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Fate of nitrogen applied as Azolla and blue-green algae (Cyanobacteria) in waterlogged rice soils – a ^{15}N tracer study

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Summary ^{15}N tracer was used to detect the extent to which nitrogen of applied *Azolla caroliniana, Anabaena variabilis* and *Nostoc muscorum* was available for assimilation by the growing rice plants in pots under 4 cm flood water for 60 days. The rate of release of nitrogen from the above biofertilizers, the amount of nitrogen remaining in the soils and the amount that was lost from the soils during this period were also examined. Previously ^{15}N-labelled biomass of Azolla, Anabaena and Nostoc to provide 40 mg N was mixed thoroughly with 0.5 kg silt loam Bangladesh soil (Sonatola series) in each of three pots used for a single treatment. Each pot received four 16 days old IR8 rice seedlings. A parallel set of experiments was conducted without rice plants.

It was found that nitrogen uptake in the rice plants was increased by 91, 176 and 215% on using Azolla, Anabaena and Nostoc which resulted in increased total dry matter yields (shoot plus root) of 74, 105 and 125%, respectively. Of the total ^{15}N applied at the start, 26, 49 and 53% was released from Azolla, Anabaena and Nostoc; about 7, 14 and 13% was lost by denitrification and 74, 51 and 47% remained in the soils as the undecomposed part of the biofertilizers, respectively, after 60 days. Of 15.76, 22.72 and 25.92 mg N assimilated by the rice plants, 48, 61 and 62% was supplied by Azolla, Anabaena and Nostoc, respectively. The rest was obtained from the soil used.

In the absence of the rice plants 30, 43 and 45% of applied ^{15}N of Azolla, Anabaena and Nostoc was released, respectively, in 60 days of which 93–96% was lost as N_2 through denitrification.

Introduction

It is now well documented that the Azolla-Anabaena symbiosis[5] and free-living blue-green algae[8,12] significantly increase lowland rice yields. It is also generally accepted that such yield increases ensue due to an additional supply of fixed nitrogen by these biological agents. However, information on how much of the fixed nitrogen is actually available to the rice plants is still scarce[8]. A recent report by Tirol *et al.*[11] showed that about 28% of Nostoc-N was available to a IR32 rice crop. Mian and Stewart[6] found that IR8 rice plants received 26–32% of applied ^{15}N of *Azolla caroliniana* in 60 days. A more detailed study is still necessary to look into the overall

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transformations of Azolla and blue-green algae in flooded rice soils. Therefore, in this study ^{15}N was used to trace the amount of released NH_4-N from decomposing biofertilizers in flooded soils, nitrification of NH_4-N to NO_3-N, any denitrification loss, the amount of nitrogen remaining undecomposed in the incorporated biomass and the amount of nitrogen which was assimilated by the growing rice plants in 60 days.

Materials and methods

Nitrogen biofertilizers

Azolla Azolla caroliniana, initially supplied by the Dundee University botanic garden, was routinely maintained for three years in N-free medium[7]. For labelling with ^{15}N, the plants were grown in medium containing 1 g l^{-1} Na $^{15}NO_3$ (30.4 atom % excess ^{15}N) for three weeks. After washing with distilled water the fronds were allowed to grow for another 24 h in N-free medium to remove any traces of NO_3-N. They were again washed with distilled water and air-dried prior to use.

Blue-green algae *Anabaena variabilis* (CCAP 1403/4b) was obtained from the Culture Centre of Algae and Protozoa, Cambridge, England. A pure culture of *Nostoc muscorum* was available in the laboratory. They were labelled with ^{15}N by growing them in BG11 medium[10] containing 0.25 g l^{-1} Na $^{15}NO_3$. The cells were collected by centrifugation, washed 3–4 times with distilled water and resuspended in N-free medium for another 24 h. They were then repelleted, washed, and air-dried.

Analysis of biofertilizers

The air-dried Azolla contained 4.25% N and 19.87 atom % excess ^{15}N. *Anabaena variabilis* contained 8.56% N and 21.87 atom % excess ^{15}N; *Nostoc muscorum* contained 9.08% N and 14.49 atom % excess ^{15}N.

Soil

The soil was silt loam from the top 0–15 cm of Sonatola series of non-calcareous dark-grey flood-plain in Bangladesh. The initial characteristics of the soil were as follows: pH 7.6, organic-C 0.60%, total-N 0.06%; 2.3 μg NH_4-N, 14.5 μg NO_3-N, 64 μg available P and 116 μg available K in each gram of soil.

