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Biological N_2 Fixation in wetland rice fields: Estimation and contribution to nitrogen balance

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Key words: acetylene reducing activity, ARA, Azolla, cyanobacteria, green manure, heterotrophic BNF, methods, N_2 fixation, review, rice

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Abstract

This paper 1) reviews improvements and new approaches in methodologies for estimating biological N_2 fixation (BNF) in wetland soils, 2) summarizes earlier quantitative estimates and recent data, and 3) discusses the contribution of BNF to N balance in wetland-rice culture.

Measuring acetylene reducing activity (ARA) is still the most popular method for assessing BNF in

rice fields. Recent studies confirm that ARA measurements present a number of problems that may render quantitative extrapolations questionable. On the other hand, few comparative measures show ARA's potential as a quantitative estimate. Methods for measuring photodependent and associative ARA in field studies have been standardized, and major progress has been made in sampling procedures. Standardized ARA measurements have shown significant differences in associative N_2 fixation among rice varieties.

The ^{15}N dilution method is suitable for measuring the percentage of N derived from the atmosphere (% Ndfa) in legumes and rice. In particular, the ^{15}N dilution technique, using available soil N as control, appears to be a promising method for screening rice varieties for ability to utilize biologically fixed N. Attempts to adapt the ^{15}N dilution method to aquatic N_2 fixers (Azolla and blue-green algae [BGA]) encountered difficulties due to the rapid change in ^{15}N enrichment of the water.

Differences in natural ^{15}N abundance have been used to show differences among plant organs and species or varieties in rice and Azolla, and to estimate Ndfa by Azolla, but the method appears to be semi-quantitative.

Recent pot experiments using stabilized ^{15}N-labelled soil or balances in pots covered with black cloth indicate a contribution of 10–30 kg N ha^{-1} $crop^{-1}$ by heterotrophic BNF in flooded planted soil with no or little N fertilizer used.

Associative BNF extrapolated from ARA and ^{15}N incorporation range from 1 to 7 kg N ha^{-1} $crop^{-1}$. Straw application increases heterotrophic and photodependent BNF. Pot experiments show N gains of 2–4 mg N g^{-1} straw added at 10 tons ha^{-1}.

N_2 fixation by BGA has been almost exclusively estimated by ARA and biomass measurements. Estimates by ARA range from a few to 80 kg N ha^{-1} $crop^{-1}$ (average 27 kg). Recent extensive measurements show extrapolated values of about 20 kg N ha^{-1} $crop^{-1}$ in no-N plots, 8 kg in plots with broadcast urea, and 12 kg in plots with deep-placed urea.

Most information on N_2 fixed by Azolla and legume green manure comes from N accumulation measurements and determination of % Ndfa. Recent trials in an international network show standing crops of Azolla averaging 30–40 kg N ha^{-1} and the accumulation of 50–90 kg N ha^{-1} for two crops of Azolla grown before and after transplanting rice. Estimates of % Ndfa in Azolla by ^{15}N dilution and delta ^{15}N methods range from 51 to 99%. Assuming 50–80% Ndfa in legume green manures, one crop can provide 50–100 kg N ha^{-1} in 50 days. Few balance studies in microplots or pots report extrapolated N gains of 150–250 kg N ha^{-1} $crop^{-1}$.

N balances in long-term fertility experiments range from 19 to 98 kg N ha^{-1} $crop^{-1}$ (average 50 kg N) in fields with no N fertilizer applied. The problems encountered with ARA and ^{15}N methods have revived interest in N balance studies in pots. Balances are usually highest in flooded planted pots exposed to light and receiving no N fertilizer; extrapolated values range from 16 to 70 kg N ha^{-1} $crop^{-1}$ (average 38 kg N). A compilation of balance experiments with rice soil shows an average balance of about 30 kg N ha^{-1} $crop^{-1}$ in soils where no inorganic fertilizer N was applied.

Biological N_2 fixation by individual systems can be estimated more or less accurately, but total BNF in a rice field has not yet been estimated by measuring simultaneously the activities of the various components in situ. As a result, it is not clear if the activities of the different N_2-fixing systems 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.

Introduction

More than half of the world population is dependent on rice. The crop was planted to 145 million hectares of land in 1988, producing 468 million tons. About 75% of rice land are wetlands where rice grows in flooded fields during part or all of the cropping period.

From the point of view of yield sustainability, traditional wetland rice cultivation has been ex-

tremely successful. Moderate but stable yield has been maintained for thousands of years without adverse effects on soil (Bray, 1986). This is because flooding allows the establishment of environmental conditions that maintain soil N fertility. In particular, flooding leads to the differentiation of a wide range of macro- and micro-environments that differ in their redox potential, physical properties, light status, and nutrient sources for the microflora. As a result, all groups of N_2-fixing microorganisms find environments suitable for their growth in wetland rice fields. Those include photosynthetic bacteria and blue-green algae (BGA) developing in the photic zone (floodwater, soil-water interface, and submerged plant biomass), heterotrophic bacteria in the soil and associated with rice, and Azolla and legumes used as green manure.

An additional 300 million tons of rice will be needed in 2020 to meet the need of a fast-growing human population. This requires a 65% production increase within 30 years without much expansion of actual cultivated area (IRRI, 1989a). However, increased rice production should not be at the expense of future generations and should fulfill the concept of sustainability. A major issue is managing nutrients in ways that reduce agrochemical use. Increased use of inorganic fertilizer is inescapable, but, as pointed out by Postgate (1990), a parallel return to greater exploitation of BNF, still responsible for providing 60–70% of the new N in the biosphere, is sensible. The development of economically feasible technologies to increase the contribution of BNF to N nutrition of rice in highly productive systems is one major challenge faced by rice scientists.

Biological N_2 fixation in rice fields and its use have been reviewed by Watanabe and Roger (1984) and Roger and Watanabe (1986). Specific reviews deal with BGA (Roger, 1991), heterotrophs (Yoshida and Rinaudo, 1982), BNF associated with straw (Ladha and Boonkerd, 1988), rice genotypic differences in stimulating BNF (Ladha et al., 1988b), Azolla (Watanabe, 1982), and leguminous green manures (Ladha et al., 1988c). The present paper reviews recent improvements and new methodological approaches to estimate BNF in the wetlands, summarizes earlier and recent quantitative estimates, and discusses research needs.

