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EPIPHYTIC NITROGEN FIXATION ON DEEPWATER RICE

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Epiphytic N_2-fixation in deepwater rice was studied by (1) microscopic examination of the rice plants at different growth stages, (2) measurement of the acetylene-reducing activity (ARA), and N_2-fixing algal enumeration at the heading and maturing stages, and (3) bacterial enumeration at the maturing stage.

A high rate of N_2-fixing activity (5.1 μmol·plant^{-1}·hr^{-1}) was observed mainly due to blue-green algae (BGA) which developed preferentially on submerged, decaying tissues of the host. A unique finding was the presence of BGA inside the leaf sheaths. The observations support the idea that the algal epiphytism and endophytism observed on rice are probably related to a mechanical effect, in relation to the roughness of the decaying tissues, rather than biotic relationships.

Extrapolation of the N_2-fixing activity to the field scale corresponded to an input of about 10–20 kg N·ha^{-1}·crop^{-1}. It can therefore be concluded that epiphytic N_2-fixation on deepwater rice makes a substantial contribution to the ecosystem.

Key Words: deepwater rice, blue-green algae, epiphytism, N_2-fixation.

Studies on epiphytic N_2-fixation in rice and in weeds of wetland rice fields (*2*, *4*) have indicated that the epiphytic microorganisms make a limited contribution to this ecosystem. One reason for this was the low biomass of host available for colonization under the wetland field conditions. On the other hand, in the case of deepwater rice, a large part of the plant remains under water and offers a much greater biomass for colonization by aquatic microorganisms. Furthermore, the submerged stems produce clusters of nodal roots which grow freely into the floodwater. There have been brief reports on the presence of N_2-fixing blue-green algae (BGA) in deepwater rice (*3*) and on photodependent nitrogen-fixing activity associated with their nodal roots (*1*). Extracellular products from the epiphytic microflora on such roots may constitute an appreciable source of nutrition and supply part of the N_2 requirements of these plants.

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Studies were therefore undertaken to examine the epiphytic microorganisms and their N_2-fixing activities in deepwater rice.

EXPERIMENTAL DETAILS

Deepwater rice plants (DW 6255) were grown in pots containing 7 kg (dry weight) of Maahas clay soil (Aquic Tropudalf) with a basal dressing of 500 mg N·pot^{-1} as ammonium sulfate. Thirty-five days after transplanting, the pots were transferred to the field in a deepwater plot (14 × 38 m), of which the water level was progressively increased by 10 cm every other day to a final depth of 110 cm which was maintained until maturity. Two foliar applications of Furadan and three foliar sprayings of Azodrin were given during the cultivation cycle to control insect pests that may attack the rice plants. The water was slightly contaminated by insecticides.

The epiphytic microorganisms and N_2-fixing activity associated with them were studied by microscopic examination at 3-week intervals. Algal and bacterial enumerations and measurements of acetylene-reducing activity (ARA) were made at the heading and maturing stages. At heading, 7 plants were taken out, their aerial (non-submerged) parts and roots in the soil were cut off, and the remainder of the material was used for measuring ARA and enumerating the epiphytic algae.

ARA was measured on four replicates (30 g fresh weight each) randomly taken and incubated with 10% acetylene in air, in 900 ml plastic cylinders, at room temperature (30–32°C), either under light (800 lux) or in the dark (wrapped in aluminum

