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HETEROTROPHIC NITROGEN FIXATION (ACETYLENE REDUCTION) ASSOCIATED TO FLOODED RICE:

A MODIFIED MEASUREMENT TECHNIQUE IN THE FIELD

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KEY WORDS

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ABSTRACT

A modified *in situ* technique for measuring heterotrophic nitrogen fixing (acetylene reducing) activity associated to rice is proposed. Ethylene evolution rates measured in opaque cylinders covering the stems of rice plants which have been cut 10 cm over the water level were found independent of the diurnal cycle. Cutting of the leaves resulted in decreased variation between plants and suppression of the acceleration of ethylene evolution rate after 12 h incubation as compared to intact plants. In both systems ethylene evolved was swept by a current of methane and the molar ratio between methane and ethylene was stabilized after 12 h. Methane evolution rates remained stable during 12 h and more than 24 h in whole plants and cut plants respectively. It is suggested that alteration in the active gas transport system after 12 h incubation under 10% acetylene may lead to erroneous evaluation of the actual ethylene production in the root's environment. The average values of ethylene evolution rates by cut plants between 12 and 24 h of incubation may be used for comparative studies of nitrogen fixing activity associated to flooded rice.

INTRODUCTION

Since its first description[19], the acetylene-ethylene assay for nitrogen fixation has been used extensively to study nitrogenase activity associated to flooded rice, and different *in situ* techniques have been developed which not disturb the soil-plant system[2,4,5,25,29,33,41]. Acetylene and ethylene are transported by the gas transport system of rice[26], but in assays where algal activity was suppressed, ethylene evolution was only evidenced after several hours of incubation[6]. Variations in the ethylene evolution rates observed during long-term assays[40] might be due either to increase of the acetylene reducing activity of

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nitrogenase exposed to acetylene[9,10], or to modifications in the gas transport system of plant sealed in gas-tight enclosures under 10% acetylene. This paper reports on a modified assay technique in which the transport effect was minimized and variations of ethylene evolution rates more demonstrative of its production in the root's environment.

MATERIAL AND METHODS

Experiments were performed in Camargue (South of France) during summer 1979 in an experimental field (1 ha) where flooded rice had been grown each summer for 3 years without pesticide treatment. Physicochemical analysis of a mean sample of soil collected prior to fertilization was performed in ORSTOM laboratories of soil science.

Basal fertilizer (formula 15, 20, 10) incorporated to ploughed soil contained 120 kg/ha of nitrogen element. 200 kg of rice variety Cigalon were sowed on May 11th, 2 days after flooding and the water level maintained between 10 and 15 cm during all the crop season. A yield of 4 t/ha was obtained at the harvest (October 10th).

Several physicochemical parameters of the flooding water were determined in the field at weekly intervals.

Intact plant systems for measurement of acetylene reducing activity, consisting of plastic cylinders (30 cm high and 5 cm diameter) sealed to polyethylene bags (40 cm high and 20 cm wide) were adaptated from previously described enclosures[25]. Pieces of rubber tubing were sealed by metal clips to close same size plastic cylinders when used as enclosures for cut plant systems. A hole (1 cm diameter) drilled near the top of each cylinder to prevent over-pressure was plugged after placing the enclosures in position. The cylinders were pushed into the soil approximately 5 cm depth around rice hills and maintained by a stick deeply drived into the sediment.

Injection and collection of gas were performed through needle puncture stoppers sealed in the polyethylene bags of intact plant enclosures[5] or the rubber tubing of cut plant cylinders. Unless otherwise indicated assays and controls were run in 10 replica.

Cut plant assay

Pure acetylene was injected to a concentration of approximately 10% of the total volume enclosed in whole plant and cut plant systems. 1 ml of diluted propane (25 ml propane and 670 ml air) was used as internal standard[3].

Transport of gas through rice plants

Time course of ethylene transport through whole plants (4 replica) and cut plants (4 replica) were compared using polyethylene bags (40 cm high and 20 cm wide) and modelling clay as described earlier[26]. 10 ml of ethylene were injected in the root bag and gas concentration in the upper bag determined every 3 h during 40 h.

The effect of ethylene concentration in the root bag on its transport through cut plants was determined during 28 h after injection of 0.1 ml, 1 ml and 10 ml of pure ethylene (3 replica each).