Improvements and new methodological approaches

There are currently three possible approaches in estimating BNF during a crop cycle. The first possibility is offered by N balance studies where balance is defined as the difference between easily measurable outputs (N in the exported parts of the plant and soil N at the end of the experiment) and inputs (N fertilizer applied and soil N at the beginning of the experiment). In such experiments, N losses by leaching, denitrification and volatilization, and atmospheric deposition are not recorded. Therefore such balance values usually provide an underestimation of BNF during the crop cycle. The second approach involves making and integrating short-term measurements at regular intervals during the crop cycle. This could be done with acetylene-reducing activity (ARA) and short-term ^{15}N incorporation measurements to determine BNF by specific agents or groups of organisms. Currently, only ARA has been used in field studies at the crop cycle level. The third approach is to determine the maximum biomass and the percentage of N derived from the air (% Ndfa) of the N_2-fixing agents. This method is valid when the organism being studied builds its maximum biomass with little turnover. In case of organisms with rapid turnover, such as BGA, the method may lead to a marked underestimation of the N_2 fixed. This method, therefore, has been used only for estimating % Ndfa in rice and macrophytic green manures (Azolla and legumes).

Acetylene-reducing activity measurements

Many variations of the acetylene reduction method have been used for in-situ studies in rice fields and the associated methodological problems were reviewed by Watanabe and Cholitkul (1979). These include a) variability of the conversion factor of acetylene reduced to N_2 fixed, b) different diffusion rates and solubilities of acetylene and N_2, c) incomplete recovery of the ethylene formed, d) disadvantage of short-term assays in assessing overall activity, and e) disturbance of the environmental conditions for the N_2-fixing organism during assays.

Recent studies confirm limitations that may make quantitative extrapolations risky. ARA

was linear with time with aquatic legumes (Ladha and Tirol-Padre, 1990) but not with associative (Barraquio et al., 1986) and algal BNF (Roger et al., 1991). C_2H_2/N_2 conversion factor values varied from 1.6 to 7.9 with Azolla, depending on species, P_{N_2}, assay duration, and age of culture (Eskew, 1987). With dense algal mats, it varied from 3.9 to 30, depending mostly on P_{N_2} used for incubation under $^{15}N_2$ (Roger et al., 1991). But when P_{N_2} was similar to that of air and when all other factors were similar to those used for C_2H_2 exposure, the C_2H_2/N_2 ratio was close to the value of 4.4 derived from regression analysis by Peterson and Burris (1976). Most incubations are done under 10% C_2H_2 in the air, but ARA increases with up to 25% C_2H_2 in the air with thick BGA blooms (Roger et al., 1991) 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., 1991) 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 since 1985. To overcome the limitations and to obtain reproducible values, methods have been developed where composite and/or standardized samples collected in situ are incubated under controlled laboratory conditions (Barraquio et al., 1986; Roger et al., 1991). In field studies, emphasis has been on sampling strategies.

Estimation of associative BNF

Several methods have been developed for in situ measurement of ARA associated with the rice plant (Lee et al., 1977). Laboratory methods, such as water culture (Watanabe and Cabrera, 1979) have been developed to overcome problems encountered in field assays, especially those dealing with gas transfer.

Earlier studies were mostly aimed at associative BNF quantification. Then, emphasis has moved toward screening techniques based on ARA to examine variations in associative N_2 fixation among rice genotypes. Hirota et al. (1978) and Sano et al. (1981) developed a screening method that required assays under anaerobic conditions and was only suitable for pot-grown rice plants, but an immediate and linear ARA with time was obtained. Barraquio et al. (1986) developed a short-term laboratory assay using the root biomass and the adhering soil from cut plants incubated in the dark. The method is destructive but relatively simple, rapid, and reduces problems encountered in the whole-plant and excised root assays.

In a subsequent study, Tirol-Padre et al. (1988) developed a sampling strategy for measuring differences among genotypes. A complete randomized block design with at least three blocks was recommended. ARA measurements were performed at heading for three consecutive days, taking several plants per genotype daily. In most trials, day-to-day variation in ARA for three consecutive days of measurements was not significant. There were also no significant differences in ARA among plants sampled at 0800, 1100, and 1600 h of the same day, but greater variability among three consecutive days of measurements was observed when sampling was done at 1100 and 1600 h. Sampling six plants day^{-1} for three consecutive days 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 μmol for long-duration varieties). Detecting a difference of 20% required a sampling of 20 plants d^{-1} for three consecutive days.

Estimation of photodependent BNF

To decrease variability among replicated measurements and to avoid the greenhouse effect usually observed in situ in the transparent enclosures used for incubation under acetylene, recent studies use composite samples of 7–13 soil cores incubated under constant and moderate temperature (about 25°C) and light intensity (about 30 klux) (Roger et al., 1988; IRRI, 1989b).

From a study of 440 groups of replicated measurements in plots where a wide range of agronomic practices was used, Roger et al. (in IRRI, 1989b) drew general conclusions on 1) the distributional ecology of ARA, 2) the implication for sampling density within a plot, and 3) the number of replicates needed for a given accuracy. The study of correlation between means and variances of the replicated measurements showed a slope of the regression curve close to 2 (Fig. 1), indicating a log-normal distribution of the data (Roger et al., 1978). This

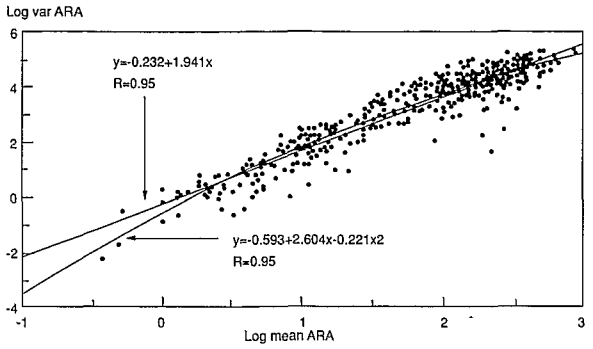

Fig. 1. Correlation between means and variances of 442 groups of ARA measurements in 5 replicated plots (4 × 4 m) (Roger et al., unpubl.).

type of distribution was observed for 1) single-locus samples collected in the same plot and 2) single-locus and composite samples collected in replicated plots.

In such a distribution, the accuracy of the mean of n measurements (P_e), defined as half of the confidence interval expressed as a fraction of the mean, is calculated as

$$P_e = \frac{1}{2}\left(10^{(tS_y/\sqrt{n})} - 10^{-(tS_y/\sqrt{n})}\right) \qquad (1)$$

where t is the statistic of Student-Fischer, n is the number of replicates, and S_y is the standard deviation of the logarithms of the data (Roger et al., 1978).

