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Biological N_2-fixation and its management in wetland rice cultivation

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Abstract

The review summarizes the current status of the utilization of N_2-fixing organisms as biofertilizer in rice cultivation. Heterotrophic bacteria, free-living cyanobacteria, *Azolla*, and legume green manures are considered with regard to their potential for increasing yield, their current use and the prospects for their use with regard to the identified limiting factors.

Biological N_2 fixation has been the most effective system for sustaining production in low-input traditional rice cultivation. On the other hand, the utilisation of N_2-fixing organisms in intensified rice production encounters serious limitations. The utilization of free-living bacteria and cyanobacteria is refrained by their modest potential and the non establishment of inoculated strains. *Azolla* and legumes used as green manures have a high potential as N source, but their utilization is severely limited by socio-economic factors.

Introduction

More than half the world population is dependent upon rice, which provides 20% of global human per capita energy and 15% of per capita protein. In 1991, rice occupied $148 \cdot 10^6$ ha-10% of world's arable land – for a global production of $420 \cdot 10^6$ t [31].

Rice grows in flooded conditions during part or all the cropping period in about 88% of rice land. Flooding changes the chemistry, microbiological properties, and nutrient supplying capacity of soil. It leads to the differentiation of a range of macro- and microenvironments differing by their redox, physical properties, light status, and nutrient sources for the microflora. As a result, all kinds of N_2-fixing organisms can find conditions favorable for their growth in ricefields. They include (1) indigenous organisms: heterotrophic aerobic, microaerophilic and anaerobic bacteria – in soil and associated with rice –, photosynthetic bacteria, and cyanobacteria; and (2) introduced green manures: *Azolla* and legumes. Traditional wetland rice cultivation has been extremely sustainable because biological N_2 fixation (BNF) has permitted a moderate but stable yield to be maintained for thousands of years without N fertilizer addition and without deterioration of the environment [9].

Reviews on BNF in ricefields deal with its estimation and contribution to N balance [66], agronomic use of N_2-fixing biofertilizers [70], and the microbial management of wetland ricefields [71]. Specific reviews on N_2-fixing organisms in ricefields deal with heterotrophs [101], BNF associated with straw [34], rice varietal differences in stimulating BNF [36], cyanobacteria [62], *Azolla* [89], and legume green manures [33, 37].

This paper summarizes quantitative data on BNF estimates in wetland ricefields and considers for each of the major groups of N_2-fixing organisms: the N_2-fixing potential, the potential to increase rice yield, the current status of their utilization, and the prospects with regards to identified limiting factors.

Assessment of biological N_2-fixation in ricefields

Methods used to estimate BNF by during a rice crop cycle include:

- Balance studies in long-term fertility experiments, or in pots.
- Acetylene reducing activity (ARA) measurements performed at intervals during a crop. This method, despite recognized limitations, was used in about 2/3 of the 38 quantitative BNF studies related to rice published since 1985 [66].
- The determination of the maximum biomass of the N_2-fixing system and the % N derived from the air (Ndfa) of this biomass. The ^{15}N dilution method was used to estimate Ndfa of the rice plant and legume green manures. Difference in natural ^{15}N abundance ($\partial^{15}N$) was used to estimate Ndfa in *Azolla*.

N-balance is currently the only method that provides an estimate of total BNF in ricefields, but the values are underestimated because losses are usually not taken into account. 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, after the early measurements in long-term field experiments [25]), there has been an increased interest in pot studies [4, 75, 77, 83].

In a bibliographic compilation of 211 N-balance estimates [66] values ranged from −102 to +171 kg N ha^{-1} crop cycle^{-1} and averaged 24 kg N ha^{-1} crop cycle^{-1} (Table 1). The balance was influenced by N-fertilizer application, the presence of rice, and light availability. An average balance of 30 kg N ha^{-1} crop cycle^{-1} was obtained when no N-fertilizer was used. This shows that the average potential of BNF in nonfertilized fields can ensure, on a long-term basis, a yield of about 1.5 t ha^{-1} (assuming that all N_2 fixed is absorbed by the rice plant and 50 kg grain is produced per kg N absorbed). Balance was negligible in the presence of N-fertilizer (4 kg N ha^{-1} crop cycle^{-1}). This results from the two known processes of BNF inhibition by N-fertilizer and N losses by NH_3 volatilization and denitrification [63]. Mean balance values estimated in the presence of light (31 kg N ha^{-1} crop cycle^{-1}) and in the absence of light (13 kg N ha^{-1} crop cycle^{-1}) indicates that, on an average, photodependent BNF contributes 2/3 of the balance.

Heterotrophic N_2-fixation

The presence of N_2-fixing bacteria in rice rhizosphere was reported as early as 1929 [76], but the study of the potential of N_2-fixing heterotrophs started only in 1971, when it was demonstrated that some BNF is associated with wetland rice roots [61, 100]. Early inoculation trials were conducted with *Beijerinckia* [17] and *Azotobacter* [80]. Most reports on bacterial inoculation of rice (40 of 44), however, have been published since 1976. Research on BNF in the rice rhizosphere has also revealed differences in the ability of rice genotypes to stimulate associative BNF and N uptake [36]. This suggests that N utilization by rice can be improved by selection and breeding of varieties that can stimulate the development of a more efficient associated microflora.

Estimations of heterotrophic BNF

Total heterotrophic BNF

Estimates from N balance in unfertilized planted pots covered with black cloth averaged 7 kg N ha^{-1} [3]. Similar trials showed balances negatively correlated with the amount of N applied [83]. 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, it was estimated that, when no N-fertilizer was applied and photodependent BNF was restrained, heterotrophic BNF contributed 16–21% of rice N, or 11–16 kg N ha^{-1} crop^{-1} [103].