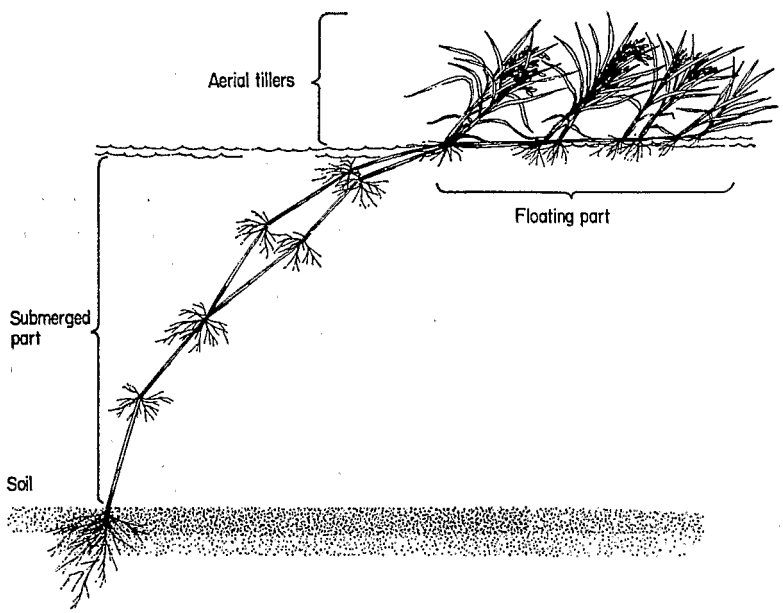

Fig. 1. Diagram of a deepwater rice plant at maturity.

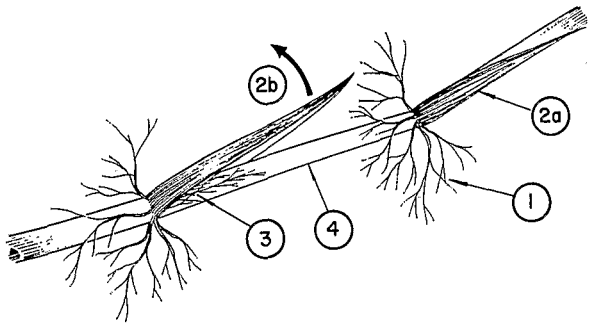

Fig. 2. Component parts of deepwater rice available for epiphytism.
1. Exposed roots. 2. Leaf sheath: (a) in position, (b) opened out.
3. Inner roots. 4. Culm.

foil). Gas samples were removed after 30 and 90 min of incubation, for chromatographic analysis. Algal enumerations were carried out as described previously (*4*). At maturity, the plants exhibited a form in which the lower part of the stem grew vertically in the water (submerged part) followed by the upper part growing horizontally just beneath the surface of the water (floating part), from which aerial tillers grew upwards (Fig. 1). The sampling methods for ARA and microbial enumerations were therefore modified, taking into account the probable distribution of epiphytic algae in relation to light availability. After removing the tillers and roots in the soil, each plant was separated into floating and submerged parts. Fresh weights of aerial tillers, floating parts, submerged parts and the roots in the soil were obtained from 8 separate plants. Light and dark ARA measurements were carried out using randomly selected, triplicate samples (100 g fresh weight) of intact floating and submerged parts.

Since microscopic examinations revealed an unequal distribution of epiphytic algae among the different organs of the plants, the exposed roots, leaf sheaths, inner roots (enclosed by leaf sheaths) and stem portions (Fig. 2), were separated from each of the floating and submerged parts of one plant. Their corresponding fresh weights were obtained, and aliquot triplicate samples were taken randomly from each of these parts for ARA measurement and algal and bacterial enumerations (*6*).

RESULTS

1) Distribution of epiphytic microorganisms

Microscopic examinations for epiphytic algae revealed the presence of BGA, green algae and diatoms growing attached to the surface of exposed roots, leaf sheaths, inner roots and the culm. The predominant BGA were N_2-fixing types, notably *Nostoc, Anabaena, Calothrix* and *Gloeotrichia.* As regards the relative distributions of these epiphytic species, no differences could be seen between submerged and floating parts of the plant. However, *Nostoc* colonies were frequently present at the points