Experimental set up

With rice plants Four treatments *viz.* control, *Azolla caroliniana, Anabaena variabilis* and *Nostoc muscorum* were considered in this study. The desired amount of air-dried biomass of Azolla, Anabaena or Nostoc to supply 40 mg N was mixed thoroughly with 0.5 kg soil contained in each of the three plastic pots of 900 cm^3 capacity used for a single treatment. About 150 cm^3 distilled water was required to saturate the soil of each pot. Four 16 days old IR8 rice seedlings were transplanted to each pot and sufficient distilled water was added to raise the water level 4 cm above the soil surface. This water level was maintained during the entire growth period, adding more distilled water when necessary. The pots were then placed at random in a growth cabinet at 25°C light (12 h, 100 $\mu mol\, m^{-2}\, sec^{-1}$) and 18°C dark (12 h) cycles. The positions of the pots were randomized each week.

Without rice plants A parallel set of experiments was carried out without the rice plants. Here 10 g soil containing 2 mg N of Azolla or blue-green algae were incubated in suba-sealed air-tight glass bottles with 4 cm overlying flood water under air. Exactly 1 ml gas samples were drawn from each bottle at 60 days and analyzed for NO, N_2O, O_2 and N_2 by mass spectrometry.

Harvest and data collection

The plants were harvested at 15, 30, 45 and 60 days of growth and carefully separated into shoots and roots. The dry matter yields were recorded after drying at 65°C to constant weight.

Soil analysis

The NH_4-N and NO_3-N contents of 2*M* KCl extracts of soils after each harvest were determined[1]. After each harvest the entire 0.5 kg soil was air-dried, powdered and total N determined[2].

Plant analysis

The dried shoots and roots of each pot were ground separately in a micro Hammer mill C580 (Glen Creston, England) to pass a 0.5 mm sieve. Total-N in shoots and roots was determined by the micro-Kjeldahl digestion and distillation method[4]. The same procedure was also followed to determine total-N in Azolla and blue-green algae.

Measurement of ^{15}N in various samples

Each nitrogen sample was converted to NH_4^+, acidified and reduced to 1–2 cm^3 volume by slow heating in a sand bath. The NH_4-N in a sample was converted to N_2 by reacting with helium gassed highly alkaline lithium hypobromite solution in Rittenberg-Y tubes (for samples with over 100 μg N) or in 16 mm standard glass tubes (for samples less than 100 μg N) under complete vacuum. The liquid part in the tubes was kept frozen for at least five minutes before allowing the N_2 gas to enter into the inlet system of a VG Micromass 601 spectrometer (Winsford, Cheshire, England). The ^{15}N concentration in the sample was then determined[3].

Results

Yield response and nitrogen uptake

Total dry matter yield of IR8 rice plants increased significantly (P = 0.01) on using Azolla and blue-green algae. The effects of Anabaena and Nostoc were similar but significantly different from that of Azolla (Table 1a). The use of Azolla, Anabaena and Nostoc increased total dry matter yields (shoot plus root) by 74, 105 and 125%, respectively, over the untreated controls in 60 days. A similar situation was also observed in nitrogen uptake by the rice plants. It is seen that the rice plants growing in Nostoc-treated soil received the highest amount of nitrogen and the lowest amount was received by the control plants. The rice plants of Anabaena-treated soils obtained much higher amounts of nitrogen than the plants of Azolla-treated soils. Overall, the rice plants grown in Azolla, Anabaena and Nostoc-treated soils assimilated 91, 176 and 215% more nitrogen, respectively, than the plants of control series during their 60 days of growth in pots (Table 1b). Table 1c shows that the rice plants assimilated 19, 35 and 40% of the total ^{15}N applied as Azolla, Anabaena and Nostoc, respectively, in 60 days. That is, more ^{15}N was received by the Nostoc and Anabaena-treated plants compared to Azolla-treated plants.

