

# EPIPHYTIC NITROGEN FIXATION ON LOWLAND

## RICE PLANTS

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### ABSTRACT

*Epiphytic nitrogen fixation on the submerged part of the rice stems was examined by:*

- studying the distribution of acetylene-reducing activity (ARA) and epiphytic algae among the hills at tillering stage;
- enumerating and identifying epiphytic microorganisms on the outer and inner leaf sheaths;
- measuring ARA and evaluating algal populations at seedling, tillering, heading and maturity stages of rice growth.

Dark and light ARA ( $\mu\text{mole C}_2\text{H}_4 \text{ h}^{-1} \text{ hill}^{-1}$ ) exhibited a log-normal distribution (L-shaped histogram; mean = standard deviation) while the total algal flora had an asymmetrical histogram, indicating the presence of several dominant epiphytic species.

Total and  $\text{N}_2$ -fixing algal populations on the outer parts of the stems ( $3.5 \times 10^5$  and  $1.2 \times 10^5$  cells (g fresh weight)<sup>-1</sup> respectively) were about twenty times higher than those of the inner parts. A similar distribution was observed with  $\text{N}_2$ -fixing bacteria (outer parts:  $2.9 \times 10^7$  cells (g fresh weight)<sup>-1</sup>; inner parts:  $1.0 \times 10^5$  (g fresh weight)<sup>-1</sup>) where the dominant types were related to the Enterobacteriaceae, associated with Azospirillum-like organisms. A macroscopic epiphytism by Gloeotrichia sp. was observed at seedling (2 t ha<sup>-1</sup>, fresh weight) and tillering stage (0.5 t ha<sup>-1</sup>), whereas only a microscopic epiphytism was present at heading and maturity stage, with Nostoc spp. as dominant species.

Light ARA declined along the cultivation cycle from 51  $\mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$  at seedling stage to 2.5  $\mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$  at maturity whereas dark ARA remained low throughout (0.3 - 2.5  $\mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ ). This corresponds to an input of 2 kg N ha<sup>-1</sup> crop<sup>-1</sup>.

### INTRODUCTION

Epiphytic nitrogen-fixing activity in a rice field ecosystem can develop on rice plants and weeds within a submerged habitat, in which the epiphytic microorganisms are protected from certain adverse environmental factors like desiccation and high light intensities. In the previous paper (Kulasooriya *et al.*, 1981), we have dealt with epiphytic nitrogen fixation associated with weeds. Watanabe & Barraquio (1979) and Watanabe *et al.* (1979) have reported on bacteria associated with rice stems. This paper reports on nitrogen fixation by blue-green algae and bacteria epiphytic on lowland rice.

### MATERIALS AND METHODS

Experiments were conducted on field-grown rice plants (IR26) without algal inoculation and fertilization. Epiphytic microorganisms and their nitrogen-fixing activities (NFA) were examined by:

- studying the distribution of acetylene-reducing activity (ARA) and epiphytic algae among the hills at tillering stage;
- enumerating and identifying epiphytic microorganisms on the outer and inner leaf sheaths;
- measuring ARA and evaluating algal populations at seedling, tillering, heading and maturity stages of rice growth.

#### *Assessment of the epiphytic microbial populations*

*Algae.* Depending on the quantity of algae present on the host, different methods were used for their evaluation. At seedling stage, when a very dense growth was observed the direct

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fresh weight was determined of the epiphytic algae dislodged from their host. At tillering, a visible growth was still present but insufficient for direct weighing. Biomass was calculated from chlorophyll measurements on algal material removed from the stems. Chlorophyll was measured after acetone extraction using Mackinney's (1941) specific absorption coefficient. Fresh weight was calculated using a ratio of 30.5 mg chlorophyll-*a* per gram fresh weight determined from the same algal material. These measurements were done separately on 35 hills, harvested from the same plot, in order to study the variability of algal epiphytism among rice hills.

At heading and maturity stages where epiphytism was not observable by the naked eye, algal enumerations were done on BG11 media (Allen & Stanier, 1968) with and without combined nitrogen for total and N<sub>2</sub>-fixing algae respectively, as described earlier (Kulasooriya *et al.*, 1981).

