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USE OF ^{15}N IN THE STUDY OF BIOLOGICAL NITROGEN FIXATION IN PADDY SOILS AT THE INTERNATIONAL RICE RESEACH INSTITUTE

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Abstract

Nitrogen fixation studies form an important aspect of the research programme in the Soil Microbiology Department at the International Rice Research Institute, particularly as dinitrogen fixation (N_2-fixation) is a key factor in determining nitrogen supply in wetland rice soils of developing areas. The ^{15}N technique has been used - (i) to detect dinitrogen fixation by the $^{15}N_2$ incorporation, (ii) to follow the behaviour of nitrogen fixed by dinitrogen-fixing organisms and (iii) to assess the contribution of N_2-fixation by the ^{15}N dilution method. Since the introduction of emission spectroscopic apparatus in 1972, the volume of studies using ^{15}N has increased.

Nitrogen fixation studies form an important aspect of the research program in Soil Microbiology Department at the International Rice Research Institute, particularly as dinitrogen fixation (N_2-fixation) is a key factor in determining nitrogen supply in wetland rice soils of developing areas. The ^{15}N technique has been used -- (i) to detect dinitrogen fixation by $^{15}N_2$ incorporation (1, 2, 3, 5, 6, 7, 10); (ii) to follow the behavior of nitrogen fixed by N_2-fixing organisms (8, 9, 11, 12); and (iii) to assess the contribution of N_2-fixation by the ^{15}N dilution method (10, 13).

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1) DETECTION OF N_2-FIXATION BY ^{15}N INCORPORATION

Before the routine use of acetylene reduction technique, the $^{15}N_2$ feeding technique was the only sensitive assay technique for dinitrogen fixation.

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11) Soil Samples

The effects of light and organic matter application to N_2-fixation in flooded soils were studied in the laboratory (1, 2). A first trial to estimate phototrophic nitrogen fixation was conducted in 1968 using soil from pot experiments incubated in test tubes under an atmosphere enriched with ^{15}N. The data (Table 1) shows that phototrophic NFA was dominant in this soil and that the addition of N fertilizer remarkably depressed the amount of nitrogen fixed. In soils exposed to the light, without nitrogen fertilizer, NFA corresponding to 30 kg $N.ha^{-1}.month^{-1}$ was estimated (IRRI, 1968). However, it has been reported that small scale experiments favor the growth of blue-green algae (Roger and Kulasooriya, 1980) and may largely overestimate photodependent N_2-fixation.

The effect of various fertilizers on N_2-fixation can be seen in Table 2. Inhibition was observed on both phototrophic and heterotrophic N_2-fixation with almost complete inhibition at 160 ppm N. Luxuriant growth of algae occurred in pots receiving ammonium N but the amount of fixed N was not appreciable. This indicated that besides an inhibitory effect on nitrogenase activity, a stimulation of the growth of non-fixing algae by mineral nitrogen can limit N_2-fixing blue-green algae growth by competition and antagonistic effects (IRRI, 1968).

12) Comparision with acetylene reduction method

The relation between acetylene reduction activity and $^{15}N_2$ uptake activity was examined for soil samples[3]. With the isotope method, nitrogen fixation was 2.61, 2.54 and 2.59 µg/10 g/day after incubating the soil under 0.2 atm O_2 + 0.25 atm N_2 for 1, 3, and 5 days, respectively. With the acetylene reduction method, evaluated nitrogen fixation was 2.64 µg/day after 6 h incubation (3:1 conversion ratio). The two methods gave similar results despite the difference of incubation period (3).

A similar comparison was made with Azolla. Azolla pinnata fixed 0.65 μmol $^{15}N_2$/hr/g fresh weight for a 24 h exposure to ^{15}N under a 10 klx light intensity. With the acetylene reduction method, activity was 2.18 μmol C_2H_4/hr/g fresh weight. Therefore, the ratio of C_2H_4 reduced to $^{15}N_2$ fixed was 3.3 (close to theoretical value) (O. Ito, unpublished).

13) N_2-fixation associated with rice

131) Dinitrogen fixation associated with wetland rice was confirmed by exposing rice plant to an atmosphere containing $^{15}N_2$ gas for 7 days (Table 3a, b). It was shown that both root and basal part of shoot are sites for N_2-fixation. N_2-fixing activity measured by $^{15}N_2$ incorporation was close to the values estimated by acetylene reduction assays (7).