BNF associated with rice rhizosphere

Reported ARA values in rice rhizosphere range from 0.3 to 2 μmol C_2H_4 plant^{-1} h^{-1}. They are usually highest at or near heading stage. Extrapolated values range from 0.8 to 6 kg N ha^{-1} crop cycle^{-1} [70]. The theoretical maximum associative BNF can be calculated by assuming that all rhizospheric bacteria are N_2 fixers and they use all C flux in rhizosphere (1 t ha^{-1} crop cycle^{-1}) with a high efficiency of 40 mg N g^{-1} C. This would be equivalent to 40 kg N ha^{-1} crop^{-1}. But bacterial enumerations in rice rhizosphere often show a ratio higher than 10 between N_2-fixing and total bacteria [66].

BNF associated with straw

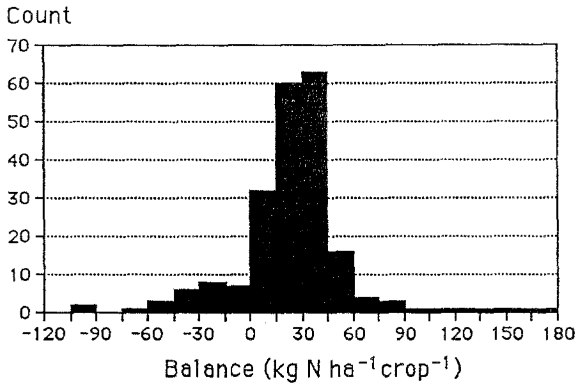
Early estimates of BNF after straw incorporation range from 0.1 to 7 (mean 2.1) mg N g^{-1} straw added, in 30 days [70]. Most data originate from laboratory incubations in darkness of soil enriched with 1 to 100% straw (average 22%) which simulates composting rather than the field situation. Moreover, dark incubation allows heterotrophic BNF only, whereas straw

Table 1. Bibliographic study of N-balance estimates in wetland ricefields* (adapted from [66])

Major statistic of the set of data analyzed

Number of data: 211
Unit: kg N ha^{-1} crop cycle^{-1}
Minimum: -102
Maximum: 171
Mean: 24.2
Median: 27.0
Standard deviation: 33.1
Coefficient of variation: 136%

Histogram of the data



Effect of various factors on N-balance

Factor	Number of data	Mean (kgN ha^{-1} crop cycle^{-1})	Standard error	Level of significance of the difference
N-fertilizer application				
–	166	29.7	25.4	1%
+	45	4.0	47.6	
Planted versus unplanted				
+	193	26.5	30.7	1%
–	18	-0.5	46.2	
Effect of soil exposure to light (treatments where no N-fertilizer was applied)				
+	152	31.2	25.7	1%
–	14	13.2	13.8	

*The set of data is temptatively exhaustive. Data originate from pot and field experiments. Data from pot experiments are extrapolated in kg N ha^{-1} crop cycle^{-1} based on the surface of the pots.

incorporation also may increase populations and N_2-fixing activity of photosynthetic organisms [34]. A few semi-quantitative data and laboratory data suggest that straw might increase BNF by 2–4 kg N t^{-1} applied [34].

These data suggest that the N-potential of heterotrophic BNF is the lowest among the N_2-fixing agents discussed in this review.

Potential of heterotrophic BNF for agronomic utilization

No method adoptable by farmers has been yet designed to enhance on purpose heterotrophic BNF in ricefields. Research has mostly aimed at promoting associative BNF by inoculating selected strains of N_2-fixing bacteria. More recently it was found that there is a potential for selecting/breeding rice varieties more efficient in stimulating associative BNF [36].

Rice inoculation with N_2-fixing heterotrophs

Genera of N_2-fixing bacteria isolated from rice rhizosphere include *Agromonas, Alcaligenes, Aquaspirillum, Azospirillum, Beijerinckia, Citrobacter, Enterobarter, Flavobacterium, Klebsiella* and *Pseudomonas* [70]. Strains most frequently isolated using exudates of rice seedlings as C source were *Enterobacteriaceae, Azospirillum* spp. and *Pseudomonas paucimobilis* [5, 50, 81].

Bacterial inoculation of rice has been studied by dipping seeds in bacterial cultures or coating them with various carriers, dipping seedlings in bacterial cultures, and inoculating nursery soil and/or the field. The analysis of data from 23 articles reporting 210 trials shows an average increase in yield of 19.8%, but the response varied from -33% to +125% [71]. Average increase was higher in pots (27.6%) than in the field (14%). Average yield increase in field experiments (+14%) (Table 2) was close to the minimum detectable difference (14.5%) which can be expected from the experimental design most commonly used (16-m^2 plots, with 4 replicates) [23]. Thus, experiments with no statistical analysis should be interpreted with caution. The distribution of the differences in yield between controls and inoculated plots was very asymmetrical and the histogram, which exhibited an abrupt rise of the first class of positive values, strongly suggested a bias (Table 2). At least, it indicated that unsuccessful trials were often not reported.

The beneficial effect of bacterial inoculation can be attributed to a combination of (1) increased associative BNF, (2) production of plant growth regulators (PGR) that favor rice growth and nutrient utilization, (3) increased nutrient availability through solubilization of immobilized nutrients by inoculated bacteria, and (4) competition of inoculated strains with pathogens in the rhizosphere. The relative importance of these components has not yet been determined. Current estimates of BNF in rice rhizosphere are insufficient to explain the average 0.5 t ha^{-1} increase in yield reported in field experiments. Assuming that all N fixed is absorbed by the plant, such a yield increase would at least require an increase in BNF of 10 kg N ha^{-1} $crop^{-1}$; but no data demonstrate a marked and durable increase of BNF in inoculated rice. The hypothesis that PGR production by inoculated bacteria increases nutrient absorption does not agree with the absence of significant difference in N fertilizer efficiency between control plots (18.7 kg grain per kg N applied) and inoculated plots (19.1 kg) (Table 2). Similar harvest index values (grain yield/straw yield) in the controls and inoculated plots (0.57) may indicate that the effect of inoculation probably takes place early in the crop cycle.