**Bacteria.** Bacterial enumerations were conducted by the MPN method as described by Watanabe *et al.* (1979) for N<sub>2</sub>-fixing Enterobacteriaceae and *Azospirillum*-like organisms. Total heterotrophic bacteria were enumerated by plating according to Watanabe & Barraquio (1979).

#### Host biomass measurements

After harvesting the whole plant, the root system and the aerial parts above the flood water level were cut off; the remaining material was used for ARA and fresh weight measurements and algal enumeration.

#### ARA measurements

Light and dark ARA measurements were carried out in the laboratory as previously described (Kulasooriya *et al.*, 1981) using cut rice stems. At seedling stage, parallel measurements were done *in situ* and in the laboratory to compare ARA under these different conditions.

At tillering stage, cut stems of 35 rice hills from the same plot were separately incubated to study the variability of the ARA among rice hills.

At heading and harvesting stages ARA measurements were done on 10 g triplicates randomly selected from the mixed material from the entire harvest of a plot of 35 hills.

At heading stage, the outermost leaf sheaths (outer parts) were separated from the inner parts of the tillers. Samples from these two sets were used separately for ARA measurements and enumerations of epiphytic microorganisms.

## RESULTS

### Epiphytic organisms

Of the epiphytic algae, *Gloeotrichia* sp. produced a visible growth on the rice stems at seedling and tillering stage. This growth could be observed irrespective of whether the host material was living or dead. Furthermore *Gloeotrichia* colonization was also observed on synthetic material such as nylon strings.

*Gloeotrichia* epiphytism decreased from seedling to tillering stage, mainly due to algal masses getting detached from their hosts as a result of gas bubble formation within the colonies. It was also noticed that colonies attached to the living parts were more easily dislodged than those attached to the dead parts.

At heading and harvesting stages, algal epiphytism was observable only under the microscope and during these stages the dominant N<sub>2</sub>-fixing species was *Nostoc*, together with *Calothrix*, *Tolypothrix* and *Gloeotrichia* as associated species. At heading stage, N<sub>2</sub>-fixing blue-green algae constituted 36% of the total epiphytic algal flora (Table 1).

Bacterial enumerations done at heading showed the presence of N<sub>2</sub>-fixing acid-gas producing bacteria (Enterobacteriaceae) as well as *Azospirillum*-like organisms. The presence of these bacteria on rice has been already reported by Watanabe *et al.* (1979).

Results of the comparison of epiphytism on outer and inner leaf sheaths (Table 1) indicated that both ARA and microbial colonization of the outer parts was much higher than on the inner parts irrespective of the type of microorganisms. Experiments using labelled N<sub>2</sub>-gas have also shown a higher N<sub>2</sub>-fixing activity on the outer surface of stems than on the inner parts (Ito *et al.*, in press).

In the case of algae this may be related to light availability. N<sub>2</sub>-fixing algae present on the inner leaf sheaths (5.3 x 10<sup>3</sup> (g fresh weight)<sup>-1</sup>) were mainly spores or inactive forms as demonstrated by the negligible difference between dark and light ARA measurements on the inner leaf sheaths. The much higher density of bacteria on the outer parts may be interpreted on the basis that outer parts contain partially decomposing material that provides suitable substrates for bacterial growth.

Table 1. Distribution of ARA ( $\text{nmole C}_2\text{H}_4$  ( $\text{g fresh weight}^{-1} \text{h}^{-1}$ ) and epiphytic microorganisms (number ( $\text{g fresh weight of host material}^{-1}$ ) between outer and inner parts of rice stem at heading stage

