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Isotopic Studies on N_2-Fixation in *Azolla* and the Availability of its Nitrogen to Rice

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Abstract

The use of *Azolla* as a nitrogen fertilizer depends primarily upon its ability to fix nitrogen efficiently and on the availability of this nitrogen to an associated crop. In this study, ^{15}N-labelled material was used to evaluate N_2 fixation by *Azolla* in the field and to assess N-uptake from *Azolla* by rice with respect to the time of incorporation and in relation to its quality. *Azolla* derived 50–60% of its nitrogen through fixation and and this was equivalent to 11–14 $kgNha^{-1}$ during a 14-day growth period. N-uptake was better when *Azolla* was incorporated at tillering than at transplanting and the recovery of nitrogen by rice from *Azolla* was more efficient than from urea, except when the fibre:nitrogen content of *Azolla* was high.

Keywords: ^{15}N uptake, *Azolla*, N_2 fixation

1. Introduction

The aquatic fern *Azolla* possesses an endosymbiotic cyanobacterium *Anabaena-azollae*, which fixes nitrogen. This plant which grows luxuriently in rice fields, has been used traditionally as a biofertilizer in China and Vietnam for centuries (Dao and Tran, 1979; Lumpkin and Plucknett, 1982), but scientific investigations on this plant are a relatively recent development (Watanabe, 1982).

Nitrogen fixation by field grown *Azolla* measured by the acetylene reduction technique, gives only approximate values and numerous problems are encountered in the conversion of the acetylene reduced to nitrogen fixed. A method using the heavy isotope ^{15}N was developed by Fried and Middleboe (1977) for the quantitative estimation of nitrogen fixation by field grown legumes and this has been applied to *Azolla* in certain preliminary studies (Eskew, 1987). Although the beneficial effects of *Azolla* on rice is largely attributed to the supply of its nitrogen, direct evidence for such transfer is provided only in a few reports (Kumarasinghe et al., 1986). The availability of nitrogen from *Azolla* depends upon its rate of decomposition after incorporation into soil and this is related to its quality, especially its C:N ratio (Liu Chung Chu, 1979). This paper reports on field experiments where ^{15}N was used to quantify nitrogen fixation by *Azolla* and to determine the availability of its nitrogen to rice with respect to the time of incorporation and in relation to its quality. The uptake of nitrogen from *Azolla* was also compared with that from urea fertilizer.

2. Materials and Methods

Quantitative estimation of nitrogen fixation

The ^{15}N-dilution technique of Fried and Middleboe (1977) was adopted in this study. The N_2-fixing test plants used were, *Azolla pinnata* var. *imbricata* (indigenous) and *Azolla microphylla*, (introduced from the Philippines) with *Salvinia molesta* and *Lemna perpusilla* as the non-fixing reference plants. The experiment was carried in 1 m^2 microplots bunded by 25 cm levees lined with plastic sheets, in monoculture as well as in dual culture with rice transplanted at 20×20 cm spacing. After land preparation and flooding to a 5 cm flood level, each microplot was subdivided into 4 equal quadrats using pieces of bamboo fixed at the level of the floodwater surface (Fig. 1). ^{15}N-labelled urea (11.3% atom excess) was added to each microplot to give an initial concentration of 40 ppm N in the floodwater. Five g (fresh weight) of each plant material was introduced into each subplot as initial inoculum. In this manner, the test and reference plants were kept immersed in the same soil solution containing ^{15}N, but effectively separated from one another. Each treatment was replicated 4 times and arranged in a randomized complete block design. ^{15}N-labelled urea solution was added every 3 days during the period of the experiment. The plants were harvested separately after 2 weeks, dried to a constant weight at 60°C, the dry weight recorded and then pow-