Figure 2 presents a graphic representation of this function for values of S_y ranging from 0.2 to 1.0. When S_y estimates are available from previous measurements performed under similar conditions, this graph can be used to determine the number of replicated plots needed for a given accuracy or the number of subsamples needed for a given representativeness of a composite sample within a plot. Figure 3 summarizes 496 S_y values obtained from photodependent ARA measurements under a wide range of conditions. The median of 653 estimates of S_y is 0.317. Applying this value to Equation 1 shows that the accuracy of averages of ARA measurements in replicated plots is usually low. For example, measurements in 10 replicated plots provide an accuracy of 0.5, for which the confidence interval is equal to the mean (Fig. 2).

The utilization of composite samples markedly increases the representativeness of measurements within a plot. However, as shown in Figure 2, on the average (i.e. $S_y = 0.3$), 10 core subsamples are needed for a representativeness of 0.5. A representativeness of 0.3 requires 26 core subsamples and one of 0.2 requires 55 core subsamples. These data show that the number of subsamples collected from a plot is often determined by methodological limitations (i.e. the maximum number of subsamples that can be reasonably collected or handled) rather than a chosen accuracy.

However, rather than the accuracy of a mean, the major concern in field experiments is the number of replicates needed to establish a significant difference between two means, X_a and X_b. Assuming that $n_a = n_b$ and $S_{ya} = S_{yb}$, the ratio X_a/X_b required to establish a significant

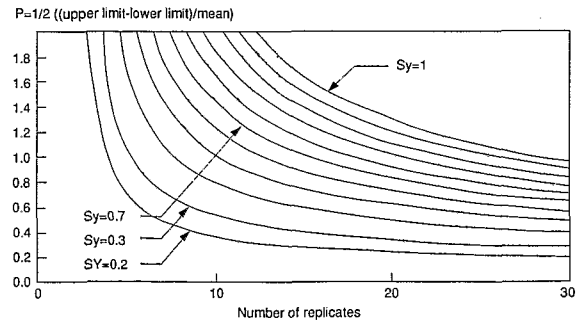

Fig. 2. Accuracy of the mean of n replicated ARA measurements according to the number of replicates and the standard error of the logarithms of the data (s_y).

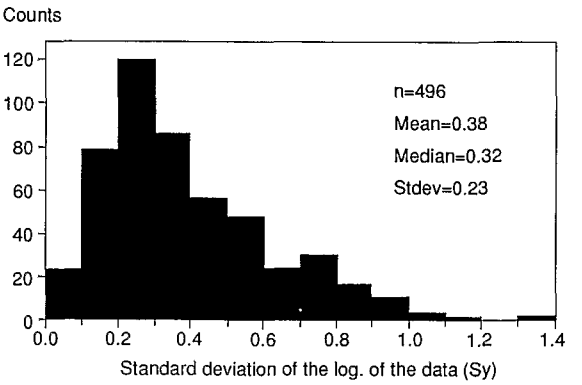

Fig. 3. Histogram of 496 estimates of the standard error of the logarithms of replicated ARA measurements performed in 4 or 5 replicated plots using composite samples of 8 to 16 soil cores, 2 cm in diameter.

difference between X_a and X_b ($p = 0.05$) is given by

$$\frac{\overline{X_a}}{\overline{X_b}} = 10^{\sqrt{(2S_y^2/n)} \cdot t_{(2n-2)}} \quad (2)$$

where n is the number of replicates, S_y^2 is the variance of transformed data, and t_{2n-2} is the t value of Student-Fischer statistic at $(2n-2)$ degrees of freedom (Roger et al., 1978). Figure 4 presents the calculation of X_a/X_b for $2 < n < 20$ and $0 < S_y^2 < 1$. The values of X_a/X_b corresponding to the median value of $S_y(0.3)$ show that, on an average, 5 replicated plots will only permit to find significant differences between values whose ratio is about 3. Ten replicates would permit to establish a significant difference between two values whose ratio is two.

Most field experiments on rice usually have 3 or 4 replicates, which is adequate for normally distributed data such as rice yield, but might often be insufficient for estimating ARA with good accuracy.

A coefficient of variation of 100% is observed among single-locus measurements because of their log-normal distribution. This coefficient of variation rapidly decreases in composite sampling when the number of subsamples increases. Therefore, the accuracy of the mean of measurements on composite samples in replicated plots depends mainly on the number of plots. In particular, it does not improve when the value of the accuracy of the measurements in each plot decreases beyond the value of the accuracy that can be expected from the number of replicated plots. Therefore, the determination of the optimal number of subsamples to be collected in a plot should take into account accuracy or representativeness for the lower limit and the number of replicated plots for the upper limit.

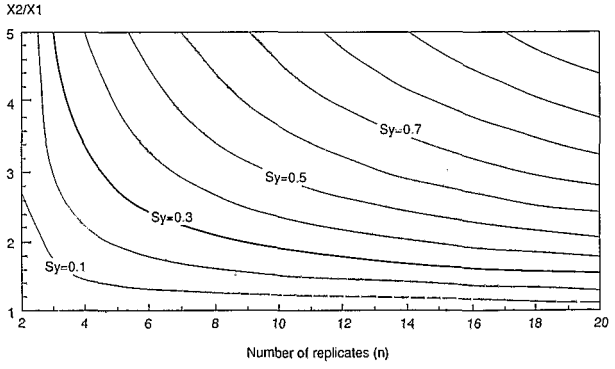

Fig. 4. Ratio (X_1/X_2) between two means of n replicated field ARA measurements ensuring a significant difference at $p = 0.05$ according to the number of replicates and the standard error of the logarithms of the data (s_y).

Interplot variability of daily ARA measurements cannot be reduced, but ARA values integrated for the crop cycle are less variable than daily values.

These data show the importance of sampling strategies in performing field measurements of ARA. Such measurements are extremely tedious and an adequate sampling and measurement strategy can avoid either superfluous sample collection or measurements (that will not improve significantly the accuracy of the data), or measurements that have little chance of producing conclusive results.

Estimation of BNF by aquatic legumes

The ARA method has been widely used for the measurement of N_2 fixation by root-nodulated upland legumes. The methodological aspects were discussed by Hardy et al. (1973) and Turner and Gibson (1980). This method was recently applied for aquatic legumes used as green manure in lowland rice, such as *Sesbania rostrata* (Ladha and Tirol-Padre, 1990). ARA measurement for such legumes may require special consideration because of their growth habit under submerged condition and their ability to produce root and stem nodules. Ladha and Tirol-Padre (1990) showed that when cut plants (aerial parts) were incubated in a plastic bag, ARA was linear from 0 to 2.5 h of incubation and did not significantly differ, regardless of whether incubation was under light or in the dark. ARA was highest in plants harvested between 1300 and 1600 h, and the optimum acetylene concentration was 10–15%. A critical factor in assaying large numbers of field-grown plants is the prolonged time interval between sampling and the assay. It was found that a 4-h interval between sampling and assay, compared with a 1-h interval, significantly reduced ARA.