	Outer sheath	Inner sheath	Whole stem (leaf sheaths + culm)
ARA Light	2.5	0.14	1.9
Dark	0.5	0.11	0.47
Total algal flora	$3.5 \times 10^5$	$1.7 \times 10^4$	$1.4 \times 10^5$
$\text{N}_2$ -fixing algae	$1.2 \times 10^5$	$5.3 \times 10^3$	$4.8 \times 10^4$
Total aerobic heterotrophs	$4.7 \times 10^8$	$3.0 \times 10^6$	$1.8 \times 10^8$
$\text{N}_2$ -fixers on glucose (Enterobacteriaceae)	$2.0 \times 10^7$	$9.5 \times 10^4$	$7.5 \times 10^6$
$\text{N}_2$ -fixers on malate ( <i>Azospirillum</i> -like)	$9.5 \times 10^6$	$9.5 \times 10^3$	$3.7 \times 10^6$

#### Variation of epiphytism among rice hills

Light and dark ARA among 35 hills from the same plot are depicted in Fig. 1A and B, in the form of histograms. Both histograms exhibited a characteristic L shape; mean and standard deviation of the variables were very close to one another. These features indicate a log-normal distribution of ARA in the light and in the dark. Similar results have been reported for ARA by soil algae and bacteria (Roger *et al.*, 1977).

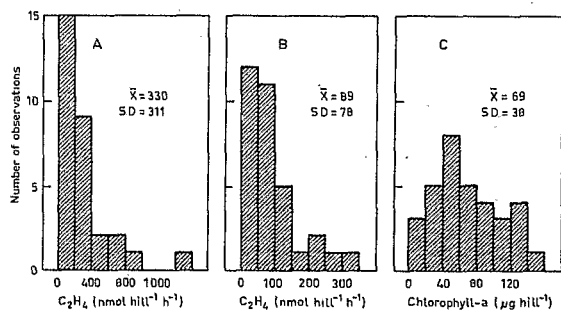


Fig. 1. Histograms showing the variations of: (A) light ARA; (B) dark ARA and (C) epiphytic algal chlorophyll, among 35 hills from a rice field at tillering stage.

This large variability of ARA among the hills implied that subsequent measurements should be done on replicates obtained from mixed material from the complete harvest of a plot and not on a few randomly selected hills. The distribution of epiphytic algae on the rice plants, determined as chlorophyll-*a* per hill was not log-normal (Fig. 1C). The asymmetrical histogram indicates that algae other than *Gloeotrichia* had also contributed to these pigment measurements. This was confirmed by plating dislodged algal material, which showed the presence of several associated blue-green algae, mainly *Oscillatoria*, *Pseudoanabaena* and *Nostoc*.

#### Variations of epiphytism and ARA along the cultivation cycle

A remarkable change was found in the algal epiphytism along the developmental cycle of the rice plant, with a corresponding change in the light ARA (Table 2). At seedling stage, when the rice stems had an epiphytic *Gloeotrichia* biomass of about 2 t fresh weight  $\text{ha}^{-1}$ , ARA in the light was  $51 \mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ . At tillering, when this biomass had diminished to 0.5 t fresh weight  $\text{ha}^{-1}$  it still had an activity of  $15 \mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ . The algae exhibited the same specific activity at these two stages (about  $2.4 \text{ nmole C}_2\text{H}_4$  ( $\text{mg protein}^{-1} \text{ min}^{-1}$ )). A similar specific activity was reported by Finke & Seeley (1978) for *Gloeotrichia* epiphytic on *Myriophyllum*. At heading and maturity, algal epiphytism was not visible to the

naked eye and the light ARA had decreased to low values: 1.2 and 2.5  $\mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ , respectively. Nevertheless, enumerations done on the rice stems showed the presence of several epiphytic  $\text{N}_2$ -fixing algae with *Nostoc* as dominant species.

These results show that the algae, though present during these stages, probably existed to a large extent as quiescent cells or propagules and contributed very little  $\text{N}_2$  to the crop.