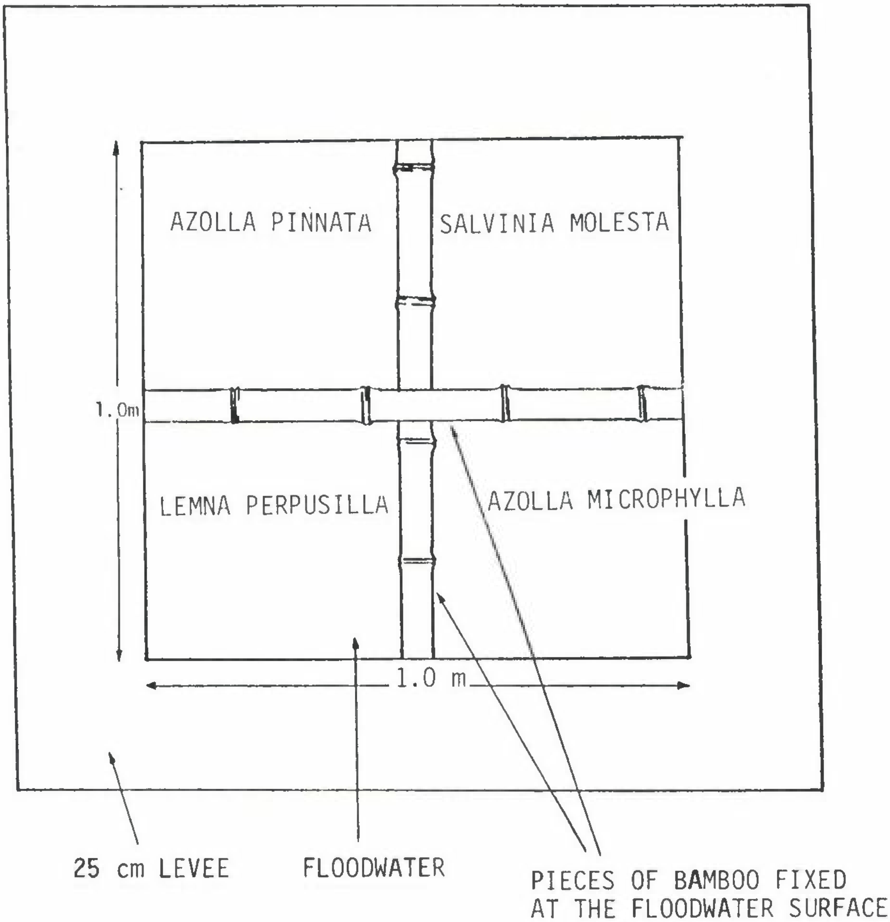

Figure 1. Diagrammatic representation of the 1 m^2 microplot used for the field measurement of N_2-fixation by *Azolla*

dered. Aliquot samples of this material were analysed for total nitrogen by micro-kjeldahl analysis and for ^{15}N enrichment by emission spectrometry.

The availability of Azolla-N to rice with respect to the time of incorporation

Uptake of nutrients from soil incorporated fertilizers depends upon their availability as well as the growth stage of the crop. In this experiment ^{15}N-labelled material was used to determine the uptake of *Azolla*-N and urea-N by rice when incorporated at transplanting and at tillering, in comparison to the application of urea according to the recommended best split method.

The species of *Azolla* used in this experiment was *A. pinnata* var. *imbricata*. The isotopic treatments were:

1. Labelled *Azolla* (equivalent to 30 $kgNha^{-1}$) incorporated at transplanting, followed by unlabelled *Azolla* (30 $kgNha^{-1}$)incorporated at maximum tillering.
2. Unlabelled *Azolla* (30 $kgNha^{-1}$) incorporated at transplanting, followed by labelled *Azolla* (30 $kgNha^{-1}$) incorporated at maximum tillering.
3. Labelled urea (30 $kgNha^{-1}$) incorporated at transplanting, followed by unlabelled urea (30 $kgNha^{-1}$) incorporated at maximum tillering.
4. Unlabelled urea (30 $kgNha^{-1}$) incorporated at transplanting, followed by labelled urea (30 $kgNha^{-1}$) incorporated at maximum tillering.
5. Labelled urea (60 $kgNha^{-1}$) applied according to the recommended best split method (20% at basal and 40% each as top dressing at maximum tillering and 2 weeks before panicle initiation).
6. Control without any N-fertilizers added, but soil incorporations simulated at transplanting and at maximum tillering.

The above treatments were given in 1 m×1 m microplots. Each treatment was replicated 6 times and the plots were arranged in a randomized complete block design.

As it would be inaccurate to extrapolate yield data obtained from 1 m^2 microplots, the following treatments were given in 5 m×4 m yield plots to obtain data on grain yield.

1. 60 $kgNha^{-1}$ unlabelled urea applied according to the recommended best split method.
2. 30 $kgNha^{-1}$ of unlabelled *Azolla* incorporated at transplanting, followed by 30 $kgNha^{-1}$ of unlabelled *Azolla* incorporated at maximum tillering.
3. 30 $kgNha^{-1}$ of unlabelled urea incorporated at transplanting, followed by 30 $kgNha^{-1}$ of unlabelled urea incorporated at maximum tillering.
4. Control without N-fertilizers added, but incorporations simulated at transplanting and at maximum tillering.

