ESTIMATION OF BIOLOGICAL N_2 FIXATION AND ITS CONTRIBUTION TO NITROGEN BALANCE IN WETLAND RICE FIELDS

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INTRODUCTION

Flooding ricefields leads to the differentiation of a range of macro- and micro-environments differing by their redox, physical properties, light status, and nutrient sources for the microflora. As a result, all N_2-fixing groups can grow in ricefields: photosynthetic bacteria and blue-green algae (BGA), indigenous heterotrophic bacteria in soil and associated with rice, and introduced *Azolla* and legumes for green manure. Biological N_2 fixation (BNF) in ricefields and its use were reviewed by Watanabe & Roger (1984) and Roger & Watanabe (1986). Specific reviews deal with BGA (Roger 1990), heterotrophs (Yoshida & Rinaudo 1982), BNF associated with straw (Ladha & Bonkerd 1989), rice genotypic differences in stimulating BNF (Ladha et al. 1988c), *Azolla* (Watanabe 1982), and legume green manures (Ladha et al. 1988b). This paper reviews recent improvements and new methodological approaches to estimate BNF in wetlands, summarizes earlier and recent quantitative estimates, and briefly discusses research needs.

IMPROVEMENTS AND NEW METHODOLOGICAL APPROACHES

Acetylene reducing activity (ARA)

Recent studies confirm limitations that may make quantitative extrapolations risky. ARA was linear with time with aquatic legumes (Ladha et al. 1988a) but not with associative (Barraquio et al. 1986) and algal BNF (Roger et al. in press). C_2H_2/N_2 conversion factor varied from 1.6 to 7.9 with *Azolla*, depending on species, P_{N_2}, assay duration, and age of culture (Eskew 1987). It varied from 3.9 to 30 with dense algal mats, mostly depending on P_{N_2} used for incubation under ^{15}N, but when P_{N_2} was close to that of air and all other factors were close to those used for C_2H_2 exposure, C_2H_2/N_2 ratio was close to the theoretical value of 4 (Roger et al. in press). Most incubations are done under 10% C_2H_2 in air, but ARA increases with up to 25% C_2H_2 in air with thick BGA blooms (Roger et al. in press) and associative BNF (Barraquio et al. 1986). *In situ*, the greenhouse effect in enclosures used for incubation reduced photodependent ARA of soil (Roger et al. in press) and *Azolla* (Li et al. 1987). Despite its limitations, ARA was used in about 2/3 of the 38 quantitative BNF studies related to rice published since 1985.

To overcome some limitations and obtain reproducible values, methods have been developed where composite and/or standardized samples collected *in situ* are incubated under controlled laboratory conditions (Roger et al. in press; Barraquio et al.

1986). In field studies, emphasis has been on sampling methods. Using 400 groups of four and five replicated ARA measurements in plots with various agronomic practices, Roger et al. (in press) drew general conclusions on sampling density and replicates needed for a given accuracy. The distribution of photo-dependent ARA approximates a log-normal pattern. On the average, three replicated plots are needed to show significant differences between means whose ratio is more than 5. Ten plots are needed for a ratio of 2. Interplot variability cannot be reduced, but ARA values integrated for the crop cycle are less variable than daily values. Using composite samples markedly reduces intraplot variability. In a 16-m^2 plot, composite samples of 10 cores (2 cm in diameter) each have an average c.v. of 20%. To assess rice varietal differences in BNF, Tirol-Padre et al. (1988) measured ARA of several plants for 3 consecutive days at heading. Six plants day^{-1} allowed detection of a 40% difference (the least significant difference was 1.3 µmol $plant^{-1}$ 6 h^{-1} for short-duration varieties and 2.1 for those with long duration). Detecting a difference of 20% required 20 plants d^{-1}.

^{15}N incorporation

^{15}N incorporation was used for short-term studies to assess BNF by various agents, to identify active sites in soil or rice plant, and to establish the C_2H_2/N_2 conversion factor in BGA and *Azolla* (Watanabe & Roger 1985; Eskew 1987).