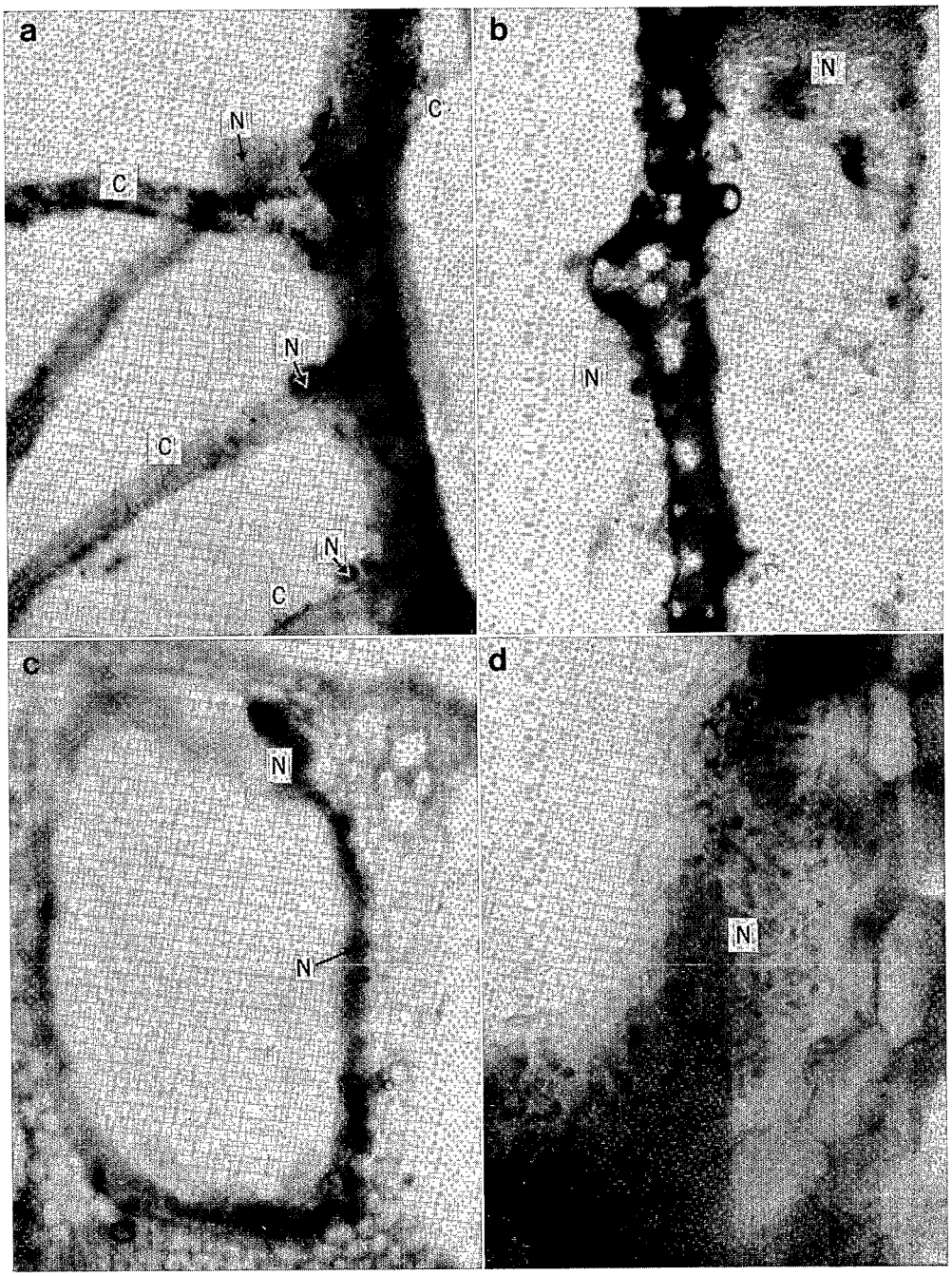

Plate 1. a–d

of lateral branches of roots as reported previously by Watanabe (*1*), while *Calothrix* did not show any such preference (Plate 1a). *Gloetrichia* was more common on the decaying leaves and leaf than on other parts. Observations on dissected parts and sections indicated that: (1) species of *Nostoc* and *Calothrix* were present on the leaf sheaths (Plate 1b), (2) the algae were also present inside the air cavities of the leaf sheaths (Plate 1c) but not within the host cells (Plate 1d), and (3) this "endophytism" was common within senescent or dead material but absent in living tissues.

The results of algal and bacterial enumerations carried out on the component parts of plants harvested at maturity are summarized in Table 1. Within the limits of accuracy of the method of algal enumeration used (*5*), no significant differences were observed between submerged and floating parts. Among the different components of the plant, the culm supported the lowest number of BGA. Bacterial enumerations on tryptic-soy agar (*6*) gave high populations of total aerobic heterotrophs. Compared to the heterotrophs, N_2-fixing populations were low and similar to those reported for wetland rice (*4*, *6*). The algal densities on the different components

Table 1. Epiphytic microorganisms enumerated from deepwater rice at maturity.