Field site preparation

The site selected has not been used for two rice growing seasons and was generally poor in soil nitrogen. Both the isotope plots and the yield plots were laid across a soil fertility and water flow gradient. The isotope plots were arranged in three blocks and the yield plots in two blocks, across the gradient. Each isotope plot of 1 m^2 had 30 cm levees between them, and 45 cm bunds between the blocks. All the bunds were lined with thick polythene sheets to prevent leaching and translocation. The 20 m^2 yield plots also had 30 cm and 45 cm bunds like the isotope plots, but without polythene linings.

Field nursery of unlabelled Azolla

The field nursery plot was prepared adjacent to the experimental site. An inoculum of 1.5 kgm^{-2} (fresh weight) of *Azolla pinnata* var. *imbricata* from material grown in a green house was used. Concentrated superphosphate fertilizer and carbofuran insecticide was regularly added to obtain healthy *Azolla* material.

Labelling of Azolla with ^{15}N

Azolla was labelled in concrete tanks of 1 m depth, to which soil from the *Azolla* nursery was added to a depth of 4 cm and water added to a further depth of 4 cm. *Azolla* from the unlabelled nursery was used as inoculum for these tanks. A solution of ^{15}N labelled urea (10% a.e.) was added daily to maintain a concentration of 50 ppm N. Concentrated superphosphate fertilizer and carbofuran were added regularly to ensure healthy *Azolla* growth.

Experimental procedure

A high nitrogen responsive, high yielding, short duration variety of rice (BG 276-5) recommended for this locality was used in this experiment. Twelve-day old rice seedlings raised in a 'dapog nursery' were transplanted at 20 × 20 cm spacings. Labelled and unlabelled urea was incorporated into the corresponding plots prior to transplanting. Basal P and K fertilizers were added respectively, at the rates of 46.56 kg $P_2O_5ha^{-1}$ as concentrated superphosphate and 19.64 kg K_2Oha^{-1} as muriate of potash. The transplanted rice was allowed to establish overnight and floodwater was introduced the following day. Rice plants were harvested at grain maturity avoiding the border rows. The harvested material was separated into panicles and straw and representative sub-samples from these were dried at 60°C to a constant weight and the dry weights were recorded. Aliquot samples were powdered and separated for total N-determinations and ^{15}N-analyses.

Uptake of nitrogen from different species of Azolla in comparison to urea fertilizer

As in the case of other green manure, soil nutrients incorporated in *Azolla* become available to the associated crop, after their death and decomposition. It has also been reported that such availability is dependent upon the quality of the incorporated material (Liu Chung Chu, 1979). The present experiment was carried out to compare the uptake of nitrogen by rice, from four different species of *Azolla*; *A. pinnata* var. *imbricata*, *A. pinnata* var. *pinnata*, *A. caroliniana* and *A. microphylla*. All the *Azolla* species were pre-labelled with ^{15}N and applied at the rate of 30 $kgNha^{-1}$ and compared with the application of 3 levels (15, 30 and 45 $kgNha^{-1}$) of ^{15}N-labelled urea (10% a.e.).

All fertilizers were added as a basal dressing, prior to transplanting of rice. The treatments were as follows:

1. Control, without any added N, but incorporations simulated.
2. 15 $kgNha^{-1}$ of labelled urea
3. 30 $kgNha^{-1}$ of labelled urea
4. 45 $kgNha^{-1}$ of labelled urea
5. 30 $kgNha^{-1}$ of labelled *A. pinnata* var. *pinnata*
6. 30 $kgNha^{-1}$ of labelled *A. pinnata* var. *imbricata*
7. 30 $kgNha^{-1}$ of *A. caroliniana*
8. 30 $kgNha^{-1}$ of *A. microphylla*

The treatments were given in 1 m^2 microplots, bunded by 30 cm levees, lined with polythene sheets. All the treatments had 6 replicates each, arranged on a randomised complete block design.

Preparation of ^{15}N-labelled Azolla

Four nursery plots, each 6 m×1.5 m were arranged alongside one another separated by 30 cm levees. Thirty cm drains were dug out to drain off excess water in case of heavy rain. Coconut leaf woven cadjans were erected alongside the bunds to prevent the mixing of *Azolla* species during rain. The floodwater level was maintained at 5 cm. An improved method of adding ^{15}N-labelled fertilizer stepwise was adopted in order to obtain enriched *Azolla* material. To each microplot 2260 g of labelled, fresh *Azolla* was added. The different amounts of urea were also incorporated to the corresponding microplots and soil incorporation was simulated in the control plots.

Rice plants were harvested at grain maturity, the panicles and straw were separated, dried to a constant weight at 60°C and the dry weights recorded. Representative aliquot samples were powdered and prepared for total-N and ^{15}N-analysis.