^{15}N dilution method

This method is attractive because one sampling can provide an estimate of BNF integrated over time. It is used to estimate BNF in plants but not in soil. The major assumption is that the $^{15}N/^{14}N$ ratio of N absorbed from soil (or water) is the same for the N_2-fixing plant and the non-fixing control. It is satisfied when ^{15}N enrichment of soil N available to the N_2-fixing system is constant during the experiment (it implies that ^{15}N added is equilibrated with soil N and labeling is constant with soil depth) or the N_2-fixing plant and the non-fixing control have similar N uptake patterns. Labeling soil and stabilizing ^{15}N are easier in flooded than in upland soils because the plow sole delineates an Ap horizon, which is relatively easy to homogenize and where most roots are located. Either a non-fixing plant or available soil N can be used as control, but the validity of the control depends upon the % of N derived from the air (Ndfa). Zhu et al. (1986) used available N of a ^{15}N labeled and stabilized soil as control for estimating associative BNF. In a study of *Sesbania* spp. where non-fixing plants and soil available N were used as controls, stabilization of added ^{15}N obtained in 100 d was sufficient to obtain similar Ndfa with the 4 controls (Pareek et al. in press). With aquatic N_2 fixers, problems caused by fast changes in ^{15}N enrichment over time, which results in large errors in %Ndfa estimation (Witty 1983), can be solved by the sequential addition of ^{15}N in water (Kulasooriya et al. 1988). But, with BGA, the N level in water sufficient for growth of non-fixing control algae may inhibit BGA growth directly or through competition (Roger 1990).

Natural ^{15}N abundance

Difference in natural ^{15}N abundance ($\partial^{15}N$) was used to test differences among rice plant organs and varieties, and to estimate Ndfa in *Azolla* (Watanabe et al. 1987; Yoneyama et al. 1987). This method, which uses the fact that soil has a higher $\partial^{15}N$

than air, is advantageous because of the stable isotopic composition of N sources. But *in situ*, a ^{15}N gradient observed with soil depth is a serious source of error. Growing plants in pots avoids this problem.

QUANTIFICATION OF BNF BY VARIOUS AGENTS

Heterotrophic BNF

Total heterotrophic BNF estimated from the N balance in unfertilized planted pots covered with black cloth averaged 36 mg N $crop^{-1}$ pot^{-1} or 7 kg N ha^{-1} App et al. (1980). In similar trials, Trolldenier (1987) found balances negatively correlated with the amount of N applied and ranging from - 440 to + 418 mg N $crop^{-1}$ pot^{-1}. Extrapolated values averaged 19 kg N ha^{-1} $crop^{-1}$ with 65 kg N ha^{-1}, - 0.3 with 112 kg N, and -14 with 146 kg N. Using available N of a stabilized ^{15}N-labelled soil as control, Zhu et al. (1986) estimated that, when no N-fertilizer was applied and photodependent BNF was controlled, heterotrophic BNF contributed 16-21% of rice N, or 11-16 kg N ha^{-1} $crop^{-1}$.

Associative BNF and varietal differences

ARA associated with rice is usually measured at heading because it is highest at this stage. Data summarized by Roger & Watanabe (1986) range from 0.3 µmol to 2 µmol C_2H_4 $hill^{-1}$ h^{-1}. Ladha et al. (1987) screened 16 varieties and found activities ranging from 0.4 to 2 µmol C_2H_4 $hill^{-1}$ h^{-1}. Assuming 1) that ARA measured at heading lasts 50 days, 2) a C_2H_2 /N_2 ratio of four, and 3) a plant density of 25 m^{-2}, N_2-fixing rate would be 1-5 kg N ha^{-1} $crop^{-1}$. Extrapolations from ^{15}N incorporation experiments range from 1.3 to 7.2 kg ha^{-1} $crop^{-1}$ (Roger & Watanabe 1986). Differences in the ability of rice varieties to support associative BNF (Nfs trait) were suspected from N balance experiments in pots with soil exposed to light (App et al. 1986). Varietal differences in Nfs were shown by ARA assays (Ladha et al. 1987, 1988b) but little is known about their physiological basis. The idea of breeding varieties higher in Nfs is attractive because such varieties would enhance BNF without additional cultural practices. However, a rapid screening technique is needed.

