POTENTIAL OF BIOLOGICAL NITROGEN FIXATION IN DEEPWATER RICE

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SUMMARY

Aquatic roots, leaf sheaths and, to a lesser extent, culms of deepwater rice under water are colonized by epiphytic N_2-fixing blue-green algae (BGA). BGA were also within the air cavities of decaying leaf tissues. Rice was grown in pots containing ^{15}N-labeled ammonium sulfate in shallow and deep (<110 cm) water in fields in the Philippines and Thailand. Rice plants in deep water had lower ^{15}N enrichment, suggesting that nitrogen in floodwater either as molecular nitrogen or combined nitrogen, or both, contributes to nutrition of deepwater rice. Submerged leaf sheaths and roots shaded by black cloth had higher ^{15}N content than nonshaded ones, suggesting also epiphytic N_2-fixation by BGA.

Direct evidence of N_2-fixation associated with deepwater rice was obtained by exposing deepwater rice to $^{15}N_2$ gas for 9 days. Higher enrichment of ^{15}N from molecular nitrogen was found in the aquatic root and leaf sheath where blue-green algae grow epiphytically. Eight mg N/plant was fixed during this period and about 40% of fixed nitrogen was found at maturity in the portions not directly exposed to $^{15}N_2$.

For many years, deepwater rice has been grown without the benefit of fertilizer nitrogen but with good biomass production. Little is known about the sources of nitrogen for deepwater rice. The submerged stems produce clusters of aquatic roots, which grow freely and may absorb nutrients from the floodwater. However, the extent to which deepwater rice can absorb nitrogen directly from the floodwater is not known; neither is the contribution of biological nitrogen fixation to its nitrogen nutrition.

We studied the algal biomass attached on the surface of the submerged and floating parts of deepwater rice and used ^{15}N to see if nitrogen fixation by epiphytic blue-green algae (BGA) contributes to the nitrogen nutrition of deepwater rice.

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EPIPHYTIC NITROGEN FIXATION ON DEEPWATER RICE

Studies of epiphytic N_2-fixation in rice and weeds in a shallow-water rice field by Roger et al (1981) and Kulasooriya et al (1981b) have indicated that epiphytic microorganisms make a limited contribution of nitrogen in this ecosystem. This was related to a limited biomass offered to epiphytism. In deepwater rice, on the other hand, a large part of the deepwater rice plant remains under water and offers greater biomass for colonization by aquatic microorganisms. Surveys of the BGA population and N_2-fixing (acetylene reduction) activity were made for the deepwater rice. The results were published by Kulasooriya et al (1981a).

Methods

A deepwater rice (DW6255) was grown in pots and transferred to an IRRI deepwater plot (14 × 38 m) 30 days after planting. Water depth was increased 10 cm every other day to a final depth of 110 cm, which was maintained. Epiphytic microorganisms in the plot were examined microscopically every 3 weeks. Algae and bacteria were counted and measurements of acetylene-reducing activity (ARA) were made at rice heading and maturity.

At heading, 7 plants were removed from the plot, their aerial parts and roots cut off, and the remainder of the material was used for measuring ARA and enumerating the epiphytic algae. ARA was measured on 4 replications (30 g fresh weight) randomly taken and incubated with 10% acetylene in air (in 900-ml plastic cylinders) at room temperature (30-32° C), either in light (800 lx) or in darkness (wrapped in aluminum foil). Gas samples were removed after 30 and 90 minutes of incubation for chromatographic analysis. Algal enumerations were as described by Roger et al (1981).

At maturity, the lower part of the plant grew vertically in water (submerged part) followed by the upper part growing horizontally just beneath the water surface (floating part). Aerial tillers grew upward from the floating parts.

After removing the aerial tillers and roots, each plant was separated into floating and submerged parts. Fresh weights of aerial tillers, floating parts, submerged parts, and roots were obtained from 8 separate plants. Light and dark ARA measurements were made for randomly selected triplicate samples (100 g fresh weight) of intact floating and submerged parts. The exposed roots, leaf sheaths, inner roots (enclosed by leaf sheaths), and culm portions were separated from each of the floating and submerged parts of one plant. Aliquot triplicate samples were taken randomly from each of these separated parts for ARA measurement and algal enumerations.