Short-term transport of gas through cut plants was evidenced by detecting ethylene and acetylene every 20 min during 140 min respectively from stem bag and root bag after injection of 1 ml ethylene in the root bag (4 replica) or 40 ml acetylene in the stem bag (4 replica).

Effect of preincubation with ethylene

It has been shown earlier[33] that the lag observed *in vitro* in the time course of ethylene evolution was suppressed by injecting ethylene in the enclosure. The effect of 12 h incubation under 0.1 m mole/l ethylene prior to acetylene injection was measured in cut plant systems *in situ*.

Effect of Propanil

12 h before the start of the experiment the water inside cylinders was removed and replaced by a solution (40 mg/l) of active Propanil[5]. The enclosures were only sealed prior to injecting acetylene.

Gas analysis

The samples collected in evacuated 3 ml Vacutainer tubes were analysed by ionization detection using a GIRDEL 3000 gas chromatograph on a Spherosyl XOB 075 column (1 m long). Injector, column and detector were at room temperature (20°C). Methane, ethylene, propane, and acetylene retention times were 27 sec, 48 sec, 63 sec, and 87 sec respectively. Calculations were based on a corrected value of ethylene leakage of 0.9 times the propane leakage when polyethylene bags were used[3,5].

RESULTS

Some physicochemical parameters of the plough layer soil are reported in Table 1. The experimental field is a silty soil rich in organic matter. The total nitrogen content of 10 m mole N/100 g dry weight is similar to paddy field soils from tropical countries[16,17]. One week after flooding the redox potential dropped to -200 mV at 3 cm depth ($+30$ mV at the interface) and the pH was neutral to slightly acidic (pH = 6.8) in the plough layer. The temperature remained stable around 22°C during night and day. The average total nitrogen content of the

Table 1. Some physicochemical parameters of the plough layer soil. Analysis of a mean sample after mixing of 30 samples of 100 g

Apparent density	1.38
Actual density	2.63
Porosity (%)	47.5
Clay, 0–2 μm (%)	20
Silt, 2–20 μm (%)	39
Silt, 20–50 μm (%)	22
Sand, 50–200 μm (%)	11
Sand, 200–2000 μm (%)	3
Total organic matter (% d.w.)	3.7
Total nitrogen (% d.w.)	0.14

flooding water was 1 mg/l, ammonium nitrogen was only 0.15 mg/l. Nitrate could not be detected in the water after 6 days of submersion.

As found previously[34], an appropriate transformation of data was necessary to satisfy the assumptions of the usual statistical methods. The regression of log S^2 on log $\bar{x}$ was used to determine the parameters of the Taylor's power law[38]. For all data of *in situ* measurements of ethylene evolution rates (48 groups of 10 values corresponding to whole plants, cut plants and Propanil treatment) the regression was $\log s^2 = -0.46 + 1.76 \log \bar{x}$, $r = 0.92$. When values from cut plant experiments without Propanil were used (28 groups of 10 results), the regression was $\log s^2 = -0.19 + 1.96 \log \bar{x}$, $r = 0.94$. The log normal distribution model could not be rejected and normalization of data was achieved by the transformation $y = \log x$, which has been used throughout this study.

Gas evolution rates during 45 h 30 incubation under acetylene in polyethylene bags (whole plants) or plastic cylinders (cut plants) are reported in Fig. 1 and Fig. 2. 95% confidence limits were calculated assuming a log normal distri-