$^{15}N_2$ *incorporation*

$^{15}N_2$ incorporation was used in short-term studies

to assess BNF by various agents, to identify active sites in the soil or rice plant, and to establish the C_2H_2/N_2 conversion factor in BGA, Azolla (Eskew, 1987 Watanabe and Roger, 1982), and stem-nodulating legumes (Becker et al., 1990).

^{15}N dilution method

This method is attractive because a single measurement 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 the soil and water in flooded conditions, is the same for the N_2-fixing and the non-N_2-fixing plant. This assumption is met when ^{15}N enrichment of soil N available to the N_2-fixing system is constant during the experiment (this implies that ^{15}N added is equilibrated with soil N and labeling is constant throughout the soil) or when the N_2-fixing and the non-N_2-fixing plant have similar N uptake patterns (this implies that the ratio of soil N [S] to that of fertilizer N [F] assimilated by N_2-fixing and non-N_2-fixing plants are equal).

The approach that ensures a constant $^{15}N/^{14}N$ ratio is promising in wetland rice fields in as much as it is easier to label soil and stabilize ^{15}N here than in upland soils; this is because the plow layer delineates an Ap horizon in which most roots are located and which is relatively easy to homogenize. Either a non-N_2-fixing plant or available soil N (Zhu et al., 1984) can be used as control, but the validity of the control depends on the level of % Ndfa.

To ensure a similar S/F uptake in the non-N_2-fixing and N_2-fixing plants, the approach requires a method to test S/F values in both plants. Wagner and Zapata (1982) attempted to indirectly determine S/F values by comparing the uptake of ^{35}S-labeled sulphate and indigenous soil sulphate. However, this method proved unsatisfactory because plants differ in their relative uptake of N to sulphate. Ledgard et al. (1985) estimated S/F in N_2-fixing and non-N_2-fixing plants by growing them in soils which received different levels of ^{15}N while keeping the amount of N constant. The proportions of S and F were determined from the intercept and slope of regression between ^{15}N enrichment of plant N and added N.

Pareek et al. (1990) quantified BNF by *Sesbania rostrata* and *S. aculeata* in two subsequent crops. The first crop was grown in soil having a fast decline of ^{15}N in available soil N and the second crop was grown in soil having a slow decline of ^{15}N. Estimates were computed using 1) three non-N_2-fixing reference species, 2) ^{15}N enrichment of available soil N as reference, and 3) the varying ^{15}N level technique. The study concluded that accurate estimates can be obtained after about 100 days of stabilization of ^{15}N applied to the soil under flooded, preferably planted conditions. The need for a control having similar S/F as that of the N_2-fixing plant becomes less critical, if soil with a fairly good stabilization of ^{15}N is used.

The accuracy of the ^{15}N dilution method is correlated with the proportion of N derived from BNF. This was shown by modelling the %Ndfa in relation to ^{15}N enrichment of N_2-fixing and non-N_2-fixing crops (Hardarson et al., 1988) or the S/F of N_2-fixing and non-N_2-fixing crops (Pareek et al., 1990). In their model, Hardarson et al. (1988) used an arbitrary value of 0.2 atom % ^{15}N excess in the N_2-fixing crop and various assumed values of atom % ^{15}N excess in the non-N_2-fixing crop with an error of $\pm 10\%$ for each atom % ^{15}N excess value. The model showed that when %Ndfa is low, small differences in ^{15}N enrichment of the non-N_2-fixing crop result in large changes in %Ndfa estimates. On the other hand, when %Ndfa is high, the error is low (Fig. 5).

Whereas the ^{15}N dilution method was found suitable for estimating Ndfa in leguminous green manure and rice, trials with aquatic N_2 fixers, such as Azolla and BGA, have shown that fast changes in ^{15}N enrichment in the water over time (Eskew, 1987), due in particular to losses by ammonia volatilization, result in large errors in %Ndfa estimation (Witty, 1983). These problems can be solved, in the case of Azolla, 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 the non-N_2-fixing control algae may inhibit BGA growth directly or through competition (Roger, 1991).

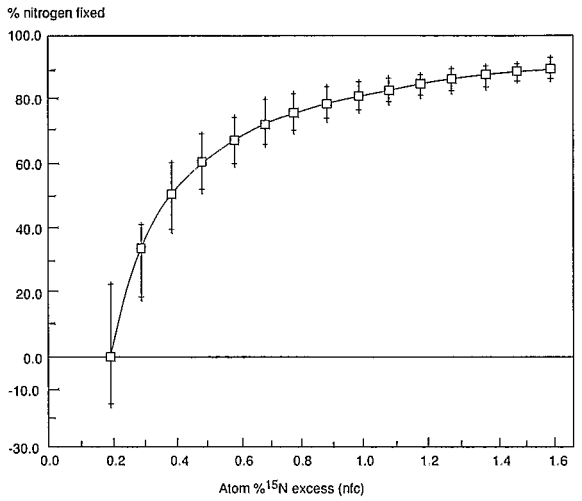

Fig. 5. Estimated values of % N_2 fixed in a plant containing 0.2 atom % excess ±0.02 SD and a range of assumed atom % ^{15}N excess ±10% SD in nonfixing reference crop (after Hardarson et al., 1988). The bars represent the upper and lower limits of standard deviation of the estimates.

Difference in natural ^{15}N abundance ($\delta^{15}N$)

The $\delta^{15}N$ method uses the fact that soil has a higher $\delta^{15}N$ than air and can serve as a naturally labelled medium. The method is advantageous because of the stable isotopic composition of N sources, but in situ a ^{15}N gradient observed with soil depth can be a serious source of error. Growing plants in pots with well-mixed soil avoids this problem (Watanabe et al., 1987). This method was used to test differences among rice plant organs and varieties, and to estimate Ndfa in Azolla (Watanabe et al., 1987, 1990; Yoneyama et al., 1987). It is currently supposed to be suitable only for semiquantitative estimations, but its potential for quantitative measurement has not been fully explored (Peoples and Herridge, 1990).