Distribution of epiphytic microorganisms

Microscopic examination for epiphytic algae revealed the presence of BGA, green algae, and diatoms attached to the surface of exposed roots, leaf sheaths, inner roots, and culm. The predominant BGA were N_2-fixing ones, notably *Nostoc*, *Anabaena*, *Calothrix*, and *Gloeotrichia*. These epiphytic species were seen on submerged and floating parts. *Nostoc* was frequently present at the points of lateral branches of roots, whereas *Calothrix* did not show any such preference. *Gloeotrichia* was more common on the decaying leaf blades and sheaths than on other parts. Observations on dissected parts and sections indicated that:

- species of *Nostoc* and *Calothrix* were present on the leaf sheaths,
- the algae were also present inside the air cavities of the leaf sheaths, but not within the host cells, and
- this *endophytism* was common within senescent or dead material but absent in living tissues.

Within the limit of accuracy of the method of algal enumeration used (Roger and Reynaud 1978), no significant difference in the population size of BGA was found among submerged and floating parts. Among the different components of the plant, the culm supported the lowest number of BGA (Kulasooriya et al 1981a). Integrated algal densities on the different components corresponded to an N_2-fixing population of 33×10^4 colonies per gram fresh plant material available for epiphytism. The value was of the same order as that obtained with intact host material at heading (23×10^4 colonies/g fresh weight), possibly indicating that algal colonization had not changed between heading and maturity of the crop. It should be noted, however, that the enumeration method does not distinguish between propagules and active cells.

Nitrogenase activity

ARA measurements at heading and maturity revealed that activity in the light was 3 times more at heading and more than 13 times higher at maturity than in darkness, indicating that photodependent N_2-fixation, probably by BGA, was more active than heterotrophic N_2-fixation (Table 1). Relating the specific activities to the rice plant biomass, total photodependent activity per plant was 3.9 and 4.6 μmol C_2H_2/hour at heading and maturity. These were equivalent to 1.2 and 1.4 μmol C_2H_2/m^2 per day when extrapolated to the field on the basis of 12/12 hours day-night cycle, and a

Table 1. Acetylene-reducing activity in the component parts of a deepwater rice plant at maturity (Kulasooriya et al 1981a).

Component parts of plants	Biomass (g fresh weight/ plant)	Light		Dark	
		Specific ARA (nmol C_2H_4/g fresh weight per h)	ARA[a] (nmol C_2H_4/h)	Specific ARA (nmol C_2H_4/g fresh weight per h)	ARA (nmol C_2H_4/h)
Floating					
Exposed roots	2.5	24	61	0.06	0.15
Leaf sheath[b]	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	0.5	35	0.08	5
Leaf sheath[b]	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

[a] Specific ARA x component biomass. [b] Covered by leaf sheath.

planting density of 25 plants/m^2.

This level of activities (more than 1 μmol C_2H_2/m^2 per day) was observed in the field when algal biomass was visible in the floodwater (Watanabe et al 1978). ARA measurements associated with separated parts of the plants (Table 1) clearly demonstrated that activity in the light was always higher than that in darkness and that specific activities associated with the floating parts were higher than the corresponding activities on the submerged parts. Among the different components, highest specific activity was associated with the inner root on the floating part followed by the leaf sheath, exposed root, 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 root.

SOURCES OF NITROGEN TO DEEPWATER RICE OTHER THAN SOIL

To estimate the contribution of nitrogen from the floodwater, DW6255 was grown in pots containing a small amount of ^{15}N ammonium sulfate. Rice was grown either by shallow flooding of the pots or by putting the pots in a deepwater plot. The experiments were at IRRI and in Thailand.