Little information is available on the establishment of inoculated strains. In most cases, variations in population densities were too small to be significant [71]. The inoculation of a mutant of *Azospirillum lipoferum* resistant to two antibiotics showed survival of the strain for 50–70 days but no establishment [48]. Inoculated *Azospirillum* was about 500 times less abundant than putative indigenous populations of *Azospirillum*, but inoculation increased the dry weight and total N of the plant.

Trials have been conducted to select the most efficient combination of a N_2-fixing strain and a specific rice cultivar, by using a two-step process in which bacterial strains were first isolated from the rhizosphere of actively N_2-fixing rice plants [26]. Strains were then tested with rice cultivars using a gnotobiotic system known as the spermosphere model [81] in which an axenic seedling is grown in darkness in a Pankurst tube on a medium without C and N source. This approach has produced both erratic increases in yield [12] and significant increases (6 to 21%) which were higher at the highest level of N-fertilizer (76–96 kg N/ha^{-1}) [50]. If the validity of the method is confirmed, its potential for practical utilization will strongly depend upon the degree of specificity required to select an efficient bacteria for given agro-ecological conditions.

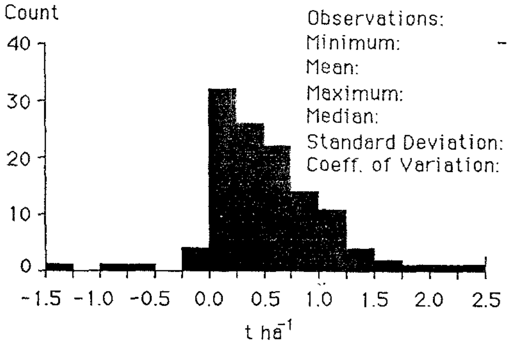
Utilization of varietal differences in promoting associative BNF

The existence of varietal differences in the ability to support associative BNF was demonstrated by N balance studies [4], ARA measurements [82], and N isotope ratios [95]. The plant traits associated with associative BNF were, by decreasing importance: dry weight of roots and submerged portions of the plant at heading, dry weight of shoots at heading, N uptake at heading, and N uptake at maturity [36]. A ranking of 21 rice genotypes for BNF and N utilization, established from the above plant traits and ARA, was fairly reproducible in two dry season trials [35]. Nothing is known, however, about the physiological basis of varietal differences. The idea of breeding varieties with higher N_2-fixing potential is attractive because it would enhance BNF without additional cultural practices. A prerequisite is the availability of a rapid screening technique. Even short-term ARA assays are time-consuming and

Table 2. Bibliographic study of the field experiments on the effect of bacterial inoculation on rice yield (adapted from [71])

	Grain yield			Straw yield	Harvest index		N efficiency	
	Control	Difference		difference	(grain/straw)		(kg grain kg^{-1} N)	
	(t ha^{-1})	(t ha^{-1})	(%)	(%)	Control	Inoc.	Control	Inoc.
Mean	3.99	0.52	14.4	15.1	0.57	0.57	18.7	19.1
Stdev	1.64	0.52	14.1	14.9	−0.17	0.17	17.1	13.8
Maxi	11.09	2.35	59.6	64.8	0.86	0.88	78.0	54.7
Mini	1.00	−1.50	−25.0	−7.5	0.25	0.25	−20.0	−12.0
Count	121	121	123	51	51	51	60	59

Histogram and statistics of yield differences between inoculated and noninoculated treatments



do not allow the screening of a large number of genotypes [82]. ^{15}N dilution can be used for screening and genetic studies, but reference varieties with low BNF stimulation ability must first be identified.

Prospects

So far, the results of bacterial inoculation experiments are inconsistent. Reported increases in yield have not been related with an increase in BNF. Usually inoculated strains did not clearly establish. Therefore reasons for yield increases are still unclear. The potential of methods aiming at selecting the most efficient combination of a N_2-fixing bacterial strain and a specific rice cultivar needs (1) further confirmation and (2) the determination of the degree of specificity required, which will determine the feasibility for practical use. Current knowledge is insufficient to establish inoculation methods that can be used by rice farmers.

The existence of varietal differences in promoting associative BNF offers a promising way of taking advantage of heterotrophic BNF. This potential can be better utilized if screening takes into account both the ability to stimulate BNF and to utilize soil N [36].

Free-living cyanobacteria

The agronomic potential of N_2-fixing cyanobacteria was recognized in 1939 by De [14], who attributed the natural fertility of wetland ricefields to BNF by these organisms. Research on cyanobacterial inoculation of ricefields was initiated in Japan in 1951 [88] and then continued in India [84]. Research on cyanobacteria agroecology in ricefields developed during the last decade [62].

Potential of cyanobacteria as a biofertilizer for rice

N_2 fixation by cyanobacteria has been almost exclusively estimated from ARA. Estimates published before 1980 ranged from a few to 80 kg N ha^{-1} $crop^{-1}$ (mean 27 kg) [64]. About 180 crop cycle measurements in experimental plots at IRRI [67] showed extrapolated values ranging from 0.2 to 50 kg N ha^{-1} $crop^{-1}$ and averaging 20 kg in no-N control plots, 8 kg in plots with broadcast urea, and 12 kg in plots where N was deep-placed.

Biomass measurements provide a rough estimate of the N_2-fixing potential of cyanobacteria because they bloom only when the photic zone is depleted of N and most of their N can be assumed to originate from BNF. Cyanobacteria in ^{15}N-labelled plots had about 90% Ndfa [27]. A visible growth of cyanobacteria usually corresponds to less than 10 kg N ha^{-1}, a dense bloom may correspond to 10–20 kg N ha^{-1}; larger biomasses (20–45 kg N ha^{-1}) are recorded only in experimental microplots or in inoculum production plots [28, 68, 69]. The theoretical maximum BNF by cyanobacteria can be calculated by assuming that the photosynthetic aquatic biomass is composed exclusively of N_2-fixing cyanobacteria (C:N = 7) and primary production is 0.5 t C ha^{-1} $crop^{-1}$. This would be equivalent to 70 kg N ha^{-1} $crop^{-1}$.