Analysis for fibre content

Representative samples of labelled material from the four species of *Azolla* were analysed for their fibre content in the following manner. Hundred g of fresh *Azolla* material was dried at 65°C for 48 hr, and its dry weight recorded. The material was powdered and an aliquot sample was analysed for its total nitrogen. Another aliquot sample was boiled with neutral detergent solution for 1 hr, allowed to cool and the neutral detergent soluble part was removed by filtration. The residue which represents the Neutral Detergent Fibre (NDF), consisted of cellulose, hemicellulose lignin and variable amounts of silica. The residue was then boiled with acid detergent solution for 1 hr, allowed to cool and filtered. The residue, Acid Detergent Fibre (ADF) consisted of cellulose, lignin and acid insoluble ash. From these values, the fibre:N ratios were calculated.

3. Results

The percentage of nitrogen derived from fixation (% Ndffix) was calculated by the following expression.

$$\% \text{ Ndffix} = \left(1 - \frac{\% \ ^{15}\text{N atom excess in test plant}}{\% \ ^{15}\text{N atom excess in reference plant}} \times 100\right)$$

As shown in Table 1 this value varied between 50 to 61% in the different *Azolla* species examined.

The quantification of the N_2-fixed was as follows:

Azolla N-yield = Dry matter yield × % N content:
N-fixed = *Azolla* N-yield × % Ndffix

Results presented in Table 2 show that the quantities of N_2-fixed by the two species under mono and dual culture ranged between 10.8 to 14.2 kgNha^{-1}. In the experiments where the uptake of nitrogen by rice from the labelled fertilizers was evaluated, the calculations were as follows:

Table 1. Percentage of nitrogen derived from fixation by *Azolla* for 14 days in a rice field

Reference plant	Test plant	% NdfFix*	
		Monoculture	Dual culture
Salvinia molesta	*A. pinnata*	61.5±9.2	58.2±12.7
	A. microphylla	59.5±9.9	54.7±8.3
Lemna perpusilla	*A. pinnata*	54.8±13.2	56.9±8.4
	A. microphylla	51.0±6.7	50.8±6.1

* Mean value of 4 replicates

Table 2. Nitrogen fixed by *Azolla* for 14 days in a rice field

Reference plant	Test plant	Monoculture		Dual culture	
		N-yield	NdfFix	N-yield	NdfFix
		(kgha^{-1})		(kgha^{-1}	
Salvinia molesta	*A. pinnata*	23.1	14.2	23.6	13.7
	A. microphylla	21.1	12.6	21.5	11.8
Lemna perpusilla	*A. pinnata*	23.1	12.7	23.1	13.1
	A. microphylla	21.1	10.8	21.5	10.9

The percentage nitrogen derived from fertilizer (% NdfF)

$$\%\ \text{NdfF} = \frac{^{15}\text{N atom excess in plant sample}}{^{15}\text{N atom excess in the fertilizer}} \times 100$$

The N-yield = Dry matter yield × % N-content
The fertilizer N-yield = N yield × % NdfF

$$\%\ \text{N} - \text{recovery} = \frac{\text{(fertilizer N-yield)}}{\text{(fertilizer N-applied)}} \times 100$$

Results of the experiment on availability of N from *A. pinnata* var. *imbricata* in relation to the time of incorporation are shown in Figs. 2(a, b, c) and 3(a,