	Submerged parts				Floating parts			
	Exposed roots	Leaf sheath	Inner roots	Culm	Exposed roots	Leaf sheath	Inner roots	Culm
	Epiphytic N_2-fixing BGA (number g^{-1} (fw) $\times 10^{-4}$)							
Nostoc spp.	22	68	12	2.2	52	24	n.d.*	1.6
Anabaena sp.	10	48	14	0.6	28	18	n.d.	1.2
Calothrix spp.	32	34	14	2.6	48	26	n.d.	1.4
Total	64	150	40	5.4	128	68	n.d.	4.2
	Epiphytic bacteria (number g^{-1} (fw) $\times 10^{-6}$)							
N_2-fixers[1] (Glucose)	14	44	6	27	139	32	n.d.	3
N_2-fixers[2] (Malate)	27	44	26	27	11	32	n.d.	3
Total heterotrophs	1,300	2,400	1,340	300	1,180	1,360	n.d.	94

[1] Enterobacteriaceae; [2] *Azospirillum*-like. * n.d.: not determined.

Plate 1a. Part of nodal roots showing *Calothrix* (C) filaments on them and *Nostoc* colonies (N) at points of branching.

Plate 1b. Transverse section of a decaying leaf showing *Nostoc* growth on it.

Plate 1c. *Nostoc* filaments (N) lining the air cavity of a decaying leaf sheath.

Plate 1d. Part of the air cavity at a higher magnification showing the absence of *Nostoc* filaments withing the host cells.

(Table 1) when integrated, corresponded to an N_2-fixing algal population of 33×10^4 colonies per gram fresh weight of plant material available for epiphytism. This value was of the same order as that obtained with intact host material at heading (23×10^4 colonies g^{-1} fw), possibly indicating that algal colonization had not changed from heading to maturity of the crop. However, it should be noted that the method of enumeration does not distinguish between propagules and active cells.

2) Nitrogenase activity

The results of ARA measurements carried out at heading (Table 2) revealed that the activity in the light was almost 3 times that in the dark. Relating the specific activities with the host biomass, the total activity per plant was 5.1 μmol $C_2H_4 \cdot hr^{-1}$ and this was equivalent to 1.5 mmol $C_2H_4 \cdot m^{-2} \cdot day^{-1}$ when extrapolated to the field on the basis of a 12/12 hr-day/night cycle, and a planting density of 25 plants m^{-2}.

Results of ARA measurements performed on upper and lower parts of plants at maturity (Table 3) showed that the activities in the light were several times higher than those in the dark, and the specific light activity on the upper parts was 3 times that on the lower parts. Relating the specific activities on the upper and lower parts to their corresponding biomasses, the rate of ARA was 5.1 μmol $C_2H_4 \cdot hr^{-1}$. This rate was the same as that obtained at heading, although the biomass per plant of host material available for epiphytism had doubled from heading to maturity. This was because the specific activity at maturity (10.7 nmol $C_2H_4 \cdot g^{-1} \cdot hr^{-1}$) had decreased to half that at heading (20.7 nmol $C_2H_4 \cdot g^{-1} \cdot hr^{-1}$).

Table 2. Acetylene reducing activity in deepwater rice at heading.

Incubation	Specific activity (nmol $C_2H_4 \cdot g^{-1}$ (fw) $\cdot hr^{-1}$)	Biomass per plant (g(fw))	Activity per plant (μmol $C_2H_4 \cdot hr^{-1}$)	Extrapolated to field (mmol $C_2H_4 \cdot m^{-2} \cdot day^{-1}$)
Light	15.9		3.9	
		246		1.53
Dark	4.8		1.2	

Table 3. Acetylene reducing activity in deepwater rice at maturity.[1)]

Part of plant	Specific activity (nmol $C_2H_4 \cdot g^{-1}$ (fw) $\cdot hr^{-1}$)		Biomass (g(fw) $plant^{-1}$)	Activity per plant (μmol $C_2H_4 \cdot hr^{-1}$)	
	Light	Dark		Light	Dark
Upper	18.8	1.4	134	2.5	0.2
Lower	6.2	0.9	344	2.1	0.3
Whole plant	9.7	1.0	478	4.6	0.5

[1)] Average for 8 plants.