Methods

Maahas clay soil (7 kg dry weight/pot) was mixed with ^{15}N-labeled (6.948 atom % excess) ammonium sulfate at 500 mg/pot during the 1979 wet season and 600 mg N/pot (17.21 atom % excess) during the 1980 wet season. DW6255 seedlings were transplanted at 1 seedling/pot. When rice was 49 days old, one series of pots was placed on the bottom of a 19 × 38 m deepwater plot at IRRI and water depth was increased 10 cm every other day to a final depth of 110 cm, which was maintained until maturity. Other pots were placed on an elevated platform built in the deepwater plot, which gave 5-cm flooding on the pot surface. A black cloth covered the surface of half of the shallow-water pots to prevent algal growth on the soil surface. In deep water a round metal support of 20 cm diameter and 90 cm height was placed on top of the pots. The outside of the support was covered with black cloth to prevent algal growth on the portion of deepwater rice below water.

In a similar experiment in Thailand, soil from a field at Bangkhen was mixed with ^{15}N-labeled ammonium sulfate at 200 mg N/pot (12.538 atom % excess). Pots in shallow water were placed in a greenhouse at Bangkhen. Pots for the deepwater test were put in a plot at Huntra, which was flooded to 50 cm or less. Other pots were placed in a farmer's field at Ongkarak, which was flooded to about 120 cm.

There were 5 replicated pots in every site. At maturity, the plant parts were separated as in ARA assays described earlier. The dried materials were analyzed for total nitrogen by micro-Kjeldahl and for ^{15}N by emission spectrometry.

^{15}N dilution as affected by water depth

The black cloth covering the submerged portions of the rice plant adversely affected the growth of deepwater rice, resulting in less tillers and panicles than the nonshaded plants (data are not presented).

Total N and dilution of ^{15}N in plants are shown in Table 2. Content of ^{15}N in plant

Table 2. Total nitrogen and ^{15}N contents and dilution of ^{15}N in total plant portions in experiments in shallow and deep water. Philippines and Thailand.

Site	Season[a]	Water depth (cm)	Shading	Total N (mg/plant) A	Total N (mg/plant) B	Dilution A/B
Philippines						
IRRI	1979 wet	5	Yes	923 ± 31[b]	273 ± 10	3.4
		5	No	879 ± 47	235 ± 11	3.7
		110	Yes	618 ± 31	114 ± 13	5.4
		110	No	983 ± 67	212 ± 17	4.6
	1980 wet[a]	5	No	939 ± 13	380 ± 1	2.5
		110	No	777 ± 31	283 ± 10	2.7
Thailand						
Bangkhen	1979 wet	5	No	829 ± 65	112 ± 8	7.4
Huntra		<50	No	1,030 ± 115	74 ± 5	13.9
Ongkarak		120	No	988 ± 79	98 ± 11	10.1

[a]Atom % excess was 6.948, 17.210, and 12.538 in 1979, 1980 at IRRI, and 1979 in Thailand respectively. [b]Mean ± standard error.

was clearly lower in the deep than in the shallow water.

If the contribution of soil nitrogen in pots was not affected by the floodwater depth, lower atom % ^{15}N in deepwater rice would mean that nitrogen was absorbed from floodwater. If the nitrogen in the plant comes partly from biological N_2 fixation by epiphytic BGA, the deepwater rice of which the submerged parts were covered by black cloth must have higher atom % ^{15}N. The analysis of the plant, however, did not prove the expectation. Because the growth and nutrient uptake of deepwater rice was adversely affected by the black cloth, the result must be interpreted with some reservation. Atom % excess of various parts of deepwater rice showed that exposed root, inner root, and leaf sheath below water had lower ^{15}N when not shaded than when shaded (Table 3). In trials in Thailand (Table 4), the leaf sheath and root under water showed lower enrichment of ^{15}N than other plant parts. These sites showed higher specific ARA as shown in Table 1 and, hence, were the major sites of epiphytism by BGA. Therefore, it is reasonable to attribute lower ^{15}N contents in the parts under water to ^{15}N-fixation by epiphytic BGA.

In shallow water, the plant parts under water also showed lower ^{15}N content (data are not presented) and shading of those parts increased ^{15}N enrichment, indicating that even in shallow water, epiphytic N_2 fixation is associated with shoots under water.