BNF associated with straw

Early estimates of BNF after straw incorporation range from 0.1 to 7 (mean 2.1) mg N g^{-1} straw added, in 30 d (Roger & Watanabe 1986). Most data originate from laboratory incubations in darkness of soil enriched with 1 to 100% straw (average 22%) which simulates composting rather than the field situation where straw left is always less than 1% soil dry weight. Moreover, dark incubation allows heterotrophic BNF only, whereas straw incorporation also favors photodependent BNF (Ladha & Bonkerd 1988). Recent greenhouse and field experiments show that straw application may significantly increase populations and N_2-fixing activity of photosynthetic bacteria and BGA (Ladha & Bonkerd 1988). However, pot experiments by Santiago Ventura et al. (1986) showed N gains of 2-4 mg N g^{-1} straw added with no difference when soil was exposed to light or kept in darkness. Quantitative estimates of BNF in field experiments with straw are not available, but a few semiquantitative data and laboratory data suggest that straw might increase BNF by 2-4 kg N t^{-1} applied.

Blue-green algae

N_2 fixation by BGA has been almost exclusively estimated from ARA. Data published before 1980 vary from a few to 80 kg N ha^{-1} $crop^{-1}$ (mean 27 kg) (Roger & Kulasooriya 1980). About 200 crop cycle measurements in experimental plots at IRRI (Roger et al. 1988) show activities of the same order: 0 - 1200 µmol C_2H_2 m^{-2} h^{-1} for daily values and 20 - 500 µmol C_2H_2 m^{-2} h^{-1} for average ARA during a crop cycle. Extrapolated values (assuming $C_2H_2/N_2 = 4$) ranged from 0.2 to 50 kg N ha^{-1} $crop^{-1}$ and averaged 20 kg in no-N control plots, 8 kg in plots with broadcast urea, and 12 kg in plots where N was deep-placed. ARA was negligible in 75% of the plots where urea was broadcast (Roger et al. 1988). As N_2-fixing BGA usually bloom only when the photic zone is depleted of N, most of their N can be assumed to originate from BNF. Inubushi & Watanabe (1986) reported that BGA in ^{15}N-labeled plots had about 90% Ndfa. A BGA bloom usually corresponds to less than 10 kg N ha^{-1}, a dense bloom may contain 10-20 kg N ha^{-1} (Roger 1990). However, estimates of BGA biomass do not allow N_2 fixed to be quantified because no data are available on the turnover of the algal biomass.

Azolla

BNF by *Azolla* has usually been estimated from biomass measurement and the assumption that most of *Azolla* N originates from BNF. The N potential of *Azolla* was summarized by Roger & Watanabe (1986) from data obtained mostly in experimental plots. The N content in maximum standing crops ranged from 20 to 146 kg ha^{-1} and averaged 70 kg ha^{-1} (n = 17; c.v. = 58%). N_2-fixing rate ranged from 0.4 to 3.6 kg N ha^{-1} d^{-1} and averaged 2 kg N ha^{-1} d^{-1} (n = 15, c.v. = 47%). In a 4-year field trial at 37 sites in 10 countries, productivity was lower than in experimental plots (Watanabe 1987). Biomass was 5-25 t fresh weight ha^{-1} (10-50 kg N ha^{-1}) for*Azolla* grown before or after transplanting (average 15 t ha^{-1} or 30 kg N). Recent experiments focus on Ndfa determination. Using the ^{15}N dilution method with *Lemna* and *Salvinia* as non-fixing controls, Watanabe & Talukdar, and Kumarasinghe (unpublished data cited by Eskew, 1987) estimated that 80-85% *Azolla* N was Ndfa. Using similar controls and applying ^{15}N-labelled urea at 3-day intervals for 14 days Kulasooriya et al. (1988) found 51-61% Ndfa and BNF of 10-14 kg N ha^{-1} in 14 days. Using the $\partial^{15}N$ method, Yoneyama et al. (1987) estimated that 59 to 99% of N of the strains tested was Ndfa. *A. filiculoides* freed from its symbiont and the *Lemna* control had a similar $\partial^{15}N$ which was not influenced by the water level (flooded or saturated soil).