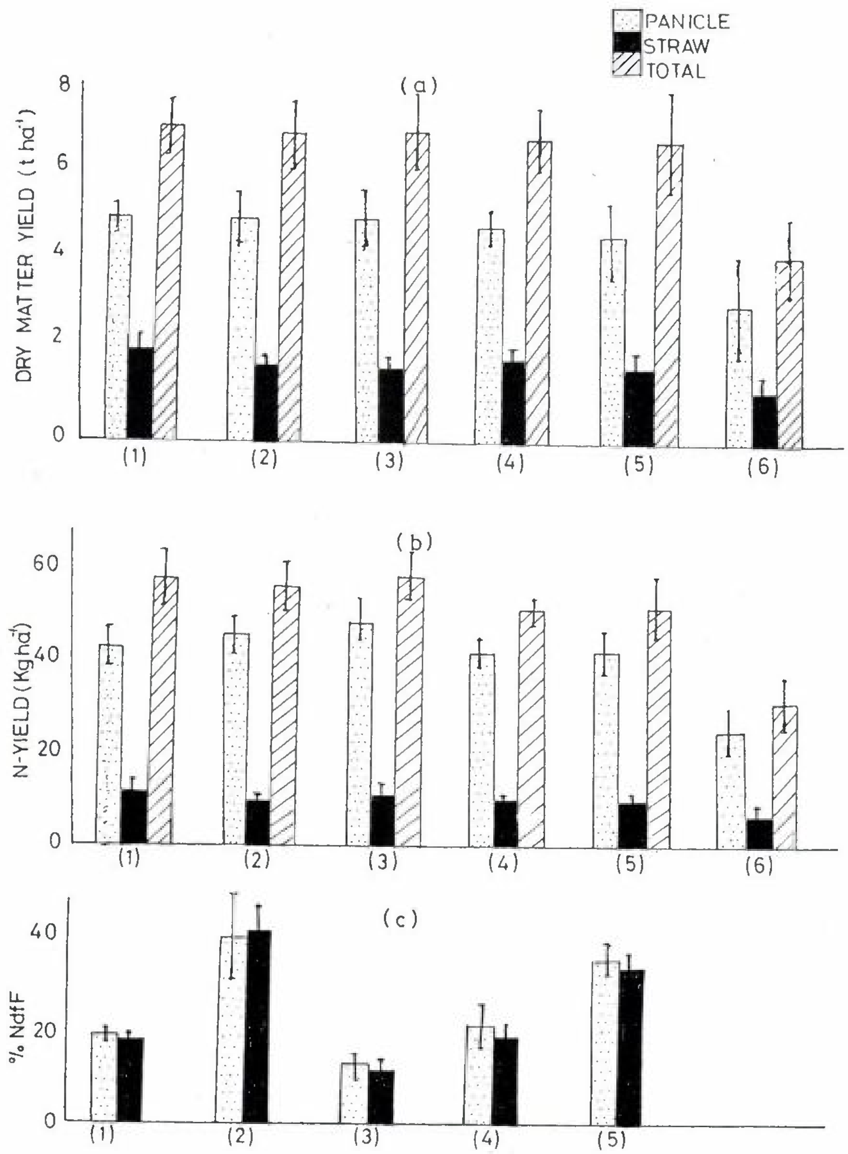

Figure 2. (a) Dry matter yield, (b) N-yield and (c) % NdfF, in a rice crop that received ^{15}N-labelled fertilizer according to the following treatments. (1) Labelled *Azolla* at transplanting; (2) Labelled *Azolla* at tillering; (3) Labelled urea at transplanting; (4) Labelled urea at tillering; (5) Labelled urea, best split; (6) Control, without nitrogen.

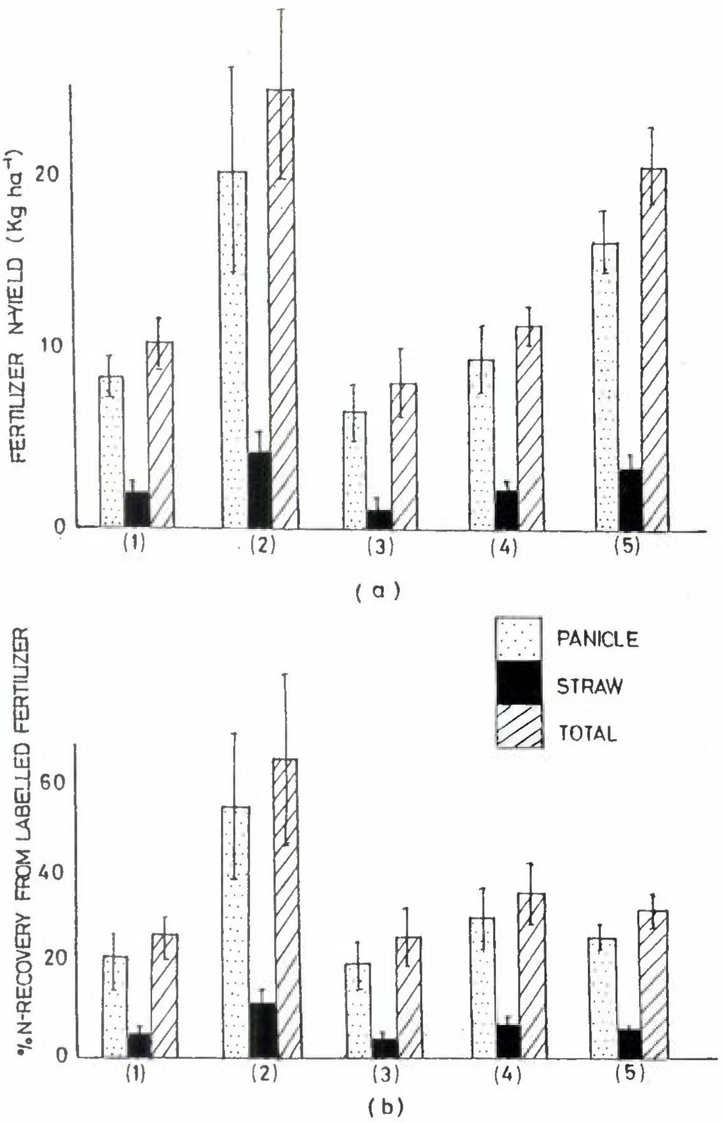

Figure 3. (a) Fertilizer N-yield and (b) % N-recovery from labelled fertilizer in a rice crop that received ^{15}N-labelled fertilizer according to the same treatments as in Fig. 2(a).

