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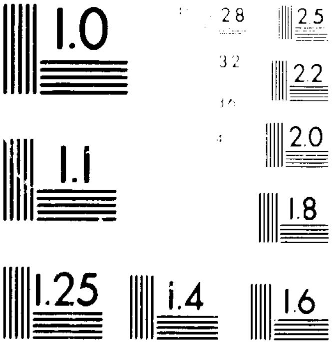

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Assignment on Biological Nitrogen Fixation

CONSULTANT'S REPORT

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1. Introduction

This assignment was undertaken to plan and conduct suitable laboratory and greenhouse experiments and to provide technical advice to trainees at the FAO/IAEA Biotechnology Laboratory at Seibersdorf, following a course of training on the use of isotope techniques in the study of Biological Nitrogen Fixation. The period of this assignment was from 7 - 28 July, 1984. During the course of this assignment I was also invited to present a paper on biological nitrogen fixation in Azolla at the First Conference of the African Association for Biological Nitrogen Fixation held in Nairobi, Kenya, from 23-27 July 1984. A copy of the abstract of the paper presented at this meeting is annexed. This trip was made at no extra cost to UNIDO, but it gave me the opportunity to exchange ideas pertaining to current developments in the field of biological nitrogen fixation.

In November 1982 the Joint FAO/IAEA Division of Isotopes and Radiation Applications of Atomic Energy for Food and Agricultural Development convened a consultants meeting to evaluate the potentiality of using blue-green algae and their associations as biofertilizer for rice. Based on recommendations made at this meeting, I was assigned the task of carrying out the preliminary investigations to work out suitable methodology employing ^{15}N to

(a) quantify biological nitrogen fixation in Azolla, and
(b) evaluate the availability of Azolla nitrogen to rice.

The experiments described in this report form a continuation of this work. Azolla is an association between a heterosporous water fern and a blue-green algae Anabaena azollae, capable of fixing atmospheric nitrogen. Biological nitrogen fixation in Azolla (A. caroliniana) was quantified by the ^{15}N substrate labelling technique using Salvinia (S. auriculata) and Lemna (L. minor) as non-nitrogen fixing reference crops. It was observed that Azolla derives about 90% of its total nitrogen, purely by biological fixation which clearly demonstrates its exceptional capacity to convert atmospheric nitrogen into amonia. The techniques involved in the quantification of biological nitrogen fixation by this method were demonstrated in detail to the trainees during the course of instructions.

However, the importance of Azolla as a biological nitrogen fixing system can be realized only if the nitrogen in it can be made available, particularly to an agronomically important crop. Fortunately, Azolla is an aquatic plant, high in its nitrogen content and as such it could form an excellent source of nitrogen to rice grown under flooded conditions. Although Azolla has been used in China and Viet Nam to increase rice yields, the scientific information available at present as regards to exploiting its full potential as a biofertilizer is scanty. In this context, an experiment was set up for the trainees in order to evaluate the availability of Azolla nitrogen to rice using ^{15}N. The guidelines for this experiment are given in the next section of this report.

2. Experimental Guidelines

Evaluation of plant-available amounts of nitrogen in *Azolla* by means of ^{15}N-isotope techniques

GREENHOUSE EXPERIMENT

Experimental Guidelines

by

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FAO/IAEA INTER-REGIONAL TRAINING COURSE ON THE USE OF ISOTOPE AND RADIATION TECHNIQUES IN STUDIES ON SOIL/PLANT RELATIONSHIPS

0455S/rr/84

CONTENTS

Evaluation of plant-available amounts of Nitrogen in Azolla by means of ^{15}N-isotope techniques

1. Introduction

Azolla is a free floating aquatic fern that is native to Asia, Africa and the Americas. The most remarkable feature of Azolla is its symbiotic relationship with the cyanobacteria Anabaena azollae. The fern provides nutrients and a protective leaf cavity for the Anabaena which in turn fixes atmospheric nitrogen, part of which is made available to the fern.

Under suitable environmental conditions Azolla can double in weight every 3-5 days and can accumulate 2-4 kg or more of N/ha/day (equivalent to 10-20 kg of ammonium sulphate). Since the Azolla-Anabaena complex grows in aquatic conditions it can provide a potential nitrogen source for crops such as rice which grow under flooded conditions. The nitrogen fixed by Azolla becomes available to the rice plant only when it is released through decomposition, following incorporation into the soil.