Possible beneficial effects of cyanobacteria other than provision of N include (1) competition with weeds, (2) increased soil organic matter content and aggregation, (3) excretion of organic acids that increase P availability to rice, (4) decrease of sulphide injury in sulfate reduction-prone soils by increased O_2 content and plant resistance to sulfide, and (5) production of PGR that enhance rice growth [64]. But this last aspect still needs to be demontrated because cyanobacteria extracts may also negatively affect rice germination [53], and despite the numerous reports on algal PGR effects, none shows the isolation and characterization of a microalgal PGR [46].

Algal inoculation technology and its current status

Experimental cyanobacteria inoculation (algalization) of ricefields initiated in Japan [88] was subsequently abandoned there. Applied research on algalization has been conducted mostly in India and, to a lesser extent, in Burma, Egypt, and China. A similar technique of inoculum production in shallow open-air ponds is used in India, Egypt, and Burma [84]. A multi-strain starter inoculum produced from laboratory cultures is propagated, on the spot, in trays or microplots with 5–15 cm water, about 4 kg soil m^{-2}, 100 g superphosphate m^{-2}, and insecticide. If necessary, lime is added to adjust soil pH to 7.0–7.5. In 1–3 weeks, an algal mat develops which is then allowed to dry. Algal flakes are collected for further use at 10 kg ha^{-1}.

Table 3 presents the analysis of 634 field experiments of algalization. The difference in yield between inoculated and noninoculated plots was very variable (C.V. > 100%). Because of the asymmetrical data distribution, the median grain yield (257 kg ha^{-1}) was considered a better index of the average effect of inoculation than the mean (337 kg ha^{-1}) [62]. While the difference in average yield between inoculated and noninoculated plots was significant at $p < 0.01$, only 17% of the 634 individual observed differences were statistically significant. This indicates a small and variable response of yield to inoculation and also an experimental error frequently larger than the response. When interpreting data from the literature, it should also be kept in mind that unsuccessful trials have often not been reported. When they were mentioned, it was usually without quantitative data that could explain the possible reason for failure. For example, a report of a multilocation trial [54] indicates that notwithstanding the 22 sets of data presented, "the results from many other locations in South India, Deccan, and the Konkan region were not received because of the failure of multiplying cyanobacteria at these locations."

Cyanobacteria inoculation is currently used on a trial-and-error basis. Methods to estimate the chance of success of inoculation in a given agroecosystem are unavailable because the factors underlying yield increases associated with successful algal inoculation are not clearly understood or quantified. No published study reporting a significant increase in yield after cyanobacterial inoculation includes estimation of inoculum quality, BNF measurement, or biomass estimates.

Reports on the adoption of algal inoculation are somewhat controversial, but even with the most optimistic evaluations, adoption seems to be restricted to a very limited area in a few Indian states, in Egypt, and possibly in Burma [71]. Farmers' limited acceptance of algalization probably reflects the low and erratic increases in yield obtained.

Prospects

Methods for utilizing cyanobacteria in rice cultivation need to be reconsidered in view of the following results of the agroecological studies of the last decade:

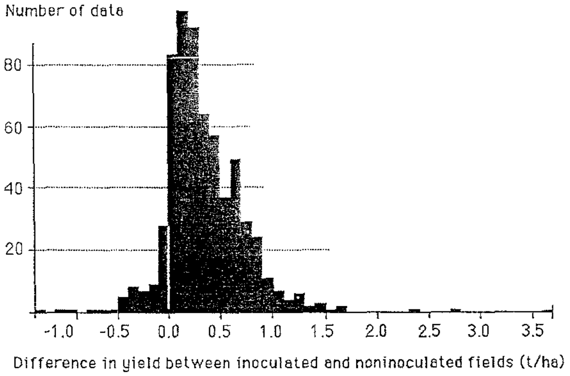
(1) N_2-fixing cyanobacteria are ubiquitous in ricefields.
(2) The study of the ratio of indigenous heterocystous cyanobacteria in 102 soils to those contained in the recommended dose of 22 soil-based inocula (10 kg

Table 3. Bibliographic study of the effect of cyanobacterial inoculation on rice yield* (adapted from [62])

1. Major statistics of the data

	Difference between control and inoculated plots	
	Absolute (k ha^{-1})	Relative (%)
Number of observations	634	634
Maximum	3700	168.2
Minimum	−1280	−19.3
Average	337	11.3
Median	257	7.9
Standard deviation	398	16.0
Coefficient of variation	118	141

2. Histogram of the data



*Data compiled from 41 references listed in [62].

ha^{-1}) showed that in 90% of the cases, indigenous cyanobacteria were more abundant than those in the inoculum [68].

(3) Results also show the infrequent establishment of nonindigenous strains inoculated in various soils, even when grazers were controlled [24, 58, 59]. While cyanobacteria inoculated in five soils persisted for at least 1 month, their growth was rare (1 out of 10 cases). Blooms developed on all soils when grazers were controlled, but were mostly of indigenous strains [58].

This suggests that attention should be paid to practices that enhance the growth of indigenous strains already adapted to the environment. Their growth is most commonly limited by low pH, P deficiency, grazing, and broadcasting of N-fertilizer. Cultural practices that alleviate limiting factors (liming, P application, grazer control, and N-fertilizer deep-placement) favor photodependent BNF and cyanobacteria growth, but their economic feasability is often low.