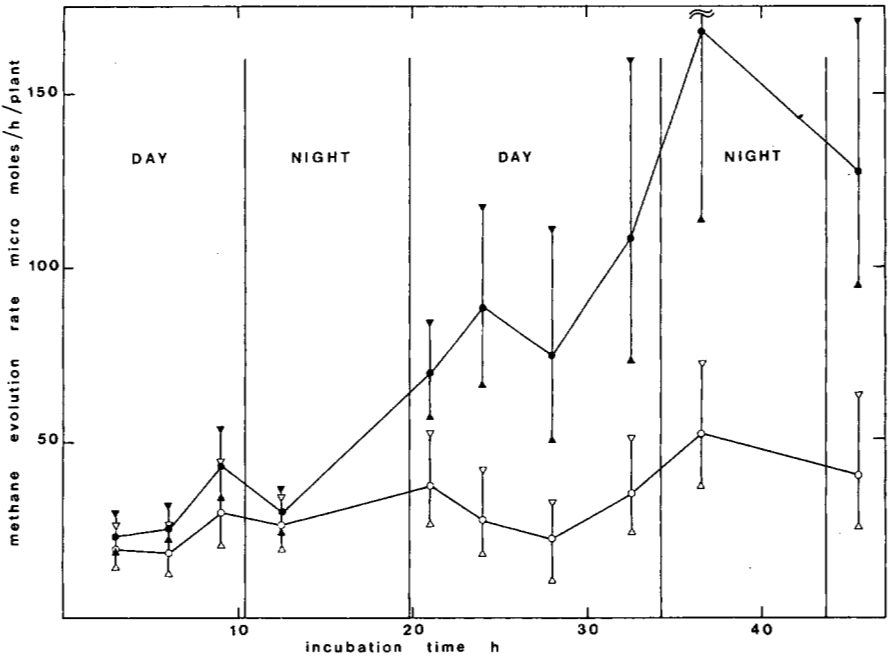

Fig. 1. Methane evolution rates in whole plant and cut plant systems incubated under acetylene. ●—● whole plants; ○—○ cut plants. Vertical lines indicate 95% confidence limits around the means of 10 replica. July 30 and 31, 1979.

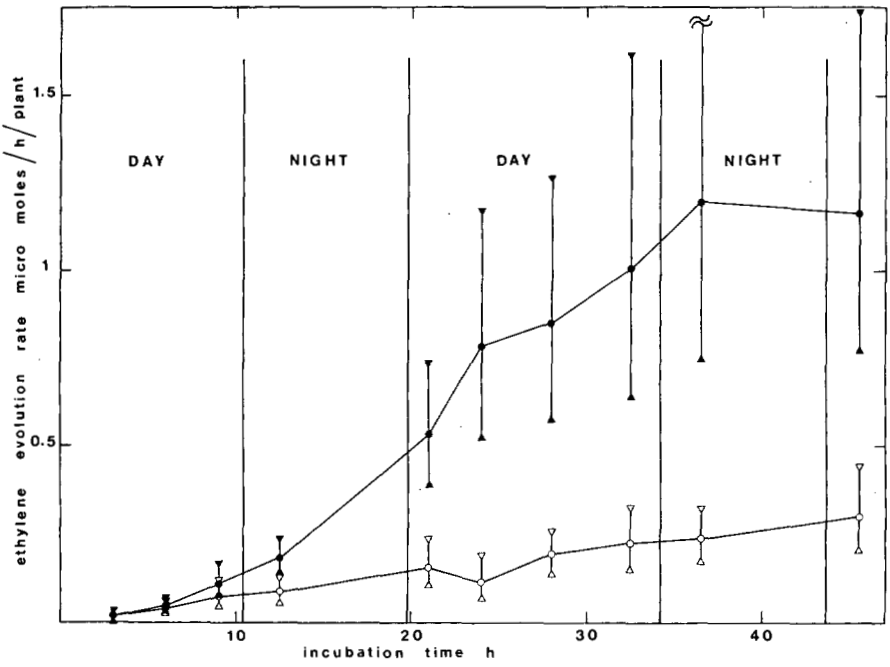

Fig. 2. Ethylene evolution rates in whole plant and cut plant systems incubated under acetylene. ●—● whole plants; ○—○ cut plants. Vertical lines indicate 95% confidence limits around the means of 10 replica. July 30 and 31, 1979.

bution of data. Acetylene concentrations decreased in both systems from 11.5% to 4% after 24 h and 3.5% after 45 h 30 but remained significantly higher in the plastic cylinders of cut plant systems.

In whole plant systems methane evolution rate increased from 24 to 169 micro moles/h/plant after 36 h incubation, but remained stable in cut plant systems during all the experiment. Comparison of variances was performed on transformed data by F-test. The variances were significantly different at the 5% level after 24 h and at the 0.1% level after 28 h. Equality of means was tested on transformed data of the first 24 h by the t-test, when variances were found not significantly different. Mean values of methane evolution rates were significantly different at the 1% level after 21 h incubation.

Similar results were obtained for ethylene evolution rates (Fig. 2). During the first 9 h variances and means were not significantly different. After 12 h 30 means were significantly different at the 1% level and after 21 h variances were significantly different at the 1% level.