Legume green manure

BNF by legume green manure (LGM) used for rice has usually been estimated from total N measurement and the assumption that 50-80% of accumulated N is Ndfa. Values of N accumulated in a prerice LGM crop summarized by Roger & Watanabe (1986) average 114 kg N ha^{-1}. Highest values (146-267 kg N ha^{-1} in 52 d) were observed for *Sesbania*. Values published after 1985 average 133 kg N ha^{-1} (Ladha et al. 1988c). Ranges in kg N ha^{-1} are 40-225 for aquatic legumes, 33-115 for grain legumes, and 24-39 for perennial trees. Assuming 50-80% Ndfa, one LGM crop can fix an average 1.0-1.6 kg N ha^{-1} d^{-1} or 60-100 kg N ha^{-1} in 50-60 d. Using ^{15}N dilution, Pareek et al. (in press) estimated that Ndfa in 25-d *Sesbania* spp. was 50% in dry season (DS) and 75% in wet season (WS). Lower values (30-50%) were reported by Rinaudo et al. (1988), and

N'Doye & Dreyfus (1988) for 53- to 63-d-old *S. rostrata*, probably because they used an uninoculated *S. rostrata* as control. A few estimates of BNF by *S. rostrata* as a prerice LGM are available from small-scale balance studies. Rinaudo et al. (1988) reported a gain of 267 kg N ha^{-1} after incorporating a 52-d crop. In a [45-d Sesbania-rice (WS)/55-d *Sesbania*-rice (DS)] sequence, Ladha et al (1988a) estimated that *Sesbania* fixed 303 kg N ha^{-1} $year^{-1}$ when uninoculated, and 383 kg N when inoculated with *Azorhizobium*.

N BALANCE AND BNF CONTRIBUTION IN WETLAND RICE

N balances in long-term fertility experiments listed by Greenland & Watanabe (1982) range from 19 to 98 kg N ha^{-1} $crop^{-1}$ (average 51) in 9 fields with no N fertilizer. In 4 fields with N fertilizer, the average is -1.5 kg N ha^{-1} $crop^{-1}$. With rice grown in alternation with an upland crop, the average was 44 kg N ha^{-1} $year^{-1}$ in 3 fields with no N fertilizer and -29 kg N in 2 fields with applied urea. At two Philippine sites, App et al. (1984) found no decrease in total soil N after 24 and 17 crops. Calculations based on yields and known inputs suggested that two crops $year^{-1}$ resulted in balances equivalent to 79 and 103 kg N ha^{-1} $year^{-1}$.