Table 3. Yield responses to *Azolla* and urea treatments given at transplanting and tillering in 4 m×5 m yield plots

Treatment	N-fertilizer applied $kg.ha^{-1}$	Grain yielded $t.ha^{-1}$	% increase over control
Control	–	1.84	–
Azolla at transplanting and tillering	47	2.81	52.5
Urea at transplanting and tillering	60	2.60	41.2
Urea best split	60	2.80	52.0

* Mean values of 4 replicates

b, c). Dry matter yield and N-yield of the crop have increased in response to N-additions, with very little differences among the different treatments (Figs. 2a & b). The percentage of nitrogen derived from fertilizer (% NdfF) show differences among the different treatments (Fig. 2C), with *Azolla* incorporated at tillering giving the highest value, followed by the urea best split application. A similar trend was seen in the fertilizer N-yield (Fig. 3a). However, the percentage of N-recovery from the applied fertilizer gave a relatively low value for the urea best split application (Fig. 3b), because the uptake from *Azolla* was from a labelled biomass equivalent to 30 $kgNha^{-1}$, whereas that in the urea best split treatment, was from 60 $kgNha^{-1}$.

Fertilizer N-yield as well as % N-recovery in all the treatments were higher in the panicles than in the straw, indicating the N-compartmentalization efficiency of this variety of rice.

The grain yield data from the yield plots and the yield component analyses are presented in Tables 3 and 4, respectively. Results on the N-availability from different species of *Azolla* in comparison to that from urea, are presented as changes in dry matter yield, N-yield and % N-recovery in Figs. 4a, b & c, respectively. The dry matter yields have increased over the control in all the treatments except the 15 $kgNha^{-1}$ treatment and the *A. pinnata* treatments. The total N-yields show significant increases in the 30 $kgNha^{-1}$ treatment and the *A. caroliniana* and *A. pinnata* var. *pinnata* treatment. In the urea treatments, however, the % N-recovery has decreased with increase in the rate of fertilizer applications. *A. pinnata* var. *imbricata* treatments which had the highest fibre to nitrogen ratio (Table 5).

Table 4. Yield component analyses from the yield plots

Treatment	Panicles per hill	Filled grains per panicle	% unfilled grain	100 grain weight (g)
Control	4.5	67.3	34.5	2.88
Azolla at transplanting and tillering	6.8	69.6	39.1	2.91
Urea at transplanting and tillering	6.1	67.7	38.1	2.83
Urea best split	6.3	69.2	39.5	2.95
LSD (0.05)	0.6	hs*	ns	ns
CV	66.0	11.1	4.5	2.30

* non significant

Table 5. Fibre and nitrogen contents of *Azolla*

Azolla species	% Fibre	% Nitrogen	F:N ratio
A. pinnata var. *pinnata*	56.61	3.44	16.4
A. pinnata var. *imbricata*	62.44	3.32	18.1
A. caroliniana	57.27	4.14	13.8
A. microphylla	57.74	3.62	15.9

4. Discussion

The percentage nitrogen derived from fixation by *A. pinnata* and *A. microphylla* as measured by the ^{15}N-dilution technique (Table 1), ranged from 50 to 61%. Eskew (1987) has discussed the possible errors in this method with reference to high values reported by certain authors. We have ourselves encountered such problems when labelled fertilizer was added in a single dose at the initial stages of setting up of this experiment. In the present study, the labelling of the floodwater was done regularly, several times during the experiment. This not only resulted in better uptake of the label by the reference plants, but also in better overall growth of all the plants. One of the assumptions concerning the ^{15}N-dilution method is that the test plants and the reference plants should have similar growth patterns. Although the