It is seen in Table 2a that the initial NH_4-N in the soil was 2.3–2.9 μg g^{-1} soil but the amount increased substantially up to 15 days and

Table 1. Response of IR8 rice to nitrogen of Azolla and blue-green algae during its 60 days of growth in pots under waterlogged conditions

Yield and nitrogen uptake	Time (days) 15	30	45	60	% increase or % of ^{15}N (at 60 days)
a) Dry matter yield of shoot and root (mg pot^{-1})					
Control	142	209	343	566a	
Azolla caroliniana	188	328	592	982b	74
Anabaena variabilis	190	330	681	1158c	105
Nostoc muscorum	189	341	657	1275c	125
b) Total-N uptake by shoot and root (mg pot^{-1})					
Control	3.24	5.24	6.84	8.24	
Azolla caroliniana	4.56	9.28	10.88	15.76	91
Anabaena variabilis	5.24	11.08	19.32	22.72	176
Nostoc muscorum	5.04	11.68	19.44	25.92	215
c) Total ^{15}N uptake by shoot and root (mg pot^{-1})					
Azolla caroliniana	0.40	0.84	1.00	1.49	19
Anabaena variabilis	0.40	1.20	2.20	3.05	35
Nostoc muscorum	0.36	1.00	1.68	2.32	40

Values of (a) at 60 days with a similar letter are not significantly different at $P = 0.01$ levels using Duncan's multiple range test.

Table 2. The amounts of NH_4-N and NO_3-N present in soils after each harvest of rice plants

Treatments	Time (days) 0[a]	15	30	45	60
a) NH_4-N ($\mu g\ g^{-1}$ soil)					
Control	2.3	4.2	2.9	1.0	0.9
Azolla caroliniana	2.3	6.8	5.3	2.1	1.7
Anabaena variabilis	2.6	12.5	5.9	2.6	2.0
Nostoc muscorum	2.9	13.0	6.4	2.7	2.1
b) $^{15}NH_4$-N (μg pot^{-1})					
Azolla caroliniana		61	43	22	8
Anabaena variabilis		206	76	31	24
Nostoc muscorum		209	97	28	21
c) NO_3-N ($\mu g\ g^{-1}$ soil)					
Control	14.5	1.4	1.7	1.2	0.3
Azolla caroliniana	14.8	0.7	2.6	0.7	0.6
Anabaena variabilis	14.8	0.3	0.7	0.5	0.2
Nostoc muscorum	14.6	0.2	0.6	0.5	0.3

[a] extracted with 2*M* KCl within 10 minutes of adding biofertilizers but the ^{15}N concentration was not measureable.

gradually decreased thereafter. The initial NO_3-N content of the soils (14.5–14.8 μg g^{-1} soil) also gradually declined to only 0.2–0.6 μg after 60 days (Table 2c). The possible reasons for this situation are discussed later.

Nitrogen balance sheet

The rice plants of Azolla, Anabaena and Nostoc-treated soils assimilated 19, 35 and 40% of the total amount of ^{15}N applied, respectively, while 74, 51 and 47% of the total amount remained in the soil and was not incorporated into the rice plants (Table 3). About 7, 14 and 13% of applied ^{15}N of Azolla, Anabaena and Nostoc was neither detectable in plants nor in soils and was probably lost as N gas. Since it was not possible to detect the kind of evolved nitrogen gas from the open pots an attempt was made to estimate this in a different way. Here, biofertilizer mixed soils were incubated under a 4 cm water layer in glass bottles with 103 cm^3 enclosed air in each. The gas samples from these incubated bottles were analysed after 60 days. It was found that the loss of ^{15}N released from biofertilizers occurred as N_2. No NO or N_2O was detectable. About 30, 41 and 42% of the applied ^{15}N of Azolla, Anabaena and Nostoc was lost as N_2 in 60 days which was equivalent to a loss of 93–96% of the total amount of ^{15}N applied. The initial 103 cm^3 enclosed air in each bottle contained 30.6 mg O_2 (20.8% O_2 v/v) which was reduced to 22.1–23.0 mg (15–16% O_2 v/v) in control soils and to 13.2–14.7 mg (9–10% O_2 v/v) in biofertilizer-treated soils.

Nitrogen derived by plants from biofertilizers and soils

The ^{15}N measurements allowed detection of nitrogen uptake by the rice plants from biofertilizers and soil (Table 4). In 60 days, the rice plants of control soils derived 8.24 mg N but the values were 15.76, 22.72 and 25.92 mg for the plants grown in Azolla, Anabaena and Nostoc-treated soils, respectively. Of 15.76 mg N, the rice plants received 7.50 mg N from applied Azolla and 8.26 mg N from soil. Similarly the rice plants received 13.95 mg N from Anabaena and 8.77 mg N from soil; 16.01 mg N was received by the rice plants from Nostoc and 9.91 mg N was derived from the soil. Therefore, in 60 days the two blue-green algae supplied about double the amount of nitrogen supplied by Azolla. A considerable amount of nitrogen was also supplied by the soil for plant use.