Estimation of BNF by various agents

Total 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., 1986). In similar trials, Trolldenier (1987) found balances negatively correlated with the amount of N applied, 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. (1984) 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 that stage. Data summarized by Roger and 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_2 $hill^{-1}$ 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 and Watanabe, 1986). Differences in the ability of rice varieties to support associative BNF were suspected from N balance experiments in pots with soil exposed to light (App et al., 1986). Varietal differences in associative BNF 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 associative BNF is attractive, because such varieties would enhance BNF without requiring 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 mg N fixed g^{-1} straw added (mean 2.1) in 30 d (Roger and Watanabe, 1986). Most data originate from laboratory incubations of soil in the dark with 1–100% straw added (average 22%). Such experiments simulate composting rather than the field situation where

straw left is always less than 1% of soil dry weight. Moreover, dark incubation allows heterotrophic BNF only, whereas straw incorporation also favors photodependent BNF (Ladha and Boonkerd, 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 and Boonkerd, 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 and 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 60 plots where urea was broadcast (Roger et al., 1988). As N_2-fixing BGA usually bloom only when the photic zone is depleted of inorganic N, most of their N can be assumed to originate from BNF. Inubushi and 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, 1991). However, estimates of BGA biomass do not allow the 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 and 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%). However, data from a 4-year field trial at 37 sites in 10 countries show lower productivities in full-scale trials 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 (Table 1). Using the ^{15}N dilution method with Lemna and Salvinia as non-N_2-fixing con-

Table 1. Ndfa in Azolla estimated by ^{15}N dilution[a]

Authors	Reference plant	Ndfa %	Remarks
Kumarasinghe et al. (1985)	*Salvinia*	92	+15 mg N/tray
	Salvinia	79	+75 mg N/tray
	Salvinia	76	+150 mg N/tray
	Lemna/Salvinia	81–83	with rice
	Lemna/Salvinia	82–79	without rice
You C.B. et al. (1987)	*Lemna*	40–59	
	Lemna	63–71	labelled *Azolla*
Kulasooriya et al. (1988)	*Salvinia/Lemna*	52–55	without rice, 40 ppm N
	Salvinia/Lemna	58–64	with rice, 40 ppm N
Watanabe et al. (1990)	*Lemna/Spirogyra*	86–93	3 crops of Azolla
	Lemna	80–81	after a rice crop

[a] After Watanabe, 1990.

trols, Watanabe and 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 $\delta^{15}N$ method, Yoneyama et al. (1987) estimated that 59–99% of N of the strains tested was Ndfa. Anabaena-free *A. filiculoides* and the Lemna control had a similar $\delta^{15}N$ which was not influenced by the water level (flooded or saturated soil).

Leguminous green manure

BNF by leguminous 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 root-nodulating pre-rice LGM crop summarized by Roger and 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, which include several stem-nodulating legumes, average 133 kg N ha^{-1} (Ladha et al., 1988c). Ranges in kg N ha^{-1} are 40–225 for aquatic LGM, and 33–115 for grain legumes. 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. (1990) quantified the contribution of BNF to total N of *S. rostrata* and *S. cannabina* grown up to 65 days in the dry season (short days) and the wet season (long days). At 25 days, the percentage of total N obtained from the atmosphere by both species was 50% during the dry season and 75% during the wet season. The contribution by BNF increased with age of the plant and ranged from 70 to 95% at 45–55 days. Although the %Ndfa in the two species were similar, *S. rostrata* showed substantially higher fixation than *S. cannabina*, and the difference was more pronounced in the wet season. This was attributed to the difference in total N uptake by the two species. The estimates of BNF per plant were converted to N_2 fixed per hectare by assuming a plant density of 500,000 ha^{-1}. At 45 days, the dry-season crop of *S. rostrata* had fixed 100 kg N ha^{-1} and the wet-season crop 140 kg ha^{-1}, while the values for *S. cannabina* were 78 and 119 kg ha^{-1}, respectively. The daily N gain was 17 kg ha^{-1} between 45 and 55 days in the wet-season crop. Almost similar % estimates of Ndfa by *S. rostrata* were reported by Yoneyama et al. (1990) using natural abundance ^{15}N dilution. Based on $^{15}N_2$-calibrated ARA, Becker et al. (1990) reported %Ndfa of 80 and 70% by 8-week-old *S. rostrata* and *Aeschynomene afraspera*, respectively. The %Ndfa did not differ in both species when grown at different times of the year.

On the other hand, Rinaudo et al. (1988) and Ndoye and Dreyfus (1988) reported a much lower contribution from N_2 fixation, ranging from 30 to 50% by 53- to 63-day-old *S. rostrata*. A possible reason for the lower estimates is that they used non-inoculated *S. rostrata* as a reference plant.

A few estimates of BNF by *S. rostrata* as a pre-rice 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.

Nitrogen balance and BNF contribution in wetland rice

Nitrogen balances in long-term fertility experiments listed by Greenland and Watanabe (1982) ranged from 19 to 98 kg N ha^{-1} crop^{-1} (average 51 kg) in 9 fields with no N fertilizer. In 4 fields with N fertilizer, the average was −1.5 kg N ha^{-1} crop^{-1}. With rice grown alternately 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, respectively. Calculations based on yields and known inputs suggested that two crops year^{-1} resulted in balances of 79 and 103 kg N ha^{-1} year^{-1}.

Compared with pot experiments, balance

Table 2. Effect of various factors on N balance[a]

Factor	No. of observations	Mean (kg N ha^{-1} crop^{-1})	Standard deviation	Level of significance of the difference
No inorganic N	166	29.7	25.4	0.000**
With inorganic N	45	4.0	47.6	
Planted	193	26.5	30.7	0.001**
Unplanted	18	− 0.5	46.2	
Light	197	25.0	33.9	0.198^{ns}
Dark	14	13.2	13.8	
No N, Light	152	31.2	25.7	0.011*
No N, Dark	14	13.2	13.8	

Coefficients of correlation between balance and N Applied (n = 211):

Inorganic N	Organic N	Total N
r = −0.320**	r = −0.157*	r = −0.365**

[a] Analysis of data compiled from: Ando (1975); App et al., (1980, 1984, 1986); De and Sulaiman (1950); Firth et al. (1973); Greenland and Watanabe (1982); Inatsu and Watanabe (1969); Konishi and Seino (1961); Koyama and App (1979); Santiago-Ventura et al. (1986); Singh and Singh (1987); Trolldenier (1987); and Willis and Green (1948).
177 values obtained in pots were extrapolated to kg N ha^{-1} crop^{-1} on an area basis.

studies in the field encounter additional difficulties 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 and Singh, 1987; Trolldenier, 1987). In a 4-crop experiment comparing organic N (Azolla and BGA) and urea, Singh and Singh (1987) found positive N balances ranging from 13 to 163 mg N crop^{-1} pot^{-1}. Balances were highest (133–163 mg N crop^{-1} pot^{-1}) in pots that received 60 kg organic N ha^{-1}, and lowest (13–29 mg) in pots that received 30–60 kg N ha^{-1} as urea. Balance 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 balance 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 balances reported by Singh and Singh (1987) 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 mg). Extrapolating values of App et al. (1986) shows N balances ranging from 16 to 70 kg N ha^{-1} crop^{-1} (average 38 kg) 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 no or low levels of N fertilizer. With high levels of N fertilizer, the balance was not significant.