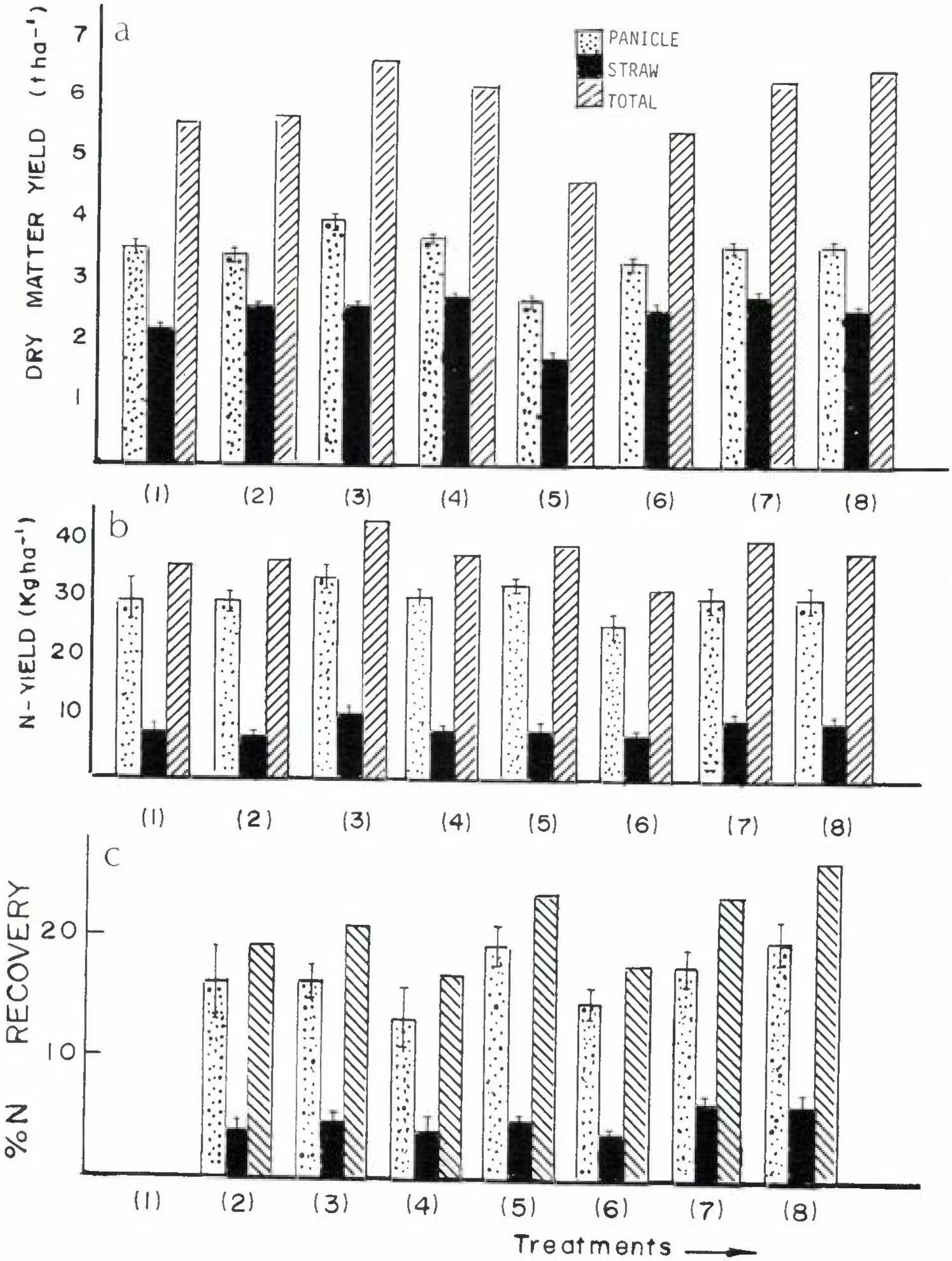

Figure 4. (a) Dry matter yield, (b) N-yield and (c) % N-recovery in a rice crop that received a basal dressing of ^{15}N-labelled fertilizer according to the following treatments: (1) Control without nitrogen; (2) 15 kgNha^{-1}; (3) 30 kgNha^{-1}; and (4) 45 kgNha^{-1} of urea respectively; and (5) (6) (7) and (8) 30 kgNha^{-1} each, as *A. pinnata* var. *pinnata*, *A. pinnata* var. *imbricata*, *A. caroliniana* and *A. microphylla* respectively.