Nitrogen derived from the floodwater (including both combined and molecular nitrogen) is estimated by the equation

$$\text{Estimated N absorbed from floodwater} \times \left(1 - \frac{\text{atom \% excess in deepwater rice}}{\text{atom \% excess in shallow water rice}}\right) \times \text{Total N of deepwater rice}$$

Table 3. Total N and ^{15}N atom % excess in various parts of rice grown in deep water, IRRI, 1979 wet season.

Parts	Submerged portions not shaded		Submerged portions shaded	
	Total N (mg/pot)	Atom % excess	Total N (mg/pot)	Atom % excess[a]
Aerial parts				
Grain	346 ± 32	1.68 ± 0.08*	234 ± 29	1.41 ± 0.14
Leaf blade	122 ± 23	1.60 ± 0.08*	65 ± 7	1.39 ± 0.08
Culm and leaf sheath	116 ± 9	1.67 ± 0.04*	105 ± 3	1.33 ± 0.11
Upper submerged parts				
Leaf sheath	58 ± 8	0.93 ± 0.07	21 ± 1	1.01 ± 0.09*
Culm	75 ± 14	1.71 ± 0.06*	43 ± 3	1.42 ± 0.11
Exposed root	20 ± 5	0.60 ± 0.33	27 ± 5	0.80 ± 0.10*
Inner root	4 ± 1	0.67 ± 0.06	3 ± 1	0.89 ± 0.09*
Lower submerged parts				
Culms	67 ± 10	1.94 ± 0.05*	15 ± 2	1.24 ± 0.07
Exposed root	130 ± 9	0.81 ± 0.06	97 ± 9	0.94 ± 0.10*
Root in soil	45 ± 7	1.58 ± 0.10*	7 ± 1	0.98 ± 0.07
Whole plant		1.84 ± 0.09		1.47 ± 0.11

[a]Shows that atom % excess indicated with * was significantly higher than that of the other treatment.

This equation is similar to the estimation of N_2-fixation by measuring ^{15}N abundance in non-N_2-fixing and N_2-fixing plant (Rennie et al 1978).

The estimates of nitrogen absorbed from floodwater were 178 and 80 mg N/pot at IRRI in 1979 and 1980 trials. These accounted for 18 and 10% of the total nitrogen in the plant. In Thailand, the values were 479 and 264 mg N/pot in Huntra and Ongkarak, corresponding to 46 and 27% of the total N in the plant. But because the shallow-water pots in Thailand were not at the same sites and some roots were attached to the soil after the water receded, the results must be considered with some reservation.

Assuming that the shaded submerged plant parts are non-N_2-fixing (photodependent) ones, photodependent N_2-fixation associated with deepwater rice was calculated by an equation similar to equation 1. In shallow water, this value was 84 mg N/plant. But in deep water, the calculation was impossible for total plant part because of higher atom % excess in nonshaded condition.

But such a calculation is possible in each part where atom % excess was higher in shaded than in nonshaded condition. The total for estimates of photodependent N_2-fixation in the leaf sheath, the exposed root, and the inner root of the upper submerged part and the exposed root of the lower submerged part was 58 mg N/plant.

Table 4. Atom % excess in different parts of deepwater rice plants grown in Thailand, 1979. Rice variety was Leb Mue Nahng 111.

Site	Water depth (cm)	Parts	Atom % excess
Bangkhen	Shallow (5)	Panicles	1.48 ± 0.10
		Leaf blade	1.82 ± 0,17
		Leaf sheath	1.87 ± 0.09
		Culm	1.53 ± 0.11
Huntra	Semideep (<50)	Panicle	1.01 ± 0.08
		Leaf blade	1.06 ± 0.07
		Leaf sheath	0.77 ± 0.04
		Culm	0.94 ± 0.08
Ongkarak	Deep (120)	Panicle	1.36 ± 0.21
		Leaf blade	1.31 ± 0.09
		Upper leaf sheath	1.36 ± 0.08
		Upper culm	1.38 ± 0.19
		Lower leaf sheath	1.07 ± 0.12
		Lower culm	1.27 ± 0.14
		Root in water	1.02 ± 0.16

$^{15}N_2$ INCORPORATION BY DEEPWATER RICE

The experiments described strongly suggest that N_2-fixation takes place in association with aquatic parts of deepwater rice. $^{15}N_2$-feeding experiments were conducted to get direct evidence of N_2-fixation and to see to what extent the fixed nitrogen is transferred to aerial parts.