Liming is rarely economically feasible. The efficiency of P fertilizer (kg N fixed per kg P applied) is usually low (2.3 g N g^{-1} P) [13]. Split P application is more efficient than basal application [28]. Grazer control can be achieved with conventional pesticides [24] but their cost is prohibitive for the result achieved. Pesticides of plant origin might be more economical. Broadcast application of N-fertilizer, which is widely practiced by farmers, inhibits photodependent BNF and causes N-losses by ammonia volatilization [21, 65]. In contrast, the deep-placement of N-fertilizer decreases its inhibitory effect on cyanobacteria and reduces N-losses by volatilization. Delaying N-fertilizer application could also possibly allow the growth of a N_2-fixing algal bloom at the early stages of the crop [99], but the resulting effects on N- losses from fertilizer applied in an algal-rich water are unknown.

The ubiquity of N_2-fixing cyanobacteria in rice soils does mean that inoculation is unnecessary. Inoculating fields with indigenous strains which are able to establish might be useful because P accumulation by the inoculum (produced with high levels of P) gives it an initial advantage over the indigenous propagules, which are usually P-deficient [69]. Since spore germination is photodependent [57], propagules applied on the soil surface might germinate better than the indigenous ones mixed with the soil. Inoculation with indigenous strains is likely to be useful after an upland crop grown before rice or after a long dry fallow because the low density of natural population density may lead to a lag of several weeks before N_2-fixation becomes significant. In fact, the positive effects of cyanobacteria inoculation observed with the method recommended in India could be due to indigenous strains, because when inoculum is multiplied on the spot in shallow trays or plots, it is probable that local strains present in the soil may outgrow the original isolates even before inoculum is added to the field.

In the absence of knowledge on factors that allows foreign strains to establish in a field, the agronomic potential of cyanobacterial inoculation is probably limited to indigenous strains used in agroecosystems favorable to cyanobacterial growth, when inoculation allows to accelerate the formation of an N_2-fixing bloom early in the crop cycle. Soil properties, climatic conditions and cultural practices needed for such conditions to occur probably limit the usefulness of cyanobacterial inoculation to a small percentage of the world's ricefields.

Azolla

Azolla is an aquatic fern which harbors the symbiotic N_2-fixing cyanobacteria *Anabaena azollae*. *Azolla* usually needs to be inoculated and grown when used as green manure. Its use dates back to the 11th century in Vietnam and the 14th century in China [44]. The N_2-fixing symbiont of *Azolla* was identified by Strasburger in 1873 but progress in *Azolla* biotechnology (i.e. recombination and sexual hybridization), is recent [38, 39, 97].

Potential as a biofertilizer for rice

BNF by *Azolla* has usually been estimated from biomass measurement and the assumption that most of *Azolla* N originates from BNF. Recent measurements show an average Ndfa of 75% in *Azolla* [96]. The maximum N potential of *Azolla*, calculated from biomass recorded in experimental plots and assuming that two crops of *Azolla* with a Ndfa of 80% are grown per rice crop is 224 kg N^{-1} crop $cycle^{-1}$. But, in a field trial at 37 sites in 10 countries, productivity was lower than in experimental plots [91]. 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). Comparisons with inorganic fertilizer showed that one *Azolla* crop incorporated before or after transplanting was equivalent to the application of 30 kg N ha^{-1} as urea.

Besides providing N to the rice crop, *Azolla*, has several other advantages. Because of its lower K absorption threshold in floodwater than rice, *Azolla* becomes a source of K for rice when incorporated [41]. *Azolla* also enhances the utilization of P fertilizer [74], decreases weed incidence [15, 44], reduces water evaporation [16], and improves soil structure, which is important where rice is grown sequentially with an upland crop [73].

Current usage

Estimates of the extent of *Azolla* use show a marked decrease during the 1980s in China and Vietnam, where it had been a traditional technology. Estimates in China are 6.5 million ha before 1978 [19], 1.34 million ha in 1979 [40], 0.7 million ha in 1982 [44] and a decrease in use was still reported in 1987 [41]. This has been attributed to the availability of cheap sources of urea and potash, and changing governmental economic policies leading to the disbanding of many agricultural communes and the reallocation of labor [73]. In Vietnam *Azolla* was used in about 500,000 ha in 1980 [70]; since then, its use has also continuously decreased.

During the 1980s, *Azolla* was tested for adoption in Brazil, India, Pakistan, the Philippines, Senegal, Sri Lanka, and Thailand. The Philippines is the only country were adoption was sufficient to be quantified during the 1986 *Azolla* Workshop [29]. In this country, farmers adopted *Azolla* on 5,000 ha in South Cotabato in 1981 [32]; success was due mainly to a high level of available P in the soils and a short dry season. *Azolla* utilization extended to 26,000 ha in 1983, and 84,000 ha in 1985 [45]. Since then, *Azolla* use has not progressed in the Philippines and has probably decreased.

Limiting factors for Azolla use and possible remedies

Water control and maintenance of inoculum

Azolla cannot withstand desiccation and requires standing water throughout its growth cycle. Because *Azolla* is propagated vegetatively, inoculum must be maintained in nurseries year-round and multiplied for distribution before field cultivation. Therefore an irrigation network and a network for inoculum conservation, production, and distribution are prerequisites for *Azolla* use. This also implies that *Azolla* adoption by farmers first depends on a government policy to establish such networks. Problems in inoculum conservation, multiplication, and transport could be solved if *Azolla* could be propagated from spores. A method for utilizing sporocarps for inoculum conservation has been developed in China but the growth of sporophytes was too slow to meet the inoculum requirement in the field; 160 kg fresh weight ha^{-1} sporocarps yielded 16 to 21 t fresh weight ha^{-1} of *Azolla* in 52 days [42]. Conditions for sporocarp formation and germination are incompletely understood [98].