Figure 6 and Table 2 summarize the balance measurements in rice soils. Data are from 14 reports, including both field and pot studies. To allow for comparisons, data from pot experi-

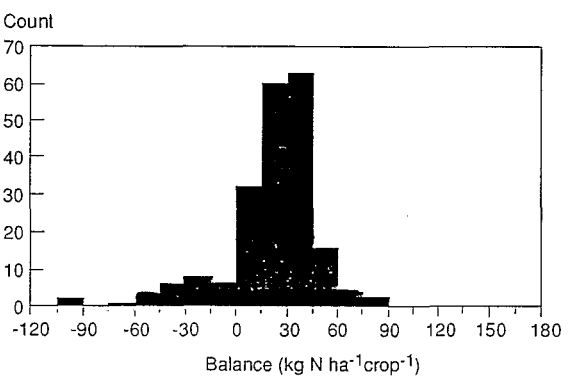

Fig. 6. Histogram of 211 N balance values from field and pot experiments dealing with rice. Data compiled from: Ando (1975); App et al. (1980, 1984, 1986); De and Sulaiman (1950); Firth et al. (1973); Greenland and Watanabe (1982); Inatsu and Watanabe (1969); Konishi and Seino (1961); Koyama and App (1979); Santiago-Ventura et al. (1986); Singh and Singh (1987); Trolldenier (1987); and Willis and Green (1948).
177 values obtained in pots were extrapolated to kg N ha^{-1} crop^{-1} on an area basis.

ments were extrapolated on an area basis and expressed in kg N ha^{-1} $crop^{-1}$. Figure 6 presents the histogram of the 211 balance values, with an average change of 24.2 kg N ha^{-1} $crop^{-1}$ (median: 27 kg). Ninety-five percent of the balance values are between −60 and +90 kg N ha^{-1} $crop^{-1}$. Extreme values are from balance experiments in pots performed over one crop cycle only (Willis and Green, 1948); other pot experiments were conducted for three to six successive crops.

A comparison of the average values of data grouped according to various criteria by the Student-Fischer *t* test (Table 2) shows a larger balance in the absence of inorganic N (29.7 kg N ha^{-1} $crop^{-1}$) than when inorganic N is applied (4.0 kg N ha^{-1} $crop^{-1}$). A study of the correlation between N balance and the level of N fertilizer applied shows highly significant ($p = 0.01$) negative values for inorganic N and total N (inorganic + organic) and a negative value ($p = 0.05$) for organic N (Table 2). This supports the general concept that inorganic N is more susceptible to losses than organic N.

Data in Table 2 also show a larger balance in planted soil (26.5 kg N ha^{-1} $crop^{-1}$) than in unplanted soil (−0.5 kg). The difference between balance in pots where soil was either exposed to light or kept in the dark is not statistically significant when considering all data. It becomes highly significant when only experiments with no inorganic N fertilizer applied are considered. The data indicate that under such conditions, photodependent BNF might contribute roughly twice more N than heterotrophic BNF.

Conclusions

Recent methodological progress in measuring BNF in rice fields 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 a promising method for screening rice varieties for ability to utilize biologically fixed N.

Ranges of reported estimates of BNF by in-

Table 3. Range of estimates of N_2 fixed by various agents in wetland rice fields (kg N ha^{-1} $crop^{-1}$) and theoretical maximum potential (value and assumptions)

Component	Reported range of estimates	Theoretical maximum potential and assumptions
BNF associated with rice rhizosphere	1–7 kg N ha^{-1} $crop^{-1}$	40 kg N ha^{-1} $crop^{-1}$ ● All rhizospheric bacteria are N_2-fixers, ● C flow through rhizosphere is 1 t ha^{-1} $crop^{-1}$, and ● 40 mg N is fixed g C^{-1}.
BNF associated with straw	2–4 kg N t^{-1} straw	35 kg N ha^{-1} $crop^{-1}$ ● 5 t of straw is applied, and ● 7 mg N is fixed g^{-1} of straw.
Total heterotrophic BNF	1–31 kg N ha^{-1} $crop^{-1}$	60 kg N ha^{-1} $crop^{-1}$ ● All C input (2 t $crop^{-1}$) is used by N_2-fixers.
Blue-green algae	0–80 kg N ha^{-1} $crop^{-1}$	70 kg N ha^{-1} $crop^{-1}$ ● Photosynthetic aquatic biomass is composed exclusively of N_2-fixing BGA (C/N = 7), and ● primary production is 0.5 t C ha^{-1} $crop^{-1}$.
Azolla	20–150 kg N ha^{-1} $crop^{-1}$ (experimental plots) 10–50 kg N ha^{-1} $crop^{-1}$ (field trials)	224 kg N ha^{-1} $crop^{-1}$ ● one Azolla standing crop is 140 kg N ha^{-1}, and ● two Azolla crops are grown per rice crop, ● Ndfa is 80%.
Legume green manures	20–260 kg N ha^{-1} $crop^{-1}$	260 kg N ha^{-1} in 55 days ● *Sesbania rostrata* is used as green manure ● 290 kg N ha^{-1} is accumulated in 50–60 d, and ● Ndfa is 90%.

dividual systems are presented in Table 3. Estimates for green manure (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 methods in small-scale trials. However, total BNF in a rice field 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 fixed N_2 to rice nutrition is still not available. The dynamics of BNF during the crop cycle are 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 considered as a nondynamic input.

Because of technological and socio-economical limiting factors, the agronomic potential of BNF is still largely underutilized in rice cultivation (Roger, 1991). Methodological progress and comprehensive evaluations of BNF in rice fields are still needed to develop and test agricultural practices that take advantage of BNF in this important agroecosystem.

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References

Ando S 1975 Effect of crude organic matter applied to the excessively draining low-yield paddy field. Bull. Kagawa Agric. Expt. Stn. 27, 1–81. (*In Japanese, English summary*).

App A A, Watanabe I, Alexander M, Ventura W, Daez C, Santiago T and De Datta S K 1980 Nonsymbiotic nitrogen fixation associated with the rice plant in flooded soils. Soil Sci. 130, 283–289.