132) Dinitrogen fixation associated with deepwater rice. It was observed that submerged parts of deepwater rice are colonized by blue-green algae. Photodependent acetylene reduction activities were found to be associated with submerged roots, leaf sheaths, and to a lesser extent, culms. To confirm N_2-fixation associated with deepwater rice and to study the availability of the fixed nitrogen to the plant, submerged parts of deepwater rice at heading stage were exposed to $^{15}N_2$ for 9 days and harvested after grain maturation (Table 4). It was found that the submerged roots and leaf sheaths colonized by blue-green algae had higher ^{15}N enrichment than the other parts of the plants. Approximately 40% of the ^{15}N in the plant was recovered from the aerial parts which were not directly exposed to $^{15}N_2$. It was estimated that 8 mg N were fixed per plant over a 9 day period. This activity is much higher than that reported for heterotrophic N_2-fixation associated with wetland rice. These results demonstrate that photodependent N_2-fixation is associated with deepwater rice, the nitrogen fixed being utilized directly by the rice plant (10, 11)

2) AVAILABILITY AND FATE OF BIOLOGICALLY FIXED NITROGEN

It is now well established that N_2-fixation by Azolla, blue-green algae and heterotrophic bacteria plays a vital role in the soil fertility and studies were undertaken to establish how much fixed nitrogen is available to the rice plant.

21) Azolla

The availability to the rice plant of Azolla N was examined in pots and in field experiments using A. pinnata labeled with ^{15}N-nitrate. The Azolla plants were either floated on the water, or incorporated in the soil. Over a 17 week period the rice plant in pot took up 53% of ^{15}N from the incorporated Azolla and only 10% from the floating Azolla. When Azolla was placed on the soil surface highest loss, amounting 60% for 6 weeks were observed; losses were 50% when it was floated on the water and only 33% when incorporated. In the field ^{15}N labeled Azolla (41 kg N/ha) was added at 30 days after transplanting. The first rice crop was absorbed 26% and 13% of N from incorporated and floating Azolla, respectively. The availability to the second crop was only 5% in both cases (9). As a consequence of multiplication of floating Azolla the ^{15}N content was diluted and the availability of ^{15}N was much lower than the availability of Azolla N per se.

22) Blue-green algae

In a preliminary experiment Gloeotrichia sp. was grown in a small pond with ^{15}N-nitrate added. Fresh algal material (1.1% N in dry matter, 8.89 atom % excess) was incorporated in a 1 m^2 plot. Recovery of ^{15}N in the grain, straw and root of the first crop was 14.7% of the ^{15}N applied as algal mass. In the second crop, the recovery was only 2.25%. Although ^{15}N remaining in soil was not analyzed, this preliminary experiment suggested a low availability of algal N to rice crop. Because the algal mass used in this

experiment had an unusual low N content and a high C/N ratio the results were of limited value (12).

In a second experiment, A. Nostoc sp. was grown under laboratory conditions, labeled with ^{15}N-NO_3, was harvested, and kept dried or fresh (7.3% N, dry weight basis). Uptake of ^{15}N from this material by rice was studied by pot and field experiments (12, 13). Availability of N from dried BGA incorporated in the soil was 23-28% for the first crop and 27-36% for two consecutive crops (Table 5). Surface application of algal material reduced ^{15}N availability to 14-23% for the first crop and 21-27% for two crops. The availability of algal ^{15}N was less than that of ammonium sulfate, but for two crops its availability was very similar. After two crops more N remained in soil from algae than from ammonium sulfate (11).

Availability of N from the incorporated fresh algae was higher than that from dried algae. It can be concluded that blue-green algal N is less susceptible to N losses than inorganic fertilizers, its low C/N ratio imparts a better N availability than such organic fertilizers as farmyard manure.

23) Heterotrophic bacteria

Experiments were designed to study the availability to the rice plant of N fixed heterotrophically in paddy soil and its subsequent transformations in soil. Soil samples enriched with glucose were incubated under $^{15}N_2$. ^{15}N-ammonium sulfate was added in other samples to compare transformations of nitrogen derived from heterotrophic dinitrogen fixation and immobilized ammonium (8). Results showed that biologically fixed-N undergoes similar transformations to that of immobilized ammonium N (8). After 42 days rice plants had absorbed 34% of the fixed-N and 8% of the soil-N. This result showed that heterotrophically fixed N remained as readily decomposable N.