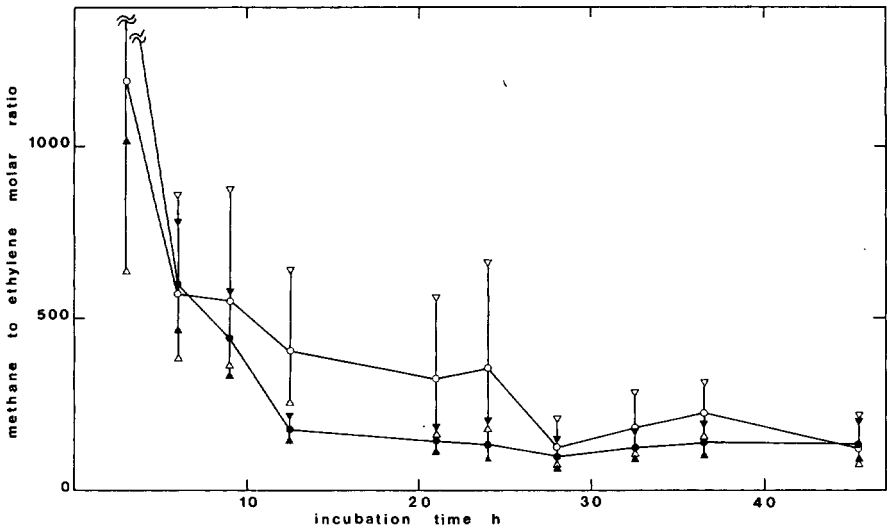

Fig. 3. Methane to ethylene molar ratio of gas evolved through whole plants and cut plants incubated under acetylene. ●—● whole plants; ○—○ cut plants. Vertical lines indicate 95% confidence limits around the means of 10 replica. July 30 and 31, 1979.

As indicated by the 95% confidence limits, variance between plants increased after 12 h incubation in whole plant systems and was very important after 24 h incubation. On the contrary variance between cut plant systems remained stable and lower during all the experiment.

Table 2. Ethylene transfer from roots of whole and cut plants. 10 ml ethylene were injected in the root bag at zero time. Results are the means and standard errors of 4 replica/treatment

Incubation time (h)	Ethylene evolution rates in the upper bag (micro moles/h)	
	Whole plant	Cut plant
3	0.38 (0.29)	0.36 (0.43)
6	0.22 (0.17)	0.38 (0.31)
10	0.31 (0.23)	0.19 (0.14)
14	0.17 (0.44)	0.20 (0.11)
17	0.10 (0.08)	0.06 (0.02)
21	0.25 (0.22)	0.14 (0.09)
24	0.17 (0.12)	0.28 (0.10)
40	0.16 (0.10)	0.18 (0.13)

Table 3. Ethylene transfer from roots of cut plants. At zero time, and after 24 h experiment, 0.1 ml, 1 ml, and 10 ml ethylene were respectively injected in the roots bags. Results are the means and standard errors of 4 replica/treatment

Incubation time (h)	Ethylene evolution rates in the stem bag (micro moles/h)		
	0.1 ml ethylene	1 ml ethylene	10 ml ethylene
3	0.01 (0.01)	0.16 (0.11)	1.98 (1.18)
6	0.03 (0.02)	0.12 (0.10)	1.52 (1.24)
9	0.02 (0.02)	0.21 (0.12)	2.06 (1.17)
19	0.01 (0.01)	0.15 (0.08)	1.47 (0.83)
24	0.02 (0.01)	0.16 (0.06)	2.30 (1.14)
25	nd*	0.16 (0.08)	2.15 (1.32)
26	nd	0.35 (0.19)	2.41 (0.94)

* nd: not determined.

Molar ratio between methane and ethylene evolved at different incubation times are reported in Fig. 3. In both systems 12 h were necessary to stabilize the relative concentrations of methane and ethylene. Unlike evolution rates, variance of the molar ratio between methane and ethylene evolved was more important for cut plant systems than for whole plant systems during the first 28 h but was not significantly different after.

Rates of ethylene transfer from roots of whole plants and cut plants are reported in Table 2. Means and variances of transformed data remained not significantly different during 40 h experiment.