Method

DW6255 was grown in IRRI's deepwater plot in the same way as in the ^{15}N dilution experiment except that 600 mg N/pot of the nonlabeled ammonium salt was added to the soil. At heading, the entire submerged portion, plus about 15 cm of the aerial part of the rice culms, was enclosed in plastic bags such as those used in ARA assays of wetland rice soil (Watanabe et al 1978). The bag opening around the culm was sealed by modeling clay. The plastic bag was completely filled with floodwater and assay chambers were submerged in deepwater plot.

^{15}N-labeled N_2 gas (Monsanto Research Corporation USA, 98 atom %) was washed by potassium permanganate solution and acidic sodium sulfate. One liter of ambient air and 1 liter of $^{15}N_2$ gas were introduced into the plastic bag, and the air was circulated through the assay chamber for 5 minutes to solubilize the introduced gas in the floodwater. Labeled N_2 in the chamber was changed every 2 or 3 days (4 changes) for 9 days. Control plants were grown beside the $^{15}N_2$-fed rice plant. Each treatment had 4 replications.

Analysis of $^{15}N_2$ in the plastic bag just after introduction and before changing gas

phase was by emission spectrometry. After 2 or 3 days, atom % ^{15}N in N_2 gas decreased to about 23, and the average of ^{15}N abundance during the 9 days was 48%.

After the assay chamber was removed, the rice plants were grown to maturity. At maturity, aerial, floating, and submerged portions were removed and ^{15}N abundance of the different parts was determined by mass spectrometer (VG Micromass 622). ^{15}N abundance in the control plant parts was also analyzed.

Results

^{15}N enrichment was found in all parts of the plant exposed to ^{15}N (Table 5). Among the parts under water, leaf sheath had the highest ^{15}N enrichment, followed by aquatic root, culm, and root in the soil.

These results agree with those of the earlier ARA and ^{15}N dilution experiment, indicating that leaf sheaths and aquatic roots and, to a lesser extent, culms were the sites of N_2-fixation in association with deepwater rice.

Labeled nitrogen was also found in aerial parts not exposed to $^{15}N_2$. About 40% of fixed nitrogen was found in the aerial parts, the leaf blade acting as sink.

Eight mg N was fixed during 9 days. This value was higher than those reported by Ito et al (1980) and Yoshida and Yoneyama (1980) by heterotrophic bacteria in association with shallow-water wetland rice.

Table 5. N_2-fixation by deepwater rice exposed to $^{15}N_2$ for 9 days. IRRI, 1980 wet season.

Plant parts	Total N (mg N/pot)	Atom % excess	Fixed N (µg N/plant)[a]
Aerial parts			
Grain	184 ± 33	0.04 ± 0.02	149
Leaf blade	75 ± 17	1.77 ± 0.88	2780
Leaf sheath	28 ± 3	0.30 ± 0.21	208
Culm	26 ± 4	0.11 ± 0.07	57
Upper parts			
Leaf sheath	36 ± 3	3.12 ± 1.14	2310
Culm	27 ± 1	0.48 ± 0.15	264
Root	6 ± 3	2.01 ± 0.77	267
Lower part			
Leaf sheath	29 ± 3	1.37 ± 0.46	831
Culm	28 ± 4	0.27 ± 0.07	153
Root	35 ± 3	0.66 ± 0.28	625
Root in soil	68 ± 6	0.23 ± 0.11	329
Whole part	542 ± 51		7973
Submerged weed	41 ± 12	0.97 ± 0.48	823

[a] Assuming an average of 48.1 % excess of $^{15}N_2$ during 9 days exposure.