At present Azolla is used as a green manure for rice mainly in China and Vietnam. Recently, however, considerable interest has developed for its use in other countries of Asia, Africa and America.

^{15}N, the stable isotope of nitrogen is being used widely to assess fertilizer-N uptake by plants. Commercial fertilizers such as urea and ammonium sulphate can be easily labelled with ^{15}N during the process of their manufacture. However, when using biological materials such as Azolla as the fertilizer source, labelling with ^{15}N may be done by growing the Azolla in a medium containing ^{15}N. Azolla labelled with ^{15}N in this manner can now be used as a labelled fertilizer by incorporating it into the soil.

2. Objectives

The Soils Section of the FAO/IAEA Joint Division is coordinating an international programme to assess the potential input of *Azolla* to flooded rice growth, with the aid of ^{15}N techniques. Preliminary experiments to test the methodologies to be employed in this programme, are being performed at the IAEA Seibersdorf laboratory and at the Research Institute for Irrigation in Szarvas, Hungary (about 500 km away). Thus initially it has been necessary to grow ^{15}N labelled *Azolla* here in Seibersdorf and freeze the labelled *Azolla* until the experiments can be set up in Szarvas. Another potential method for preserving the *Azolla* biomass for transport is drying. Either freezing or drying may change the subsequent degradation of the *Azolla* biomass with resepect to fresh *Azolla* and therefore may also affect the availability of the N contained in the biomass. Thus this experiment was set-up to evaluate these effects.

The urea treatment is included to compare the availabity of *Azolla*-N with that of a widely used commercial fertilizer.

The unfertilized control treatment is included to allow the calculation of apparent fertilizer use efficiency by the conventional difference method, and to assess yield responses.

Thus we will be able to compare actual fertilizer efficiency determined with isotopes with apparent fertilizer efficiency, to illustrate that additional information on the processes occuring in the soil-plant system can be obtained using isotopes.

3. Method

Rice seeds of the cultivar 'Nucleoryza' were pre-germinated in Petri dishes and after 6 days they were put into a 0.12 m^2 tray with a 3cm layer of Meadow clay soil (from Szarvas, Hungary). At 11 days the seedlings were transplanted into pots containing 0.5 kg (air dried basis) of Meadow clay soil on April 9. Two seedlings were transplanted into each pot. Transplanting was done after addition of Azolla or urea. A solution of KH_2PO_4 was added to each pot to provide 57 ppm P and 45 ppm K. After transplanting the pots were kept flooded with demineralized water. The pots were placed in the greenhouse in a totally randomized design. There are 5 replicates per treatment.

An amount of fresh, frozen or dried Azolla, estimated to contain 100 mg per N per pot, was thoroughly mixed with the soil before placing the soil into the pot. The actual amounts added are given in Table 2. Urea, to provide 100 mg N per pot, was also added in the same manner. A control treatment was also included to which no N was added. It should be mentioned that no unfertilized control treatment is needed to determine N availability when isotopes are used. The unfertilzed control is included in this classroom demonstration experiment to illustrate the additional information that can be derived by employing isotopes in these studies.

Labelling of Azolla

Azolla caroliniana was grown in modified IRRI nutrient media, in 0.12 m^2 plastic trays containing 5 l of solution. Initial inoculum was 15g Azolla per tray. After 7 days the Azolla had reached approximately 60g per tray and had achieved the stage where most of the water surface was covered with a single layer of Azolla. At this time 10% ^{15}N atom excess urea was added to provide an N concentration in the solution of 50 ppm.

Urea-N was not added earlier, since it tends to promote a heavy growth of green algae. At 15 days after Azolla inoculation the Azolla was harvested and rinsed thoroughly. One-third was frozen, 1/3 oven dried at 60^oC, and 1/3 was returned to the trays of nutrient solution for additional 3 days. Upon thawing, a large amount of additional water drained from the frozen Azolla, thus the weight of Azolla added to each pot was reduced in proportion to the amount of water lost. Samples of dried frozen and fresh Azolla were taken for total N and ^{15}N analysis just prior to mixing Azolla with soil. The results of these analyses are given in Table 2, p. 7

Table 1.