Table 4. Rates of gas evolution during 140 min through cut plants. 40 ml acetylene and 1 ml ethylene were injected at zero time in stem bags and root bags respectively. Results are the means and standard errors of 4 replica/treatment

Treatment	40 ml acetylene in the stem bag	1 ml ethylene in the root bag
Incubation time (min)	Acetylene evolution rate in root bag (micro moles/h)	Ethylene evolution rate in stem bag (micro moles/h)
20	2.57 (0.75)	Traces in 1 bag
40	3.45 (2.04)	0.06 (0.03)
60	2.39 (1.57)	0.12 (0.03)
80	12.37 (7.71)	0.09 (0.03)
100	14.25 (9.78)	0.12 (0.03)
120	7.91 (1.76)	0.15 (0.05)
140	7.12 (2.94)	0.12 (0.03)

Influence of ethylene concentration in the root bag on gas transfer by cut plants was tested during 26 h and results are reported in Table 3. Injection of ethylene in the root bags was repeated after 24 h to maintain concentrations of approximately 4, 40, and 400 micro moles/l ethylene in the root atmosphere. During all experiment ethylene evolution rates in the stem bags remained constant and in the proportion of ethylene concentrations in the root bags. Controls (bag without plant and plant without ethylene) remained free of ethylene showing that gas transfer from roots was responsible for ethylene evolved in the stem bags.

Short-term measurements during the first 140 min of gas transfer are reported in Table 4. Acetylene and ethylene were transported through cut plants within 20 min, but 60 min were approximately necessary to observe the highest values.

Injecting ethylene at 0.1 m mole/l in the enclosure prior to the start of the experiment was not successful. During the first 16 h after acetylene injection, a

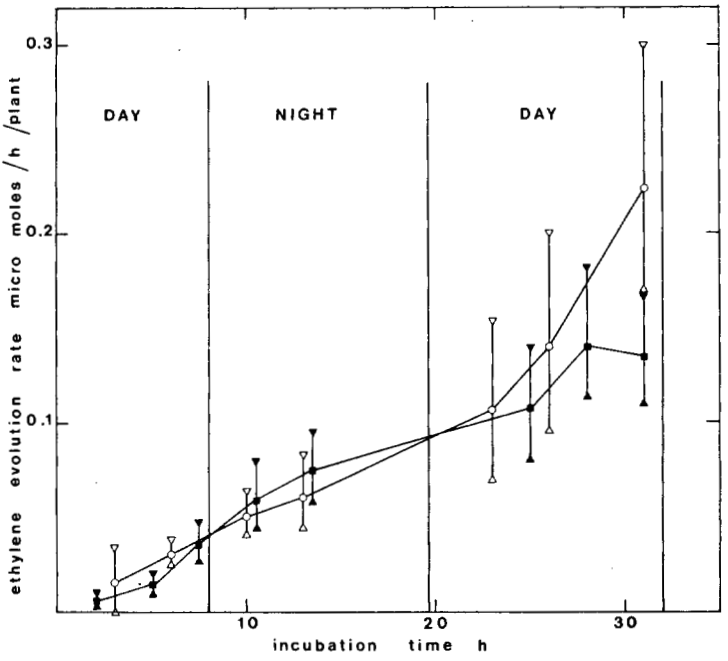

Fig. 4. Effect of propanil on ethylene evolution rate through cut plants incubated under acetylene. ■___■ Propanil treated enclosures; O___O controls. Vertical lines indicate 95% confidence limits around the means of 10 replica. September 11 and 12, 1979.

slight decrease of ethylene concentration was noticed, which might be attributed to transport to the root's environment through the rice plant. In order to decrease the remaining ethylene concentration in the atmosphere of the enclosures, it was decided to blow out the gas through the small apertures without disturbing the systems and to continue the incubation after reinjecting acetylene. Ethylene evolution rates were still erratic as compared to controls. Means of 0.05 ± 0.016 and 0.04 ± 0.015 micro moles/h/plant were measured at 22 h and 25 h respectively.

The effect of the photosynthetically active herbicide Propanil on the system is reported in Fig. 4. Variances and means of ethylene evolution rates in treated and control cylinders were only significantly different during the first 3 h of incubation, and at the last measurement after 31 h.

Ethylene evolution rates in cut plant systems without Propanil and ethylene treatment at different stages of rice growth are reported in Fig. 5. Means of 10 replica and 95% confidence limits were calculated after log transformation of data. As shown in Fig. 5, acetylene reducing activity increased during all experiments, and an acceleration occured after 24 h incubation in August 29, 30 and 31 (Fig. 5 B) and September 11, 12 (Fig. 5 C). The decrease observed between 21 h and 24 h on July 31st was not significant as indicated by variance and mean comparisons. Acetylene reducing activity was not related to photoperiodism.