DISCUSSION

Molecular nitrogen is fixed in deepwater rice by both photodependent and heterotrophic N_2-fixing microorganisms. The role of N_2-fixation in nitrogen nutrition of deepwater rice was proven by ^{15}N dilution technique and direct exposure of deepwater rice with $^{15}N_2$. Greater contribution by BGA was indicated by greater ARA in light than in darkness and by greater ^{15}N dilution in the submerged tissues not shaded. Although $^{15}N_2$ feeding experiments were not conducted with shaded plants, the assumption that $^{15}N_2$ was fixed more by photodependent N_2-fixing microorganisms than by heterotrophic microorganisms is in agreement with data obtained by ARA and ^{15}N dilution techniques.

Previous observations had been that epiphytic BGA preferentially developed on the submerged decaying tissues of the host (Kulasooriya et al 1981a). The idea that N_2 fixation by epiphytic microorganisms results in the accumulation of nitrogen in decaying tissues and this fixed nitrogen is not directly available by host plant is not supported by the results we describe here. In the $^{15}N_2$-feeding experiment, the contribution of phototrophic N_2-fixing microorganisms to the transferred nitrogen was not directly assessed. It is reasonable, however, to assume that the transferred nitrogen came partly from photodependent N_2-fixation, because ARA values associated with submerged parts were much greater in the light than in darkness and the total amount of N fixed during the $^{15}N_2$ exposure appeared to be much higher than the reported heterotrophic N_2-fixing rates associated with wetland rice (Ito et al 1980, Yoshida and Yoneyama 1980).

Extrapolating the observed N_2-fixing activity for 9 days to 100 days, which is assumed as the submerged growth period of deepwater rice, N_2-fixation explains about 10% of total nitrogen in deepwater rice at maturity (80 mg N over 800 mg N in total biomass of the host). Because BGA on deepwater rice grown in IRRI's deepwater plot grew much less profusely than BGA associated with deepwater rice in Thailand (visual observation by authors), the role of BGA in nitrogen nutrition of deepwater rice field could be much greater than those found at IRRI.

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DISCUSSION

DE DATTA: Is epiphytic nitrogen fixation greater in deepwater rice than in shallow-water wetland rice?

WATANABE: N_2-fixing activity per rice plant was much higher in deep water than in shallow water. This is due to highly photodependent N_2-fixing activity (as shown in Table 1) and to higher biomass under water.

GOMOSTA: How is the $^{15}N_2$ fixed by epiphytic algae absorbed and transported to aerial parts?

WATANABE: The possibility of nitrogen fixation by aerial plant parts is ruled out because of negligible acetylene reduction activities of the aerial parts. I suspect that $^{15}N_2$ is fixed in algae cells and released to the plant after their disintegration or decomposition. We need to determine the mechanism.

TOWNSEND: Do you see any great potential for this?

WATANABE: Our ^{15}N data show 9 mg N fixed/plant in 9 days. Assuming 90-100 days as the effective period when nitrogen fixation is going on, it becomes about 10% of the total nitrogen in the plant. I point out that our experiment at IRRI was in a shallow deepwater pond. We grow much less algae there than can be grown on farms in deepwater rice areas. Thus, I suspect the contribution in a deepwater rice area will be much higher, but I cannot say how much.

KARIM: Considering the fact that nitrogen is fixed by microorganisms in deepwater rice, what conditions would enhance or optimize the fixation rate? Do blue-green algae have preferential association with particular varieties of rice?

WATANABE: Water quality and plant morphology may affect the epiphytic growth of blue-green algae. There must be a kind of association but I have not studied varietal differences.

CATLING: Is the high amount of nitrogen fixation in deepwater rice due to a high daily rate of fixation and the large biomass of deepwater rice?

WATANABE: It is due to the large biomass of deepwater rice. But, as shown in Table 1, photodependent nitrogen fixation in aquatic roots and leaf sheaths was much higher than heterotrophic N_2 fixation. Therefore, I suspect the daily rate was also much higher in deep water than in shallow water.