Treatment Summary

Treatment No.	N-Fertilizer Treatment
1	Dried *Azolla*
2	Frozen *Azolla*
3	Fresh *Azolla*
4	Urea
5	None

4. Harvesting

The pots should be re-arranged into treatment groups. That is, all pots with dried *Azolla* should be placed in a row, etc. Each group will be responsible for harvesting the 5 pots of one treatment. Cut the rice shoots approximately 2 cm above the soil surface. Place the rice shoots into paper bags labelled with the experiment, treatment and replication number. Thus, a bag labelled 15-1-1 means experiment 15, treatment 1, replicate 1. Dry the samples for 24 hours in a forced draft oven maintained at 60°C. If the sample is larger than 500 g fresh weight it should be cut into 2 cm pieces with scissors, thoroughly mixed and quartered and a 200-300 gram sub-sample taken for drying and analysis. In this case the total fresh weight, fresh weight of sub-sample and dry weight of sub-sample must be recorded to allow total dry weight to be calculated.

5. Analysis

The dried sample will then be ground. Total N will be determined by the Kjeldahl method, and ^{15}N enrichment will be determined using mass spectrometry after Dumas combustion. Total N and ^{15}N analysis will be performed by the laboratory staff.

6. Calculations:

ISOTOPE CALCULATIONS

$$N \text{ yield} = \text{Dry weight} \times \frac{\%N}{100}$$

$$\% \text{ Ndff } (\% \text{ N derived from fertilizer}) = \frac{\% \, ^{15}\text{N a.e. of Plants}}{\% \, ^{15}\text{N a.e. fertilizer}} \times 100$$

$$\% \text{ Ndfs } (\% \text{ N derived from soil}) = 100 - \% \text{ Ndff}$$

$$\text{N fert. Yield} = \text{N yield} \times \frac{\%\text{Ndff}}{100}$$

$$\text{Fertilizer use efficiency} = \frac{\text{N fert. yield}}{\text{N fert. applied}} \times 100$$

DIFFERENCE METHOD:

Apparent N fertilizer yield = N yield treatment - N yield control

$$\text{Apparent fertilizer use efficiency} = \frac{\text{Apparent N fert. yield}}{\text{N fert. applied}} \times 100$$

Table 2. Data needed for calculations.

Treatment No	Treatment Fertilizer	Fresh Wt. g	Dry Wt. g	Total N %	N Added mg	N-15 Atom excess %
1	Dried Az.	-	2.50	4.88	122	0.884
2	Frozen Az.	30	2.18	4.94	108	0.906
3	Fresh Az.	45	1.76	4.91	87	1.289
4	Urea	-	-	-	100	3.050
5	Control	-	-	-	0	-

TABLE 3: Data Summary and Calculation Sheet

TRT	REP	Dry Wt g	% N	N Yield mg	^{15}N % a.e.	Ndff %	Ndfs %	N-Fert. Yield mg	Fert. Use Efficiency %
1 Dried Azolla	1	5.51	0.86	47.4	0.467				
	2	5.32	0.85	45.2	0.446				
	3	4.67	0.84	39.2	0.454				
	4	6.68	0.88	58.8	0.507				
	5	6.34	0.87	55.2±1.1	0.492				
	Av			49.2	0.464	52.5	47.5	25.8	21.1
2 Frozen Azolla	1	5.38	0.89	47.9	0.484				
	2	5.40	0.90	48.6	0.437				
	3	5.15	0.94	48.4	0.468				
	4	5.32	0.88	46.8	0.470				
	5	5.27	0.96	50.6	0.472				
				48.5±1.1	0.457	50.4	49.6	24.4	22.6
3 Fresh Azolla	1	6.82	0.86	58.7	0.622				
	2	6.98	0.86	60.0	0.674				
	3	7.01	0.85	59.6	0.627				
	4	6.23	0.89	55.4	0.673				
	5	6.77	0.85	57.5	0.601				
				58.2±1.9	0.630	48.9	51.1	28.5	32.8
4 Urea	1	7.70	0.79	60.8	1.748				
	2	9.41	0.77	72.5	1.799				
	3	10.09	0.73	73.7	1.754				
	4	9.09	0.74	67.3	1.731				
	5	9.08	0.76	69.0	1.733				
				68.7±5.1	1.744	57.2	42.8	39.2	39.2
5 Control	1	2.07	0.83	17.2	0.006				
	2	1.96	0.83	16.3	0.015				
	3	1.85	0.81	15.0	0.008				
	4	1.93	0.83	16.0	0.010				
	5	1.81	0.80	14.5	0.007				
					0.009				