growth patterns of the *Azolla* species and *Salvinia* and *Lemna* were not estimated, their rates of growth (observed by the development of the surface cover) were similar. The ability of *Azolla* to fix 10 to 14 $kgNha^{-1}$ in 2 weeks augments well for its potential as a biofertilizer. Thus, if a good growth of *Azolla* can be obtained within 2 weeks between land preparation and transplanting of rice, it has the potential to completely replace the amount of chemical fertilizer normally recommended as a basal application prior to transplantation. Figures 2a & b show that the rice plants have responded positively to the N-additions, irrespective of the manner by which the fertilizers were applied. Fertilizer N-yield and the % N-recovery (Figs. 3a & b) show: (1) better uptake from *Azolla* than from urea and (2) from the same fertilizer (either *Azolla* or urea), the uptake has been better when it was applied at tillering than at transplanting. Being an organic source, *Azolla* would release its nitrogen more slowly than urea. This could reduce losses due to leaching, percolation, ammonia volatalization, and perhaps act in better harmony with crop uptake, resulting in more efficient recovery by the crop. Better N-uptake at tillering than at transplanting is due to the inability of the small rice seedlings to take up available nitrogen as rapidly as vigorously growing plants at tillering. The % N-recovery from the urea applied according to the recommended best split method being lower than that from *Azolla* incorporations (Fig. 3b), may be due to an experimental artifact. The calculations based on the total amount of labelled-N added (60 $kgNha^{-1}$) applied at transplanting, tillering and panicle initiation, and comparing it with 30 $kgNha^{-1}$ of *Azolla* added either at transplanting or tillering is strictly not correct, because fertilizer losses as well as efficiency of crop uptake at different stages of crop development are not similar. A better comparison would have been with labelled *Azolla* added in the same manner as the split application of urea, but such an application schedule with *Azolla* is impracticable. As the primary objective of this experiment was to determine whether *Azolla* added at transplanting or at tillering is recovered better by rice in comparison to urea applied in the same manner, inclusion of an additional treatment was not warranted. Results from the yield plots (Table 4) show that 47 $kgNha^{-1}$ of nitrogen applied as *Azolla* has resulted in the same grain yield increase as obtained by the application of 60 $kgNha^{-1}$ of urea fertilizer. This indicates that either *Azolla* is a better fertilizer than urea, or that the effect of *Azolla* is not entirely due to nitrogen. The latter possibility is more likely, as the addition of organic mater would improve the texture, aeration, cation exchange capacity and microbial activity of a soil, leading to a reduction of nutrient losses, including nitrogen.

In the experiment where N-uptake from four different *Azolla* species was compared with that from three levels of urea fertilizer, the dry matter yield, N-yield and % N-recovery (Figs. 4a, b & c respectively), there was a gradual increase from treatments T_1 to T_3 in all three parameters as the level of urea applied increased from 0 to 30 kgNha^{-1}, but at 45 kgNha^{-1} there was a decrease. This may be due to the fact that this amount of H given as a basal dressing was in excess of what could be taken up by rice seedlings. Among the four species of *Azolla* examined, *A. imbricata* has given the lowest value for the dry matter, N-yield and N-recovery, whereas the other three species gave similar values. Analyses of the four *Azolla* species showed that *A. imbricata* had the highest fibre: nitrogen ratio (Table 5), indicating that N-uptake is also influenced by the quality of the material used.

From these studies, it may be concluded that field grown *Azolla* under favourable conditions possess a high rate of nitrogen fixation which can meet with the basal demand of a rice crop. To obtain best results, it is not only necessary to have a proper *Azolla* incorporation schedule, but also use good quality material with low fibre to nitrogen ratios.

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REFERENCES

Dao, T.T. and Tran, Q.T. 1979. Use of *Azolla* in rice production in Vietnam. In: *Nitrogen and Rice.* The International Rice Research Institute, Los Banos, Philippines, pp. 395–405.

Eskew, D.L. 1987. Use of ^{15}N in N_2 fixation and N-cycling studies of *Azolla.* In: *Azolla Utilization.* The International Rice Research Institute, Los Banos, Philippines, pp. 233–239.

Fried, M. and Middleboe, V. 1977. Measurement of amount of nitrogen fixed by a legume crop. *Plant & Soil* **47**: 713–715.

Liu Chung Chu. 1979. Use of *Azolla* in rice production in China. In: *Nitrogen and Rice*. The international Rice Research Institute, Los Banos, Philippines: pp. 375–395.

Lumpkin, T.A. and Plucknett, D.L. 1982. *Azolla* as a green manure: Use and management in crop production. Westview Press, Boulder, CO. 320 pp.

Kumarasinghe, K.S., Zapata, F., Kovacs, G., Eskew, D.L., and Danso, S.K.A. 1986. Evaluation of the availability of *Azolla*-N and urea-N to rice. *Plant & Soil* **90**: 293–299.

Watanabe, I. 1982. *Anabaena-azollae* symbiosis: Its physiology and use in tropical agriculture. In: *Microbiology of Tropical Soils and Plant Productivity*. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, pp. 169–186.