3) THE ^{15}N DILUTION TECHNIQUE

31) Experiments with deepwater rice

A ^{15}N dilution experiment was conducted simultaneously with the $^{15}N_2$ feeding experiment on deepwater rice described above. Deepwater rice was grown in pots with ^{15}N labeled ammonium sulfate (600 mg N/pot). After harvest, plants were split into various portions, as in the feeding experiment, and the ^{15}N content determined. ^{15}N contents of various portions of the plants in $^{15}N_2$ feeding experiments were negatively correlated to ^{15}N contents of corresponding portions of the plants grown in pots with ^{15}N-ammonium sulfate ($r = -0.73$) (Fig. 1). This negative correlation suggests that ^{15}N dilution, which was found to be more intense in submerged roots and leaf sheaths than other parts, was due to photodependent nitrogen fixation on these parts (10). Submerged roots and leaf sheaths of deepwater rice covered with black cloth had higher ^{15}N contents (less dilution) than the same parts exposed to light. This suggests that photodependent N_2-fixation diluted ^{15}N in the tissues of deepwater rice.

Rice was grown in deep and shallow water conditions, in the Philippines and Thailand (10). Rice grown in deepwater condition showed a lower ^{15}N content, suggesting that nitrogen in the water, occurring as combined N, dinitrogen, or both, contributes to nitrogen nutrition of deepwater rice (Table 6).

Experiments on deepwater rice suggests the potential of the ^{15}N dilution technique in identifying active sites of dinitrogen fixation, providing that control experiments are carried out to prove that the observed difference in ^{15}N contents is due to solely N_2-fixation.

32) Relationships between N balance and ^{15}N dilution

Nitrogen balance studies by total nitrogen analysis provide

an approximation of net nitrogen input in the system. Although no sophisticated techniques as $^{15}N_2$ incorporation or acetylene reduction are required, this method is labor intensive and time consuming. Principles of isotope dilution may be applied to assess the amount of plant nitrogen derived from dinitrogen by comparison of a N_2-fixing system with a non fixing, but otherwise identical, system.

^{15}N-ammonium sulfate and sucrose were added to moist soil in order to label the nitrogen fraction of the biomass. The soil was then air-dried, remoistened and planted with two consecutive crops of dryland rice or wetland rice (14). Total N balance and ^{15}N contents were measured in grains, straws and roots (Table 7). Dryland rice had a lower positive nitrogen balance and a much higher ^{15}N contents than wetland rice. When pot surfaces were covered with black cloth, to suppress photodependent N_2-fixation, no statistically significant nitrogen gain was observed. ^{15}N content of plants was higher than when photodependent N_2-fixation by blue-green algae or Azolla was allowed and a negative correlation between nitrogen balance and ^{15}N content in the plant was observed (Fig. 2). Therefore, it is reasonable to assume that the lower ^{15}N contents in rice grown in unshaded pots was due to the dilution of ^{15}N by nitrogen derived from photodependent (free-living and symbiotic) dinitrogen fixation. When wetland rice growing in pots with suppression of photodependent N_2-fixation was taken as a control system, the calculated contribution of nitrogen derived from photodependent N_2-fixation to nitrogen in the plant was 20-30%.

4) FUTURE USE OF ^{15}N IN N_2-FIXATION STUDIES

Undoubtedly $^{15}N_2$-feeding experiments are necessary for direct evidence of N_2-fixation. Unfortunately, $^{15}N_2$ gas is too expensive for general field application. Growing one rice plant in an $^{15}N_2$ atmosphere costs several thousand US dollars!

^{15}N dilution experiments are quite suitable for application to wider aspects of dinitrogen fixation studies. Providing proper control of the non N_2-fixing system is selected, the ^{15}N dilution technique could be useful in evaluating the effect of inoculation by blue-green algae, Azolla or bacteria, identifying the sites of N_2-fixation and screening highly active N_2-fixing systems (blue-green algae, Azolla, or rice in association with bacteria).

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Table 1. Nitrogen fixation under various soil treatments (IRRI, Annual Report for 1968).