Discussion

Data obtained show that the total dry matter yield of IR8 rice plants increased significantly by 74, 105 and 125% due to an increased

Table 3. Recovery of ^{15}N of Azolla and blue-green algae applied into waterlogged rice soils in plants and soils in 60 days

	With rice plants[a]			Without rice plants[b]		
^{15}N fractions	Azolla	Anabaena	Nostoc	Azolla	Anabaena	Nostoc
Applied at the start, mg pot^{-1}	7.95	8.75	5.80	0.40	0.44	0.29
Released for plant uptake						
mg pot^{-1}	2.08	4.30	3.08	0.12	0.19	0.13
percent	26	49	53	30	43	45
Received by rice plants,						
mg pot^{-1}	1.49	3.05	2.32			
percent	19	35	40			
Remaining in soils,						
mg pot^{-1}	5.87	4.45	2.72	0.28	0.25	0.16
percent	74	51	47	70	57	55
Not detected						
mg pot^{-1}	0.59	1.25	0.76	0.12[c]	0.18	0.12
percent	7	14	13	30	41	42

[a] 0.5 kg soil in each pot, [b] 10 g soil in each glass bottle, [c] Nitrogen loss measured as N_2.

Table 4. Differentiating the amount of total-N assimilated by IR8 rice plants from applied biofertilizers and that from soil in 60 days

Treatments	Total-N assimilated by the plants (mg pot^{-1})	N-derived from applied biofertilizers		N-derived from soil	
		mg pot^{-1}	%	mg pot^{-1}	%
Control	8.24			8.24	100
Azolla caroliniana	15.76	7.50	48	8.26	52
Anabaena variabilis	22.72	13.95	61	8.77	39
Nostoc muscorum	25.92	16.01	62	9.91	38

uptake of 91, 176 and 215% more nitrogen from applied Azolla, Anabaena and Nostoc, respectively, in 60 days. The rice plants of the control soil produced the lowest amount of total dry matter (566 mg) receiving the least amount of total nitrogen (8.24 mg) compared to the highest amount of dry matter of Nostoc-treated plants (1275 mg) due to the highest amount of nitrogen uptake (25.92 mg). The ^{15}N measurements also show that the rice plants assimilated 19, 35 and 40% of total ^{15}N applied as Azolla, Anabaena and Nostoc, respectively. Such a difference in nitrogen supply was dependent upon the rate of decomposition of Azolla and blue-green algae. It is seen that about 50% of total ^{15}N was released from the two blue-green algae in 60 days whereas only 26% of total ^{15}N in Azolla was released during the same period. Shi *et al.*[9] found that the rice plants received 20.4% of total Azolla-N in 85 days. Tirol *et al.*[11] reported that about 28% of applied ^{15}N of *Nostoc* sp. was assimilated by IR32 rice plants grown in pots.

The applied biomass of Azolla and blue-green algae started decomposition within a few days of their incorporation into the soils. This is evidenced by the assimilation of an appreciable amount of ^{15}N by the rice plants within 15 days and by $^{15}NH_4$-N accumulation in the soils. However, after 15 days $^{15}NH_4$-N in the soils gradually declined to a negligible amount at 60 days although nitrogen uptake by the rice plants progressively increased with time. There was no build-up of $^{15}NO_3$-N to account for the $^{15}NH_4$-N which disappeared. Besides continuous uptake by the growing rice plants, some of the released $^{15}NH_4$-N was oxidized to $^{15}NO_3$-N which did not accumulate (mainly due to plant uptake and partly due to loss through denitrification). In addition, it may be expected that the soil microbes also assimilated some of the generated $^{15}NH_4$-N and NO_3-N.

About 7% of the applied ^{15}N of Azolla was lost from soil in 60 days. The loss was 13–14% in the case of blue-green algae. Although it is not possible to measure such a loss from open pot soils, accumulation of some NO_3-N in soils at 30 days suggests that nitrification occurred in these soils which in turn initiated denitrification. Direct evidence of denitrification comes from the detection of N_2 evolved from the biofertilizer-mixed soils incubated in closed containers. It is seen that in soils without rice plants about 93–94% of released $^{15}NH_4$-N of Azolla and blue-green algae was lost as N_2 in 60 days due to nitrification-denitrification processes. Shi *et al.*[9] reported that 14.6% of Azolla-N was lost from soil in 85 days compared with an uptake of 20.4% by the rice plants. Tirol *et al.*[11] also found that 8% of ^{15}N in *Nostoc* sp. was lost from soil and that the IR32 rice crop received 28% of the total amount applied.

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