Balance studies in the field encounter additional difficulties, as compared with pot experiments, because of sampling errors, unaccounted subsoil contribution, and losses by leaching. Therefore, there is renewed interest in pot studies (App et al. 1986, Santiago Ventura et al. 1986, Singh & Singh 1987, Trolldenier 1987). In a 4-crop experiment comparing organic N (*Azolla* and BGA) and urea, Singh & Singh (1987) found N gains ranging from 13 to 163 mg N $crop^{-1}$ pot^{-1}. Gains were highest (133-163 mg N $crop^{-1}$ pot^{-1}) in pots that received 60 kg organic N ha^{-1}, and lowest (13-29) in pots that received 30-60 kg N ha^{-1} as urea. Gain in the control was 51 mg N $crop^{-1}$ pot^{-1}. In a second experiment comparing the effects of soil exposure to light, presence of rice, and flooding in nonfertilized plots, N gains ranged from 78 to 103 mg $crop^{-1}$ pot^{-1} in fallow pots not exposed to light and from 243 to 277 mg $crop^{-1}$ pot^{-1} in planted pots exposed to light. The N gains reported by Singh & Singh in flooded pots exposed to light (51 and 277 mg N $crop^{-1}$ pot^{-1}) cover a similar range than 89 values reported by App et al. (1986) (70-260 mg N $crop^{-1}$ pot^{-1}, average 153). Extrapolating values of App et al. (1986) shows N gains ranging from 16 to 70 kg N ha^{-1} $crop^{-1}$ (average 38) in unfertilized planted pots exposed to light. Santiago Ventura et al. (1986) reported balances of about 100 mg N $crop^{-1}$ pot^{-1} in pots exposed to light and receiving none or low levels of N fertilizer. With high levels of N fertilizer, balance was unsignificant.

CONCLUSION

Recent methodological progress in measuring BNF in ricefields includes improved strategies for sampling and a better understanding of the potential of the ^{15}N dilution methods (labeled substrate and natural abundance). ^{15}N dilution, using available soil N as control, is promising for screening rice varieties for ability to utilize biologically fixed N.

BNF by individual systems (Table 1) can be estimated more or less accurately. Estimates for introduced green manures (*Azolla* and legumes) are based on biomass measurements combined with Ndfa determination and are probably more reliable

than estimates for indigenous fixers based mostly on indirect methods (ARA) or balance in small-scale trials. However, total BNF in a ricefield has not yet been estimated by measuring simultaneously the activities of the various components *in situ*. As a result, the relation between the different N_2-fixing systems, especially indigenous ones, are not fully understood and it is not clear if their activities are independent or related. A method to estimate *in situ* the contribution of N_2 fixed to rice nutrition is still not available. Dynamics of BNF during the crop cycle is known for indigenous agents but the pattern of fixed N availability to rice is known only for a few green manure crops. As a result, BNF in models of N cycling in wetlands, is either not taken into account or taken into account as a non-dynamic input.

Table 1. Range of estimates of N_2 fixed by various agents in wetland ricefields (kg N ha^{-1} crop $^{-1}$) and theoretical maximum potential (assumptions and value).

BNF associated with rice rhizosphere: 1-7 kg N ha^{-1} crop $^{-1}$
If all rhizospheric bacteria are N_2 fixers, C flow through the rhizosphere is 1 t ha^{-1} crop^{-1}, and C efficiency is 40 mg N fixed g C^{-1}: 40 kg N ha^{-1} crop $^{-1}$
BNF associated with straw: 2-4 kg N t^{-1} straw applied
If 5 t of straw is applied and 7 mg N are fixed g^{-1} of straw: 35 kg N ha^{-1} crop $^{-1}$
BNF heterotrophic (total): 1-31 kg N ha^{-1} crop $^{-1}$
If all C input (2 t crop^{-1}) is used by N_2-fixers: 60 kg N ha^{-1} crop $^{-1}$
Blue-green algae: 0-80 kg N ha^{-1} crop $^{-1}$
If the photosynthetic aquatic biomass is composed exclusively of N_2-fixing BGA (C/N = 7) and primary production is 0.5 t C ha^{-1} crop^{-1}: 70 kg N ha^{-1} crop $^{-1}$
Azolla: 20-150 kg N ha^{-1} crop $^{-1}$ in experimental plots, 10-50 in field trials.
If *Azolla* maximum standing crop is 140 kg N ha^{-1}, two *Azolla* crops are grown per rice crop, and Ndfa is 80%: 224 kg N ha^{-1} crop $^{-1}$
Legume green manures: 20-190 kg N ha^{-1} crop $^{-1}$
If 265 kg N ha^{-1} is accumulated in 50-60 d and Ndfa is 80%: 212 kg N ha^{-1} crop $^{-1}$

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