Pretreatment*	Incubated in light		Incubated in dark	
	Atom % excess N^{15}**	Estimated amount of fixed N (kg/ha/month)	Atom % excess N^{15}**	Estimated amount of fixed N (kg/ha/month
PKL † No plant	.395	32.0	.038	3.2
PKD †† No plant	.493	32.8	.020	1.2
PKL IR8	.459	32.6	.026	1.9
PKD IR8	.514	36.0	.006	0.5
PKL Peta	.517	38.1	.047	3.4
PKD Peta	.410	27.2	.027	1.8
NPKL No plant	.043	5.7	.048	4.6
NPKD No plant	.020	2.0	.000	0.0
NPKL IR8	.075	6.4	.017	1.5
NPKD IR8	.070	5.1	.024	1.8
NPKL Peta	.151	13.3	.031	2.5
NPKD Peta	.089	5.6	.000	0.0

*Treatment of Maahas clay soil in greenhouse pots from which soil samples were obtained 2 months after transplanting IR8 and Peta.

**Determined after incubation of soil in glass tubes for 1 month in the greenhouse.

†L - Light (good growth of indigenous algae).

††D - Dark (no algal growth observed).

Table 2. Effect of ammonium N fertilizer on nitrogen fixation (IRRI, Annual Report for 1968).

Fertilizer	Level of N application (ppm)	Atom % excess N^{15}	
		Incubation in light	Incubation in dark
No fertilizer	0	0.150	0.100
Ammonium sulfate	80	0.042	0.036
	160	0.009	0.000
Ammonium chloride	80	0.005	0.015
	160	0.000	0.000
Urea	80	0.024	0.043
	160	0.000	0.000

Table 3a. $^{15}N_2$ incorporation into rice plant (IR26) in a growth chamber for 7 days.

Sample no.	Dry wt (g)	% of N	^{15}N content (atom % excess)	Amount of ^{15}N (μg)	Apparent N_2 fixation rate (μmol of N_2/day)[b]
1 Root	14.2	0.495	0.714	503	5.40
Outer leaf sheath	5.14	0.569	1.57	461	4.95
Inner leaf sheath[a]	17.3	1.03	0.052	92.0	0.98
Leaf blade	12.2	1.77	0.003	6.51	0.069
Young panicle	1.08	2.63	0.005	1.42	0.015
2 Root	10.89	0.554	0.818	493	5.29
Outer leaf sheath	3.20	0.510	2.57	420	4.51
Inner leaf sheath[a]	21.37	0.922	0.091	179	1.92
Leaf blade	12.80	1.60	0.001	2.40	0.026
Young panicle	1.98	1.94	0.002	0.769	0.008

[a]Basal nodes are included.

[b]Atom % excess of $^{15}N_2$ gas was 44.3%.

Table 3b. $^{15}N_2$ incorporation into rice plants in a growth chamber for 7 days.

Plant part	Dry wt (g)	% of N	^{15}N content (atom % excess)	Amount of ^{15}N fixed (µg)	Apparent N_2 fixation rate (µmol of N_2/day)[a]
Uncovered					
Root	2.33	0.765	0.501	89.3	1.77
Outer leaf sheath	2.29	0.727	0.947	158.0	3.11
Basal node	1.66	0.515	0.245	20.9	0.413
Inner leaf sheath	9.34	1.06	0.028	27.6	0.545
Leaf blade	4.02	1.97	0.031	24.6	0.486
Young panicle	3.58	1.16	0.017	7.07	0.139
Covered					
Root	2.25	0.769	0.424	73.3	1.45
Outer leaf sheath	3.22	0.544	1.86	324.0	6.40
Basal node	1.57	0.549	0.385	32.2	0.656
Inner leaf sheath	9.25	0.929	0.059	50.7	1.00
Leaf blade	5.31	1.51	0.040	32.1	0.034
Young panicle	2.72	1.33	0.039	14.1	0.279

[a]Atom % excess of $^{15}N_2$ gas was 24.2.

Ito et al (7).

Table 4. N_2-fixation by deepwater rice exposed to $^{15}N_2$ for 9 days, 1980 wet season, IRRI.

Plant parts	Total N mg N/pot	Atom % excess	Fixed N µg N/pot[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
Floating 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
Submerged parts			
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 plant	542 ± 51		7973
Submerged weed	41 ± 12	0.97 ± 0.48	823

[a]Assuming the average of 48.1 atom % excess of $^{15}N_2$ during 9 days exposure. Watanabe et al (1980).

Table 5. Recovery of ^{15}N (%) in algae after two crops of rice in soil and plants (IRRI Annual Report for 1981).

Experimental	BGA incorporated				Surface applied			
	1st crop	2nd crop	Soil	Unaccounted	1st crop	2nd crop	Soil	Unaccounted
Fresh algae pot experiment	38	5	53	4	13	8	73	6
Dried algae pot experiment	28	8.5	58	5.5	14	7	57	22
Dried algae field experiment	23.5	3.5	33*		23	4	32*	

*In the 0-15 cm layer of the soil.