As discussed below, means (M) and 95% confidence limits (CL) of ethylene evolution rates between 12 and 24 h of incubation were calculated to compare,

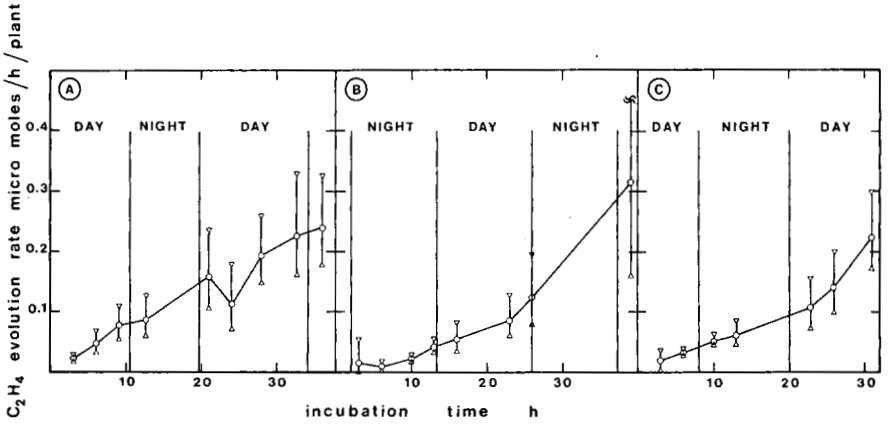

Fig. 5. Seasonal variation of nitrogenase activity in the experimental field of Camargue. O—O ethylene evolution rates measured at: A, heading stage; B, flowering stage; C, ripening stage. Vertical lines indicate 95% confidence limits around the means of 10 replica.

in the experimental field, nitrogenase activities associated to rice variety Cigalon at different growth stages (10 replica each). Significantly higher values were measured at heading stage (M = 100 nano moles/h/plant, 95% CL = 65–152) than flowering stage (M = 55, 95% CL = 40–92) and ripering stage (M = 85, 95% CL = 60–120). Using a conversion factor of 4 : 1 between acetylene and nitrogen reducing activities[9], these values correspond to 0.60, 0.33 and 0.51 micromoles nitrogen reduced/day/plant respectively.

DISCUSSION

Log normal distribution of *in situ* measurements of acetylene reducing activity in rice field has been evidenced earlier[34]. We found a better agreement with the mathematical model when only cut plant systems in opaque cylinders were considered.

Since nitrogen fixation in flooded paddy soils has been shown to be associated to the plant, relative contributions of photosynthetic and heterotrophic activities have been discussed[23,30,32,40,45,46,47], but under the climatic conditions of Camargue heterotrophic activity has been found predominent[35]. In the experimental field blue-green algae were never abondant and rare after 1 month of flooding (A. Vaquer, personal communication) as found previously in Camargue[7,22]. Therefore we were mainly interested by heterotrophic nitrogen fixation, and the final purpose of our work was to demonstrate the contribution of sulfate reducing bacteria to this process.

Algal photosynthesis is completly inhibited by 2 mg/ml Propanil (A. Vaquer, personal communication) but concentrations up to 43 mg/l have no effect on heterotrophic fixation[5]. As ethylene evolution rate was not affected in the enclosures by incubation with 40 mg/l Propanil, it can be assumed that photosynthetic nitrogen fixation is negligible in opaque cylinders.

In laboratory experiments nitrogen fixation was not correlated to the soil ammonium content[27,29], and nitrogenase activity of soil was not inhibited by 134 kg/ha of nitrogen applied as fertilizer[31]. Furthermore, fertilization improved nitrogen fixation[39] and immediate depressing effect of ammonium addition has been shown to be alleviated in few hours after NH_4^+ removal by the plant[42]. Therefore it can be assumed that nitrogen applied in basal fertilization prior to flooding had no inhibitory effect on the nitrogen fixing activity associated to the rice rhizosphere after 3 months, and that the plough layer with reducing conditions, a high organic matter content and a constant temperature of 22°C was a favorable environment for heterotrophic nitrogen fixation[27].