Need for P-fertilizer

Reported threshold values of P deficiency are 0.4% dry weight in *Azolla* and 20 ppm available Olsen P in soil [2]. Such P-rich soils are uncommon. A growth test on 972 Philippine soils showed that only 13% of the samples were highly suitable for *Azolla* growth and P fertilization would be required in most soils [11]. This was confirmed by the observation that 80% of field-grown *Azolla* were P deficient ($p < 0.4\%$). To be economically feasible, P-fertilization requires a ratio of N fixed to P applied that is greater than the ratio of the prices of the corresponding fertilizers (about 3 for most countries). Basal P application has a low efficiency and may be economically infeasible, while split application has an efficiency of 5–10 g N_2 fixed per g P applied [93]. P-fertilization limited to the inoculum production plot permits the P-enriched *Azolla* to multiply 6 to 7 times without P application in the main field and ensures a high efficiency of P applied [93].

Pests

Although commercial pesticides effectively control *Azolla* pests, their application is not economically feasible in the field [28] and should be limited to inoculum production [47]. Pesticides of plant origin might be economically feasible for field use. Alternate drainage and irrigation, cultivation in wet fields or with a thin water layer, and reduced application of organic manures, may help controlling pests [102].

Temperature requirement

The optimum temperature for most *Azolla* species (20–30 °C) is below the average temperature in the tropics [43]. Cool weather is a key to successful *Azolla* cultivation in Vietnam and China. Temperature limitations can be reduced by selecting cold- or heat-tolerant strains. Among strains tested at IRRI, *A. microphylla* #418 was most tolerant of high temperature (37 °C day/29 °C night) [94].

Economics

Technologies used in Vietnam and China are labor intensive and therefore have economic limitations. In South Cotabato (Philippines) where *Azolla* spread spontaneously and no P-fertilizer and little labor were needed, economic return from *Azolla* adoption, including cost savings in chemical fertilizers and weed control, was more than US$ 35 ha^{-1} at 1981 prices [32]. However, conditions in the study area were exceptionally favorable and should be viewed realistically. When conditions for *Azolla* growth were not favored by an exceptionally high level of available P, economics were not in favor of *Azolla* use [72]. The economic potential of *Azolla* is greatest where the opportunity cost of labor is low, and labor cost becomes critical where wage rates approach US$ 2 day^{-1} [32]. It is clear that a case study in the Philippines is not enough to allow definite conclusions regarding *Azolla* economics, which may vary according to socio-agricultural systems.

Prospects

Azolla has a N-potential similar to that of legumes but is easier to incorporate and grows well with rice in flooded conditions. Environmental, technological, and economic factors limit its use. Problems in inoculum conservation, multiplication, and transport could be solved if *Azolla* could be propagated from spores. Temperature limitations and requirements for P can be reduced by selecting cold- or heat-resistant strains with low P requirements, and by split application of P-fertilizer, limited or not to inoculum production.

Azolla strains exhibit a wide range of behavior with regard to environmental factors, P requirement, N_2 fixation, and productivity, The ability to combine favorable characters such as resistance to high temperature and pests, low P requirement, and erect growth (permitting higher productivity) would allow strains to be designed for specific conditions. For this purpose, recombination of algal and plant symbionts [38, 39] and sexual hybridization [97] between *Azolla* species proved feasible. The IRRI biofertilizer germplasm contains 23 hybrid strains obtained by algal transfer and 85 obtained by sexual hybridization, some of which exhibit improved characters [94]. However, hybrid formation requires that macro- and micro-sporocarps be obtained and no method has been yet designed to induce sporulation at will. This is a major limiting factor for *Azolla* hybridization.

The key economic costs in *Azolla* use are those of P application, labor, and pest control. Economic limitations are important and need further evaluation but calculations should also consider the long-term benefits of *Azolla* on soil fertility. *Azolla* has potential not only as a green manure but as a multipurpose biofertilizer that can be a weed suppressor, a mineral scavenger – especially for K –, an animal feed, a primary producer in rice-fish-*Azolla* culture [20] and a depollutant. This potential may renew interest in *Azolla* use [41].

Legume green manures

A broad range of legume green manures (LGM) has been used in rice cultivation (Table 4). Potentialities of LGM for rice were early recognized. In 1936 the International Institute of Agriculture reported that: "application of green manure may involve great progress in rice growing by ensuring yields higher than those at present attained". Similar statements were recorded from the proceedings of symposia in 1952, 1953 and 1954 [51]. Afterwards, it seems that less attention was paid to LGM in rice production. They were mentioned in only a few paragraphs of the proceeding of the symposium on "Nitrogen and Rice" held in 1979 at IRRI. The discovery of stem-nodulating legumes [18,1] able to grow, fix N_2, and develop large biomasses under waterlogged conditions; and the concern for agricultural sustainability, have renewed the interest of the scientific community in LGM for wetland rice. It is significant that a symposium at IRRI in 1987 was devoted to green manure in rice farming [30].

Potential to increase rice yield

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 traditional LGM crop [70] average 114 kg ha^{-1} (Table 3). Those values were most often for a crop grown until maturity, which is rarely done with a LGM. More recent values (Table 4) show that in 30–45 days a LGM accumulates 7 to 143 kg N ha^{-1} (mean 63). Values published after 1985 average 133 kg N ha^{-1} [37]. Ranges in kg N ha^{-1} are 40–225 for stem nodulating 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.

Beside N and other nutrient provision to the crop, the beneficial effects of LGM incorporation that have been reported include:

- improvement of soil properties, especially (1) total N and organic matter content, (2) available Zn, (3) water holding capacity, and (4) soil aggregation [7, 8];
- control of some rice pests, in particular nematodes [56]; and
- immobilization of nitrogen nitrified during dry fallows, that would otherwise be lost by denitrification when soils are reflooded.

Estimates of yield increase due to traditional LGM range from 30 to 100 kg grain per ton of LGM incorporated [70].