Table 2. Acetylene reduction activity (ARA), biomass and rate of  $\text{N}_2$ -fixation of blue-green algae<sup>a</sup> on rice stems, at different stages of crop growth

		Growth Stage			
		Seedling	Tillering	Heading	Maturity
ARA	$\text{C}_2\text{H}_4$ ( $\mu\text{mole m}^{-2} \text{ h}^{-1}$ )	51.0	15.0 <sup>b</sup>	1.2 <sup>b</sup>	2.5 <sup>b</sup>
	Light				
	$\text{C}_2\text{H}_4$ ( $\mu\text{mole (g fresh weight of stem)}^{-1}$ )	614.0	37.5 <sup>b</sup>	1.9 <sup>b</sup>	1.1 <sup>b</sup>
	$\text{C}_2\text{H}_4$ ( $\mu\text{mole m}^{-2} \text{ h}^{-1}$ )	-	2.2	0.3	2.5
Dark	% of light ARA	-	27	25	100
Biomass	Fresh weight ( $\text{kg ha}^{-1}$ )	2037	553	-	-
	Number (g fresh weight of stem) <sup>-1</sup>	-	-	$4.8 \times 10^4$	$5.9 \times 10^4$
$\text{N}_2$ -fixation	( $\text{nmole C}_2\text{H}_4$ (mg protein) <sup>-1</sup> min <sup>-1</sup> )	2.3	2.5	-	-

<sup>a</sup> dominant species: *Gloeotrichia* sp. and *Nostoc* spp.

<sup>b</sup> *in situ* values extrapolated on the basis of an activity under artificial light, equal to 55% of the *in situ* activity.

Along the crop cycle, dark ARA remained low (0.3 to 2.5  $\mu\text{mole C}_2\text{H}_4 \text{ m}^{-2} \text{ h}^{-1}$ ) and relatively unchanged from tillering to maturity. The range of dark ARA on rice stems (bacterial activity) was in agreement with the results reported by Watanabe *et al.* (1979).

#### DISCUSSION AND CONCLUSION

Among the epiphytic bacteria,  $\text{N}_2$ -fixing Enterobacteriaceae and *Azospirillum*-like forms corresponded to 8% of the total aerobic heterotrophs and their contribution to the epiphytic NFA was low.

Epiphytic NFA was primarily due to a visible growth of *Gloeotrichia*, which was predominant during the early stages of rice growth. The ARA decrease observed from seedling to tillering and thereafter was mainly due to a decrease of the epiphytic *Gloeotrichia* that detached from their host and became floating.

Towards the latter part of the crop cycle a "microscopic epiphytism" mainly due to *Nostoc* and *Calothrix* had a very low activity. This decrease in algal biomass and its activity was possibly related to a dramatic decrease in light availability due to the start of the rainy season and an increased rice canopy. Results obtained are insufficient to explain fully the relationship between the algal epiphytes and their host, but certain inferences can be drawn.

*Gloeotrichia* has been reported to be epiphytic on aquatic plants (Freymy, 1930; Finke & Seeley, 1978). However, according to our experience, it does not exhibit any selectivity between dead and living, organic or inorganic material, but seems to grow preferentially on rough surfaces as indicated by the following observations:

- epiphytism on *Chara*, which has a rough corticated surface was much more than on

*Najas* (Kulasooriya *et al.*, 1981).

- colonies on living, smooth rice stems get detached more easily than those on dead plant material which has rough surfaces as demonstrated by Howard-Williams *et al.* (1978).
- colonization was observed even on old, rough nylon strings but not on new smooth ones placed into the flood water. Similar colonization on polyethylene strips has been reported by Finke & Seeley (1978).

In the case of "microscopic epiphytism" it was also observed that most of the isolated epiphytic strains grew adherent to the surface of the culture vessels and rarely formed floating colonies. The results obtained do not permit confirmation of either the existence or the absence of biotic relationships between the algae and the host, but indicate that both a mechanical effect in relation to the roughness of the support and an ability of certain strains to grow attached to a support are involved in algal epiphytism. From the ARA measurements of these experiments the  $N_2$  input by organisms epiphytic on rice can be evaluated as a few (2-3)  $kg\ ha^{-1}\ crop^{-1}$ , mainly due to the activity of *Gloeotrichia*.

In terms of nitrogen supply, algal epiphytism may appear to be of little value, but it has an important role in providing an inoculum potential for the regeneration of  $N_2$ -fixing algal blooms which are affected periodically by adverse conditions.

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