)	Nancy, France	9	kg N/ha.yr	this paper
Lolium perenne sod (cv. Viktoria)	Nancy, France	2-11	kg N/ha.yr	this paper
Zea mays (INRA $F_7 \times F_2$)	Nancy, France	1.5	kg N/ha.yr	14
Loudetia simplex savanna	Lamto, Ivory Coast	12	kg N/ha.yr	35
Hyparrhenia sp. savanna	Lamto, Ivory Coast	9	kg N/ha.yr	this paper
Panicum maximum sod	Lamto, Ivory Coast	4	kg N/ha.yr	22

Table 5.
MPN counts (in bacteria per g of dry soil) of N_2-fixing bacteria in the rice rhizosphere.[18]

	Aerobic	Anaerobic
Non-rhizosphere soil (control)	3 200 000	372 000
Rhizosphere soil	21 900 000	1 350 000
Rhizosphere soil + roots	61 500 000	472 600

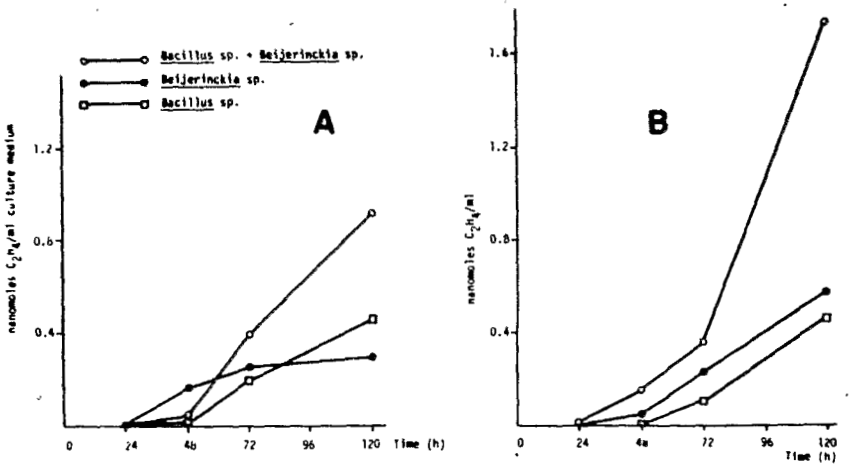

Figure 7.
Time course of C_2H_2 reduction by pure or mixed cultures growing in glucose Feodorov-Kalinnskaya liquid medium (A) or in starch Feodorov-Kalininskaya liquid medium (B).

7. CONCLUDING REMARKS

The flooded rice-soil system appears to be generally the most active asymbiotic N_2-fixing system known to date. However, our present ecological knowledge suggests that some formations growing in swamps and bogs, such as *Typha* sp. or *Cyperus papyrus* stands, could be as efficient.

Sen's hypothesis was that, beside blue-green algae, bacteria were responsible for the increase in the N content of some flooded soils when planted to rice.[47,48] This theory was validated in 1970 in both the field and laboratory[18,20,22,24,25,34,44] and more recently by studies (still unpublished) on gnotobiotic systems including heterotrophic diazotrophic bacteria but no Cyanophyceae.

The results presented here indicate that the C_2H_2-C_2H_4 assay can be used for the measurement of N_2 input in paddy fields and in laboratory rice-soil or gnotobiotic rice-diazotroph systems. However, improvements are

Figure 8.
Scanning electron micrograph of the root of a rice seedling grown in liquid medium and inoculated with *Beijerinckia* sp. (gnotobiotic system), showing mucigel around the root cap and a *Beijerinckia* sp. colony. Very few intact *Beijerinckia* cells are discernible, but the lipoid globules at the end of each cell are always visible. Gaps in the mucigel result from lyophilisation. Magnification x 13,000.

Figure 9.
Same material as Figure 8, but the picture is related to a root zone approximately one cm behind cap. The sheath of *Beijerinckia* sp. cells attached to the root is embedded in an amorphous layer, presumably of bacterial origin. Magnification x 13,000.

still required to overcome the various above-mentioned difficulties, especially those which impede field studies. The identity of the diazotrophic bacteria, their physiology and their ability to colonize roots, the respective role of photosynthetic and heterotrophic diazotrophs, the significance of primary ecological determinants (such as anaerobiosis, soil nitrogen forms, P, Mo and K availability), the balance between gross N_2 fixation and denitrification are but a few of the problems awaiting further experimental laboratory and *in situ* investigations.

From an agronomic point of view, stimulation of asymbiotic N_2 fixation is most desirable. In some conditions, algalization[49] or introduction of *Azolla* sp. in paddy fields has been a success. Thus far, no serious attempts at bacterization of rice roots have been performed. To the best of our knowledge, research on the behavior in the rice rhizosphere of derepressed mutants of diazotrophs already obtained by geneticists[50] has not yet been initiated.

Another interesting possibility would be to develop organic N fertilizers compatible with biological N_2 fixation, as was recently suggested by Hardy *et al.*[10] for legumes.

The last, and apparently most promising, though not exclusive approach would be to achieve stimulation of bacterial N_2 fixation in the rice rhizosphere by: (1) increasing the carbohydrate flux into the roots and the rhizoplane as suggested by Havelka and Hardy[26] for legumes; (2) increasing colonization susceptibility of the roots through genetic manipulation of the plant;[32,51] or (3) through any other means.[26] Future breeding programs with rice should accommodate this approach and should by no means be confined to increasing responsiveness to nitrogen fertilizers.

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