Current utilization

Despite their potential, a setback in use of leguminous green manures has been observed during the last decade. China is the only country where LGM are still noticeably used, but data show a decrease from 10 million ha in 1974 to less than 4 million ha in 1990 [79]. In other countries, usage of LGM seems to have become incidental [70]. In several rice growing areas of India 20-30% of soils were planted to LGM at mid century [78], then green manuring exhibited a strong setback with the increase in cropping intensity and the low cost and ready availability of fertilizer [85].

Table 4. N accumulated by legumes used as green manure in rice cultivation

1. Crops grown until maturity (reproduced from [70])

Species	Nitrogen accumulated	
	(kg ha^{-1})	(% fresh weight)
Astragalus sinicus	108–123	0.35–0.47
Canavalia ensiformis	98	0.47
Cassia mimosoides	97	0.44
Crotalaria anagyroides	98	0.33
Crotalaria juncea	105–129	0.30
Crotalaria quinquefolia	88	0.19
Dolichos biforus	89	0.58
Gycine koidzumii	71	0.42
Phaseolus sp.	-	0.28
Phaseolus lathyroides	90	-
Phaseolus calcaratus	42	0.22
Sesbania aculeata	96–122	0.32–0.36
Sesbania rostrata	267	-
Sesbania sesban	100–202	0.39
Sesbania microcarpa	87	0.50
Sesbania sirececa	146	-
Average	114	0.37

2. N accumulate in crops grown for definite time (calculated from data tabulated in [10])

	Dry weight	N accumulated	
	(t ha^{-1})	(kg ha^{-1})	(kg ha^{-1} day^{-1})
110 crops of 16 species grown for 30 to 178 days (average 52 days)			
Mean	4.3	99	1.9
Maximum	13.3	267	5.1
Minimum	0.2	7	0.2
32 crops of 11 species grown for 30 to 45 days (average 40 days)			
Mean	2.5	63	1.6
Maximum	6.7	143	3.2
Minimum	0.2	7	0.2

Limiting factors

Reasons for decline and constraints associated with LGM use appear in several reviews [7, 22, 70]. Some detrimental effects have been reported, mainly in temperate condition [90]. One important limiting factor is the bulkiness of the LGM and the resulting problems for incorporation. Nitrogen content in legumes varies from 0.2 to 0.6% therefore the fresh weight corresponding to 50 kg N varies from 10 to 26 tons. Incorporation of such a biomass requires draft and/or man power, which are often not available. There are also incidental reasons. For example, in certain areas of India indiscriminate cattle grazing due to inadequate social control explain farmer reluctance to raise a green manure crop [85].

However, major limitations are socio-economic. First, it is clear that LGM are not appealing because they do not yield food or cash directly. Then there are many economical limitations. In situations where

N fertilizer is available, assuming an average yield of 15 kg grain per kg N applied, the cost of inorganic N fertilizer relative to the price of rice is very favorable. Furthermore, many governments have adopted a fertilizer subsidy policy and made cheap credit available for farmers to buy N fertilizer. On the other hand, the costs of green manure seed and land preparation are not favorable. If there is residual moisture in the soil, after the harvest of the rice crop, the economic advantage is very often in favor of growing a cash crop of legumes, groundnut, maize, millet, onion etc. and to resort to application of inorganic fertilizers for the next rice crop [85].

The situation where N fertilizer is not available is most frequently that of subsistence farmers, with small land holdings, who cannot afford to release land used for food or forage crops to LGM cultivation and therefore prefer to grow a catch crop. In some areas where no N fertilizer is available and organic manure was traditionally applied to rice, green manure is now applied preferentially to vegetable cash crops rather than to the rice crop.

Furthermore, the increased availability of inorganic fertilizers and low emphasis on GM by research and extension have contributed to the decline in LGM use.

Prospects

The sustainability issues together with the discovery of stem-nodulating legumes have revived scientific interest in LGM. The formation of stem nodules has been reported in 25 species of the genera *Sesbania* and *Aeschynomene*. Stem-nodulating legumes exhibit adaptation to waterlogged or water-saturated conditions of growth. Data on N accumulated in 40–55 days show a higher N potential of stem-nodulating LGM than of traditional LGM. Several studies have shown the very high N_2-fixing potential of *Sesbania rostrata* and *Aeschynomene afraspera* [6, 37, 49, 52, 60,]. ^{15}N dilution and ∂ ^{15}N studies showed that Ndfa of 45–55 day-old *S. rostrata* was about 70% and increased to 90% at 65 days. Field experiments show that stem-nodulating LGM offer a better N potential for wetland rice than traditional LGM. In experimental plots, a gain of 267 kg N ha^{-1} was reported after incorporating a 52-d crop [60]. In a 45-d *Sesbania*-rice (WS)/55-d *Sesbania*-rice (DS) sequence, *Sesbania* fixed 303 kg N ha^{-1} $year^{-1}$ when uninoculated, and 383 kg N when inoculated with *Azorhizobium* [37]. Incorporating one 40–60 day old culture of *Sesbania* or *Aeschynomene* may increase yield by 1–3 t ha^{-1} [86, 87], which is equivalent to the application of 50 to 100 kg fertilizer N ha^{-1} [7, 37, 87].

Photoperiod sensitivity limits the utilization of *S. rostrata* while *A. afraspera* seems to be considerably less photoperiod sensitive [6]. The non availability of seeds of stem-nodulating legumes is currently the major limiting factor for their adoption by farmers. But many of the socioeconomic factors that limit the use of traditional LGM use also will limit that of stern-nodulating LGM.

Conditions for economical use of LGM are that (1) there is no alternative for a more profitable crop, (2) legume establishment does not require soil preparation and is inexpensive, (3) LGM productivity is stable in time, (4) manpower and adequate implements are available for incorporation, and (5) there is no concurrence with manpower needed for rice transplanting [22]. In particular, N_2 fixation by a LGM (and conservation of NO_3 mineralized during the dry season) may be an economically viable proposition if production costs can be kept low, and if the LGM does not compete with marketable or subsistence crops.