App A A, Watanabe I, Santiago-Ventura T, Bravo M and Daez-Jurey C 1986 The effect of cultivated and wild rice varieties on the nitrogen balance of flooded soil. Soil Sci. 141, 448–452.

App A, Santiago T, Daez C, Menguito C, Ventura W, Tirol A, Po J, Watanabe I, De Datta S K and Roger P 1984 Estimation of the nitrogen balance for irrigated rice and the contribution of phototrophic nitrogen fixation. Field Crops Res. 9, 17–27.

Barraquio W L, Daroy M L G, Tirol A C, Ladha J K and Watanabe I 1986 Laboratory acetylene reduction assay for relative measurement of N_2-fixing activities associated with field-grown wetland rice plants. Plant and Soil 90, 359–372.

Becker M, Ladha J K and Ottow J C G 1990 Growth and N_2-fixation of two stem-nodulating legumes and their effect as green manure on lowland rice. Soil Biol. Biochem. 22, 1109–1119.

Bray F 1986 The Rice Economies, Technology and Development in Asian Societies. Basil Blackwell. 254 p.

De P K and Sulaiman M 1950 The influence of algal growth in the rice fields on the yield of crops. Indian J. Agric. Sci. 20, 327–342.

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

Firth P, Thitipoca H, Suthipradit S, Wetselaar R and Beech D F 1973 Nitrogen balance studies in the central plains of Thailand. Soil Biol. Biochem. 5, 41–46.

Greenland D J and Watanabe I 1982 The continuing nitrogen enigma. Trans. Int. Congr. Soil Sci, New Delhi 5, 123–137.

Hardarson G, Danso S K A and Zapata F 1988 Dinitrogen fixation measurements in alfalfa-ryegrass swards using ^{15}N and influence of the reference crop. Crop Sci. 28, 101–105.

Hardy R W F, Burns R C and Holsten R D 1973 Application of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5, 47–81.

Hirota Y, Fujii T, Sano Y and Iyama S 1978 N_2-fixation in the rhizosphere of rice. Nature, London 276, 416–417.

Inatsu O and Watanabe K 1969 Changes of fertility in peaty paddy soils in Sorachi district. Hokuno 36, 31–38. (cited by Greenland and Watanabe, 1982). (*In Japanese*).

Inubushi K and Watanabe I 1986 Dynamics of available nitrogen in paddy soils. II. Mineralized N of chloroform-fumigated soil as a nutrient source for rice. Soil Sci. Plant Nutr. 32, 561–577.

IRRI 1989a IRRI Toward 2000 and Beyond. Manila, Philippines. 72p.

IRRI 1989b Photodependent nitrogen fixation and photosynthetic aquatic biomass. *In* Annual Report for 1988. pp 347–353. International Rice Research Institute, Los Baños, Philippines.

Konishi C and Seino K 1961 Studies on the maintenance-mechanism of paddy soil fertility in nature. Bull. Hokuriku Agric. Exp. Stn. 2 Niigata Prefecture. 41 p. (*In Japanese, English summary*).

Koyama T and App A A 1979 Nitrogen balance in flooded rice field. *In* Nitrogen and Rice. pp 95–104. The International Rice Research Institute, Los Baños, Philippines.

Kulasooriya S A, Senviratne P R G, De Silva W S A G, Abeysekera S W, Wijesundara C and De Silva A P 1988

Isotopic studies on N_2-fixation in Azolla and the availability of its nitrogen to rice. Symbiosis 6, 151–166.

Ladha J K and Boonkerd N 1988 Biological nitrogen fixation by heterotrophic and phototrophic bacteria in association with straw. *In* Proceedings of the First International Symposium on Paddy Soil Fertility, Chiangmai, Thailand, 6–13 December 1988. pp 173–187. ISSS, Paddy Soil Fertility Group.

Ladha J K and Tirol-Padre A 1990 Standardization of acetylene reduction assay with field grown aquatic legume. Int. Rice Res. Newsl. 15, 16–17.

Ladha J K, Miyan S and Garcia M 1988a *Sesbania rostrata* as green manure for lowland rice: Growth, N_2-fixation, *Azorhizobium* sp. inoculation, and effects on succeeding crop yields and nitrogen balance. Biol. Fert. Soils 7, 191–197.

Ladha J K, Padre A T, Punzalan G C, Watanabe I and De Datta S K 1988b Ability of wetland rice to stimulate biological nitrogen fixation and utilize soil nitrogen. *In* Nitrogen Fixation: Hundred Years After. Eds. H Bothe, F J de Bruijn and W E Newton, pp 747–752. Gustav Fischer, Stuttgart, New York.

Ladha J K, Tirol-Padre A, Punzalan G C and Watanabe I 1987 Nitrogen-fixing (C_2H_2-reducing) activity and plant growth characters of 16 wetland rice varieties. Soil Sci. Plant Nutr. 33, 187–200.

Ladha J K, Watanabe I and Saono S 1988c Nitrogen fixation by leguminous green manure and practices for its enhancement in tropical lowland rice. *In* Sustainable Agriculture: Green Manure in Rice Farming. pp 165–183. The International Rice Research Institute, Los Baños, Philippines.

Ledgard S F, Simpson J R, Freney J R and Bergersen F J 1985 Assessment of the relative uptake of added and indigenous soil nitrogen by nodulated legumes and reference plants in the ^{15}N dilution measurement of N_2 fixation: Glasshouse application of method. Soil Biol. Biochem. 17, 323–328.

Lee K K, Alimagno B V and Yoshida T 1977 Field technique using the acetylene reduction method to assay nitrogenase activity and its association with rice rhizosphere. Plant and Soil 47, 519–526.

Li Zhuo-Xin, Zu Shou-Xian, Mao Mei-Fei, Wang Fu-Lai and Zhao Bing-Bo 1987 Determination of amount of N_2-fixation and change in N_2-fixing activity of Azolla in natural environment. *In* Azolla Utilization. pp 223–231. The International Rice Research Institute, Los Baños, Philippines.

Ndoye I and Dreyfus B 1988 N_2-fixation by *Sesbania rostrata* and *Sesbania sesban* estimated using ^{15}N and total N difference methods. Soil Biol. Biochem. 20, 209–213.

Pareek R P, Ladha J K and Watanabe I 1990 Estimation of N_2-fixation by *Sesbania rostrata* and *S. cannabina* in lowland rice soil by ^{15}N dilution method. Biol. Fert. Soils 10, 77–88.

Peoples M B and Herridge D F 1990 Nitrogen fixation by legumes in tropical and sub-tropical agriculture. Adv. Agron. 44, 155–223.