Previous works have evidenced that in paddy field rice acts as a channel for

the diffusion of gas from the atmosphere to the root system[1,18,26]. This property is used for *in situ* measurements of nitrogenase activity and problems related to acetylene and ethylene diffusion in the system have been discussed. Among those are the lag observed *in situ* before ethylene evolution in the enclosures which can be attributed to the time course of gas transfer through the plant, and to gradual saturation of the soil and water[6,25]. In laboratory experiments different methods were successful in suppressing the lag[20,33] but cannot be used in the field. Stirring the soil would increase the amount of ethylene recovered in the vapor phase of only 15% in the conditions of our experiments[15,40], but avoid dynamic studies of the process. Preincubation under acetylene before the start of the experiment has been proposed[6], but long-term exposure of nitrogen fixing organisms to acetylene causes an increase in the specific activity and derepression of nitrogenase synthesis[9,10]. The rapid increase observed after 24 h incubation of whole plants under acetylene has been attributed to these effects.

From comparison of methane evolution through whole and cut plants we suggest that another effect is also responsible for the increase of the ethylene evolution rate. It can be seen from Fig. 1, Fig. 2, and Fig 3 that an important flow of methane (about 250 times the flow of ethylene) acts in the plant gas transport system as vector for ethylene produced in the root's environment, and that a lag of 12 h or more was necessary to stabilize the relative concentrations of methane and ethylene evolved through whole and cut plants. Therefore it is likely that ethylene evolution rates measured before 12 h are not related to the actual ethylene production in the root's environment.

Production of methane in the reducing horizon of the paddy soil and it's release through rice has been evidenced earlier[13,24]. As described previously[13] but unlike *in vitro* experiments[28] acetylene did not change methane evolution rates through plants *in situ*. It is suggested that transport of methane through rice might continue even after inhibition of methane production due to the importance of gas accumulating in the reducing horizon, as noticed by bubbles containing methane evolving if the plough layer is disturbed. However we observed a significant increase of methane and ethylene evolution rates in whole plants after 12 h as compared to cut plants (Fig. 1 and Fig. 2), while molar ratio between gas remained similar in both conditions. It is suggested that an 'active gas transport system' of whole plants was significantly affected after 12 h incubation under acetylene, unlike the 'passive transfer' through cut plants. Variance between 'passive transfer' was significantly lower during 28 h than between 'active gas transport system'.

It was previously described that detachment of the aerial part of the rice

plant did not affect the nitrogen fixing activity of the root remaining in the field[25], and that acetylene reduction in the phyllosphere of rice (excluding the stem part up to 10 cm from the soil) was negligible[41] unless inoculating practices[21].

From laboratory experiments (Tables 2 and 3) we have found that cut plants were able to transfer acetylene and ethylene from stems and roots respectively as did whole plants[26], and that ethylene transfer rate was related to it's concentration in the root's atmosphere. Short-term measurements indicate that the steady stage of gas transfer was not achieved before 1 h (see Table 4). This lag was attributed to dilution of gas in the lacunae in ^{14}C fixation experiments, in which 1 h incubation could underestimate the fixation by as much as 40%[37]. Therefore diurnal variations of nitrogen fixation observed during 1 h experiments should be more likely attributed to photosynthetic nitrogen fixing activity of blue-green algae in the water layer than to heterotrophic utilization of plant exudates in the rhizosphere[4].

Ethylene production by bacteria in reduced microsites of soil[36] and it's cooxidation by methane oxidizers[8] could interfer with assays in the rooting environment[13,14,44], though acetylene inhibition of ethylene cooxidation has been demonstrated *in vitro*[11,12].

From all considerations above we agree with previous statement[5] that 'there is unlikely to be any straight-forward relationship between *in situ* measurements of ethylene evolution rates and true nitrogen fixing activity'. Therefore acetylene-ethylene assays should be restricted to comparative studies between varieties, stages and conditions of growth of flooded rice. The cut-plant assay described was found more convenient to handle in the field than whole plant enclosures, thus allowing more replica. Moreover, it was found that variance between plants was decreased. Ethylene evolution rates were not reliable before 12 h and after 24 h, but mean values between 12 h and 24 h of incubation were calculated and a significantly higher nitrogen fixing activity was measured at the heading stage for Cigalon variety in Camargue, as for IR 26, IR 36 and IR 38 varieties under tropical conditions[6,43].

However it was not possible to evidence from our data the relationship between nitrogen fixing rates and rhizospheric bacteria, if there is any. Further experiments are necessary to evaluate the relative contributions of the stem portion and the rhizosphere, as it has been found in tropical fields that the lower part of the stem could be colonized by heterotrophs responsible for an important nitrogen fixing activity[43].

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