Summary and conclusion

Nitrogen is usually the limiting factor to high yields in rice fields. Therefore, the use of BNF as an alternative or supplementary source of N for rice has been the major approach in microbiological management of wetland rice. Whereas N_2-fixing green manures have been used for centuries in some rice-growing areas, research on cyanobacterial and bacterial inoculants for wetland rice is relatively recent, being initiated in the early 1950s for free-living cyanobacteria and in the 1960s for rhizosphere bacteria.

Currently, most bacterial strains tested for inoculation have been N_2-fixing forms, but ARA, ^{15}N, and N balance studies have not provided clear evidence that the promotion of rice growth and N uptake was due to increased BNF. Therefore, several authors refer to the production of PGRs to explain the beneficial effect of bacterial inoculation. No experiment has yet supported this hypothesis. If it is verified that the ability of inoculated strains to produce PGRs is more important than their N_2-fixing ability, it is clear that the screening of bacterial strains for inoculation should not be limited to N_2-fixing strains. The few data available on strain establishment showed that, in most cases, inoculated strains disappeared or established themselves for various periods of time, but did not multiply. That would limit the effect of inoculation to the earlier growth stage of the plant, a hypothesis that agrees with the absence of an inoculation effect on harvest index. Given the current knowledge, no definite conclusion regarding the potential of bacterial inoculation of rice can be drawn.

The selection and breeding of rice according to the variety's ability to stimulate an associative microflora that promotes BNF and soil N utilization is still limited by the absence of an efficient screening method. The relatively low N_2 fixation potential of associative BNF is not a hindrance to this promising approach, whose major advantage is that the N potential is inherent to the plant and thus requires no additional cultural practice by the farmer.

Free-living cyanobacteria have a moderate potential of about 30 kg N ha^{-1} crop $cycle^{-1}$ which may translate to a yield increase of 250–350 kg ha^{-1}. However, the technology for cyanobacterial inoculation has not progressed beyond the experimental stage of large-scale field testing. As long as inoculation is applied on a trial-and-error basis, it will have little chance of success. Recent developments indicate that foreign strains usually do not establish and more attention should be paid to promoting indigenous strains, which are ubiquitous. Cultural practices to enhance their growth are known but environments where those practices can be efficient and economically viable are probably limited. In the long term, genetic engineering may contribute to cyanobacteria management in wetland rice fields, but it is not yet known if and how engineered strains can establish and/or compete with the indigenous microflora. In-depth agroecological research is required before cyanobacteria technology can be substantially improved.

Azolla has proved useful as a N biofertilizer in some rice-growing countries. Like legumes it has a high N potential, but it is easier to incorporate and grows well with rice under flooded conditions. Environmental, technological, and economic factors limit *Azolla* use. Recent progress in strain hybridization and recombination has opened new ways to alleviate some environmental and nutritional limitations. Socioeconomic limitations are important and are probably increasing, as shown by the setback of *Azolla* in China and Vietnam where it was traditionally used. However, recent studies have shown that *Azolla* has potential not only as a green manure but as a multipurpose biofertilizer. These limiting factors and the potential of *Azolla* as a multipurpose crop, which may revive interest in its use, will decide the extent of its future utilization.

Leguminous green manures have traditionally been used in many rice-growing countries. Estimates of N accumulated in a traditional prerice LGM crop range from 42 to 202 kg N ha^{-1}. Despite this potential, a strong setback of LGM use has been observed during the last decades. In countries other than China, the use of LGM seems to have become incidental, mostly because of socioeconomic limitations. LGM are not appealing because they do not yield food or cash directly. Where N fertilizer is available, green manuring is usually more expensive than inorganic N. Situations where N fertilizer is not available concern mostly subsistence farmers, with small holdings, who cannot afford to release land used for food or forage crops to LGM and prefer to grow a cash crop.

During the last decade the discovery of the high N_2-fixing potential of some flood-resistant stem-nodulating legumes has revived the interest of rice scientists in LGM. Field experiments show that stem-nodulating LGM offer a better N potential for wetland rice than traditional LGM do. However, many of the socioeconomic factors that limit the use of traditional LGM use also will limit that of stem-nodulating LGM. N_2 fixation by a LGM (and conservation of NO_3 mineralized during the dry season) may be an economically viable proposition if production costs can be kept low, and if the green manure does not compete with marketable or subsistence crops.

BNF in rice fields has been the most effective system for sustaining production in low-input traditional cultivation. The general impression when considering the current management of N_2-fixing organisms in ricefields is that 40 years after the first inoculation experiments, the agronomic potential of BNF is underutilized and its intentional use is decreasing.

Considering that rice obtains most of its N from the soil, regardless of the amount of chemical N fertilizer applied, concerns in recent high-input, intensive rice cultivation are (1) sustainability of high yields and (2) the possible environmental impacts of intensive management on soil fertility. Knowledge on this aspect is still limited, but the key roles of the rhizosphere, the photosynthetic aquatic biomass, and their N_2-fixing components in maintaining the fertility of rice soils under intensive cultivation have been recognized and need further study [92].

An additional 300 million tonnes 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 [31]. But increased rice production should not be at the expense of future generations and should fulfill the concept of sustainability. A major challenge is managing pests and nutrients in ways that reduce agrochemical use. Increased use of inorganic fertilizer is inescapable, but, as pointed out by Postgate (1989) [55], a parallel return to greater exploitation of BNF, still responsible for providing 60–70% of the new N in the biosphere, seems common sense. Currently, BNF is intentionally used in only a few percent of the global rice-growing area. Designing economically viable methods for utilizing N_2-fixing organisms in rice cultivation still remains a major scientific challenge.

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