Peterson R B and Burris R H 1976 Conversion of acetylene reduction rate to nitrogen fixation rates in natural populations of blue-green algae. Anal. Biochem. 73, 404–410.

Postgate J 1990 Fixing the nitrogen fixers. New Scientist, 3 February 1990, 57–61.

Rinaudo G, Alazard D and Moudiongui A 1988 Stemnodulating legumes as green manure for rice in West Africa. *In* Green Manure in Rice Farming. pp 97–109. The International Rice Research Institute, Los Baños, Philippines.

Roger P A 1991 Blue-green algae in agriculture. *In* Microorganisms that Promote Plant Productivity. Eds. J O Dawson and P J Dart. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Roger P A and Kulasooriya S A 1980 Blue-green algae and rice. The International Rice Research Institute, Los Baños, Philippines. 112 p.

Roger P A and Watanabe I 1986 Technologies for utilizing biological nitrogen fixation in wetland rice: Potentialities, current usage, and limiting factors. Fert. Res. 9, 39–77.

Roger P A, Jimenez R and Santiago-Ardales S 1991 Methods for studying blue-green in ricefields: distributional ecology, sampling strategies, and estimation of abundance. IRRI Research Paper Series no. 150. The International Rice Research Institute, Los Baños, Philippines.

Roger P A, Reddy P M and Remulla-Jimenez R 1988 Photodependent acetylene reducing activity (ARA) in ricefields under various fertilizer and biofertilizer management. *In* Nitrogen Fixation: Hundred Years After. Eds. H Bothe, F J de Bruijn and W E Newton. p 827. Gustav Fischer, Stuttgart, New York.

Roger P A, Reynaud P A and Monniaux G 1978 Normalisation des données et calcul de la précision des mesures en microbiologie du sol (Normalization of data and calculation of the accuracy of measurements in soil microbiology). Cah. ORSTOM, Ser. Biol. 13, 171–180.

Sano Y, Fujii T, Iyama S, Hirota Y and Komagata K 1981 Nitrogen fixation in the rhizosphere of cultivated and wild rice strains. Crop Sci. 21, 758–761.

Santiago-Ventura T, Bravo M, Daez C, Ventura W, Watanabe I and App A A 1986 Effects of N fertilizers, straw, and dry fallow on the nitrogen balance of a flooded soil planted with rice. Plant and Soil 93, 405–411.

Singh A L and Singh P K 1987 Nitrogen fixation and balance studies of rice soil. Biol. Fert. Soils 4, 15–19.

Tirol-Padre A, Ladha J K, Punzalan G C and Watanabe I 1988 A plant sampling procedure for acetylene reduction assay to detect rice varietal differences in ability to stimulate N_2-fixation. Soil Biol. Biochem. 20, 175–183.

Trolldenier G 1987 Estimation of associative nitrogen fixation in relation to water regime and plant nutrition in a long-term pot experiment with rice. Biol. Fert. Soils 5, 133–140.

Turner G L and Gibson A H 1980 Measurement of dinitrogen fixation by direct means. *In* Methods for Evaluating Biological Nitrogen Fixation. Ed. F J Bergersen. pp 111–138. Wiley, New York.

Wagner G H and Zapata F 1982 Field evaluation of reference crops in the study of nitrogen fixation by legumes using isotope techniques. Agron. J. 74, 607–612.

Watanabe I 1982 Azolla-Anabaena symbiosis – its physiology and use in tropical agriculture. *In* Microbiology of Tropical Soil and Plant Productivity. Eds. Y Dommergues and H S Diem. pp 169–185. Martinus Nijhoff Publishers, The Hague, The Netherlands.

Watanabe I 1987 Summary report of the Azolla program of

the International Network on Soil Fertility and Fertilizer Evaluation for Rice. *In* Azolla Utilization. pp 197–205. The International Rice Research Institute, Los Baños, Philippines.

Watanabe I and Cabrera D R 1979 Nitrogen fixation associated with the rice plant grown in water culture. Appl. Environ. Microbiol. 37, 373–378.

Watanabe I and Cholitkul W 1979 Field studies on nitrogen fixation in paddy soils. *In* Nitrogen and Rice. pp 223–239. The International Rice Research Institute, Los Baños, Philippines.

Watanabe I and Roger P A 1982 Use of ^{15}N in the study of biological nitrogen fixation in paddy soils at the International Rice Research Institute. *In* Proceedings of the Consultants Meeting, FAO/IAEA Joint Project, Vienna. pp 81–98.

Watanabe I and Roger P A 1984 Nitrogen fixation in wetland rice fields. *In* Current Development in Biological Nitrogen Fixation. Ed. N S Subba Rao. pp 237–276. Oxford and IBH Pub Co., New Delhi.

Watanabe I, Yoneyama T, Padre B and Ladha J K 1987 Difference in natural abundance of ^{15}N in several rice (*Oryza sativa* L.) varieties: Application for evaluating N_2-fixation. Soil Sci. Plant Nutr. 33, 407–415.

Watanabe I, Yoneyama T, Talukdar H and Ventura W 1992 The contribution of atmospheric N_2 to *Azolla* spp. grown in flooded soils. Soil Sci. Plant Nutr. 37, 101–109.

Willis W H and Green V E 1948 Movement of nitrogen in flooded soils planted to rice. Soil Sci. Soc. Am. Proc. 13, 229–237.

Witty J F 1983 Estimating N_2-fixation in the field using ^{15}N-labelled fertilizer: Some problems and solutions. Soil Biol. Biochem. 15, 631–639.

Yoneyama T 1990 Use of natural ^{15}N abundance for evaluation of N_2-fixation by legumes and Azolla in remote fields. Vol IV. pp 126–130. Proc. 14th International Congress of Soil Science, Kyoto, Japan.

Yoneyama T, Ladha J K and Watanabe I 1987 Nodule bacteroids and Anabaena: Natural ^{15}N enrichment in the legume-rhizobium and Azolla-Anabaena symbiotic systems. J. Plant Physiol. 127, 251–259.

Yoshida T and Rinaudo G 1982 Heterotrophic N_2-fixation in paddy soils. *In* Microbiology of Tropical Soils and Plant Productivity. Eds. Y R Dommergues and H G Diem. pp 75–107. Martinus Nijhoff/Dr W Junk, Publishers, The Hague, The Netherlands.

Zhu Zhao-liang, Liu Chong-qun and Jiang Bai-fan 1984 Mineralization of organic nitrogen, phosphorus, and sulfur in some paddy soils of China. *In* Organic Matter and Rice. pp 259–272. The International Rice Research Institute, Los Baños, Philippines.