Roger and Watanabe (12).

Table 6. $^{15}N/^{14}N$ ratio in deep water rice plants growing under shallow and deep water conditions (IRRI, Annual report for 1980).

Site	Water depth	Light exposure[a/]	Total N mg/pot A	^{15}N mg/pot B	$^{15}N/^{14}N$ ratio B/A x 100
Bangkhen	Shallow	+	718	111	15.4
Hantra	Semi-deep	+	979	86.2	8.8
Bangkok	Deep	+	952	97.6	10.2
IRRI	Shallow	+	502	194	38.6
	Shallow	-	504	230	45.6
	Deep	+	737	201	27.2
	Deep	-	530	114	21.5

[a/] + : covered with sack cloth
- : not covered with black cloth.

Table 7. Balance of labeled and unlabeled nitrogen and ^{15}N content in grains, straws and roots of rice.

Treatment	Balance of nitrogen (mg/pot)		Atom % excess of ^{15}N in plant*
	^{15}N*	^{14}N	
Dryland, unplanted	- 58	125	-
Dryland, planted	- 34	216	7.14
Flooded, unplanted	- 49	- 658	-
Flooded, planted	- 27	814	3.93
Flooded, black cloth**	- 42	4^{ns}	4.35
Flooded, Azolla + P	- 41	938	3.62
Flooded, algae + P + Ca	- 28	1013	3.80
Flooded, black cloth + P + Ca	- 35	145^{ns}	4.08
Standard error	10	115	0.07

*Initial amount of ^{15}N was 196 mg. Atom % excess of ammonium sulphate was 31.06.

**Pot surface was covered by black cloth (Ventura and Watanabe, in press).

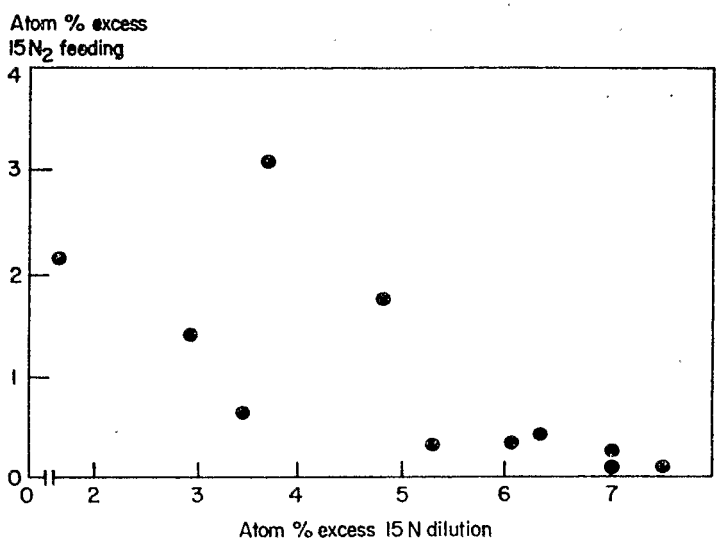

Fig. 1. Relationship between ^{15}N contents of various parts of deepwater rice, determined by $^{15}N_2$ feeding and ^{15}N dilution techniques.

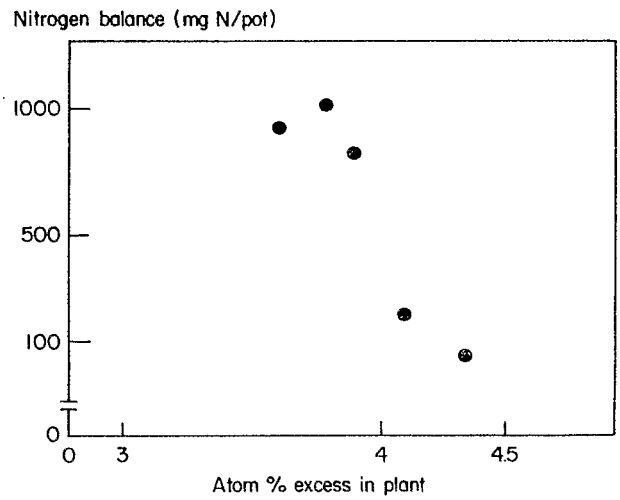

Fig. 2. Relationship between nitrogen balance in flooded soil-rice ecosystem and ^{15}N content in rice plants.