Table 4. Acetylene reducing activity in the component parts of a deepwater rice plant at maturity.

Component parts of plant	Biomass (g(fw)·plant^{-1})	Light		Dark	
		Specific ARA (nmol C_2H_4·g^{-1} (fw)·hr^{-1})	ARA[1] (nmol C_2H_4·hr^{-1})	Specific ARA (nmol C_2H_4·g^{-1} (fw)·hr^{-1})	ARA[1] (nmol C_2H_4·hr^{-1})
Floating					
Exposed roots	2.5	24	61	0.06	0.15
Leaf sheath	62	54	3,348	1.5	93
Inner roots	0.25	69	17	0.03	0.01
Culm	98	2.5	245	0.5	49
Submerged					
Exposed roots	64.3	0.54	35	0.08	5
Leaf sheath	15	12	180	0.1	1.5
Inner roots	1.3	3	4	0.05	0.06
Culm	100	0.3	30	0.02	2

[1] Specific ARA × component biomass.

Table 4 gives the results of ARA measurements carried out with separated parts of the plants. These results clearly demonstrate that the activity in the light was always higher than that in the dark, and that the specific activities associated with the floating parts, were higher than the corresponding activities on the submerged parts. Among the different components, higher specific activity was associated with the inner roots on the floating stem, followed by the leaf sheath, exposed roots and culm. However, the total activity of each part of the plant (specific ARA × fresh weight) was clearly highest in the leaf sheath, followed by the culm and exposed roots.

DISCUSSION

Compared to wetland rice (*4*), deepwater rice maintained a high N_2-fixing activity. BGA to a large extent, accounted for this since dark activity always remained relatively low. This was also reflected in the lower ARA values on submerged components of the plants at maturity (Table 4) compared to the floating components which receive more light in the field.

In contrast, results of algal enumeration for these components (Table 1) did not show such differences. The algal populations on the floating and submerged parts were of the same order. These results indicate that most of the algae present on the submerged parts were in a quiescent stage, probably due to light limitation, while those on the floating parts remained more active. Similar results have been obtained for epiphytic algae on lowland rice (*4*) in which the light ARA at maturity remained very low, despite the presence of a considerable population of enumerated N_2-fixing BGA.

The highest ARA in relation to plant biomass, obtained with the leaf sheaths, was primarily due to the high incidence of N_2-fixing BGA associated with them. A unique finding of this study was the observation that BGA were also present inside the leaf sheaths. This observation on rice plants grown in experimental plots at the International Rice Research Institute was confirmed by microscopic examinations on samples collected from deepwater rice fields in Nakornnayok (Thailand) which showed a very high density of a true-branching, heterocystous BGA within the leaf sheaths. Sufficient data are not yet available to explain the nature of this endophytism, but the observations made so far do not support the existence of biotic relationships: (1) endophytic algae were present in senescent or dead material but absent in living tissues, and (2) this phenomenon was not specific to deepwater rice, the same being observed in the decaying leaf sheaths of associated grasses.

These findings most probably indicate that the algal colonization is not host specific, but is more closely related to a mechanical effect offered by decaying surfaces (*4*).

Enumerations carried out on selective media gave high densities of epiphytic N_2-fixing bacteria (Table 1), but dark ARA measurements did not yield results compatible with such populations. Similar results have been reported for epiphytic N_2-fixation in rice (*4*) and weeds (*2*) of wetland rice fields, where the dark ARA values were always low despite the presence of heavy populations of epiphytic N_2-fixing bacteria. As all the ARA measurements were performed in air, it is possible that a substantial part of the activity due to microaerophilic organisms could have been inhibited. The presence of such microaerobic sites *in situ* is quite likely considering the very large populations of heterotrophic bacteria (Table 1) associated with the decaying host material.

The rate of ARA obtained in this study (5.1 μmol $C_2H_4 \cdot plant^{-1} \cdot hr^{-1}$) was measured under low light intensities using cut material, removed from the field. This value must therefore be considered as an underestimate of the *in situ* activity. Nevertheless, extrapolation of this activity to the field scale corresponds to an input of about 10–20 kg $N \cdot ha^{-1} \cdot crop^{-1}$. It can therefore be concluded that the epiphytic N_2-fixation in deepwater rice makes a substantial contribution to this ecosystem.

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