CONCLUSION

Fertilizer Type	Efficiency as compared to UREA (%)
Dried Azolla	54%
Frozen Azolla	58%
Fresh Azolla	83%

References - Background on Azolla

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3. Suggestions and Recommendations

The importance of the study of biological nitrogen fixation and biological nitrogen fixing systems was highlighted at this training course. However, the capacity to fix nitrogen and to make it available to other crops vary depending on the plant species, climatic conditions and other edaphic factors.

A. It is therefore suggested that trainees initiate research in their own countries to quantify using ^{15}N, biological nitrogen fixation in symbiotic as well as non-symbiotic plant systems. Special consideration should be given to Rhizosphere fixation.

B. A breeding programme should be undertaken to screen for Azolla strains resistant to local pests and tolerant to a wide range of temperature. The FAO/IAEA Agricultural Biotechnology Laboratory at Seibersdorf has already initiated some work on these lines.

C. I would like to recommend that the newly established International Centre for Genetic Engineering and Biotechnology be encouraged to initiate a programme of research on biological nitrogen fixation with Azolla and blue-green algae perhaps in collaboration with the FAO/IAEA Agricultural Biotechnology Laboratory at Seibersdorf.

4. Concluding Remarks

I believe that the experiments conducted and the training offered by me to scientists from developing nations would not only be of value to their own research work, but would also form an important contribution to the efforts made to exploit the vast potential of the biological nitrogen fixing systems with the ultimate goal of enhancing crop production throughout the world.

Finally, I would like to take this opportunity to thank the UNIDO for appcinting me as a consultant to work and advice in the field of biological nitrogen fixation. If it becomes necessary I would be happy to make my services available to UNIDO again.

KSKumarasinghe/gvd
1984-08-07

Abstract of Paper presented at the First conference of the African Association for Biological Nitrogen Fixation: K.S.K.
Nairobi 23-27 July '84

AZOLLA-ANABAENA ASSOCIATION: BIOLOGICAL NITROGEN FIXATION AND ITS POTENTIAL AS A BIOFERTILIZER FOR RICE

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ABSTRACT

A quantitative estimate of the amount of nitrogen fixed by Azolla (A.caroliniana) grown under field conditions in Hungary was made by the ^{15}N substratum labelling technique. Approximately 80-90 % of the nitrogen in Azolla was derived by biological fixation while on a area basis the nitrogen fixation rate was 20-25 KgN/ha within a growth period of 36 days. Two free-floating plant species Salvinia (S.auriculata) and Lemna (L.minor) that were tested for their suitability as non-nitrogen fixing reference crops were found to be ideal for the purpose.

Due to its aquatic habitat and the nitrogen fixing ability, Azolla is often considered as a potential source of nitrogen to rice grown under flooded conditions. In the present investigation the availability of the Azolla nitrogen to rice was evaluated by two

methods; the 'direct method' and the 'indirect method'. In the 'direct method' *Azolla* previously labelled with ^{15}N was incorporated into the paddy soil at a rate of 144 KgN/ha while ^{15}N-labelled urea was applied at a rate of 100 KgN/ha. In the 'indirect method' ^{15}N-urea (100 KgN/ha) and ^{15}N-urea (100 KgN/ha) plus unlabelled *Azolla* (250 KgN/ha) were incorporated into the soil and the amount of nitrogen in rice that originated from unlabelled *Azolla* was evaluated using the 'A' - value of unlabelled *Azolla*. The data from both methods show that *Azolla* under the present experimental conditions is about 120% as efficient as urea in providing nitrogen to rice grown under flooded conditions. If conditions are favourable for growth, *Azolla* could therefore at least partially substitute for urea fertilizer in rice cultivation.