

22 N₂-fixing Cyanobacteria as Biofertilizers in Rice Fields

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Cyanobacteria constitute the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes (Stanier & Cohen-Bazire, 1977). N₂-fixing forms contribute by maintaining the fertility of natural and cultivated ecosystems. Currently, research on their agronomical use has almost exclusively focussed on wetland rice. In the 110 000 000 ha of wetland ricefields, N₂-fixing cyanobacteria occur as indigenous ubiquitous free-living organisms. Research on ricefield inoculation with cyanobacteria was initiated in Japan by Watanabe *et al.* (1951). N₂-fixing cyanobacteria also occur in symbiosis with the aquatic fern *Azolla*, which has been used as green manure for rice since the eleventh century in Vietnam and the fourteenth century in China (Lumpkin & Plucknett, 1982). *Azolla* biotechnology by recombination and sexual hybridization is recent (Wei *et al.*, 1986; Lin *et al.*, 1988).

22.1 Free-living cyanobacteria

22.1.1 Ecology in ricefields

Contrary to early beliefs, N₂-fixing cyanobacteria are ubiquitous in ricefields. Counts in 396 soils of ten countries ranged from 10 to 10⁷ g⁻¹ dry soil (median: 2 × 10⁴) (Roger *et al.*, 1987). In ricefields, cyanobacteria are subjected to large diurnal and seasonal variations in insolation (0–120 k lux), temperature (5–60°C), pH (5–10), O₂ concentration (0–30 ppm), and nutrient status. In arid tropics, high light intensities and temperatures may inhibit their growth at the beginning of the crop cycle. In wet monsoonal zones, they may develop early in the crop cycle during the dry/warm season, but can be inhibited during the wet season because of light deficiency and disturbance by heavy rain. The abundance of N₂-fixing forms is positively correlated with soil pH and available P (Roger, 1996).

The major biotic factor limiting cyanobacteria growth is grazing by invertebrates, mostly ostracods, mosquito larvae, and snails. A dense ostracod population may ingest 34 kg ha⁻¹ d⁻¹ (dry weight) of cyanobacteria, ingesting about 1.7 kg N and excreting 1 kg N (Grant *et al.*, 1986). Strains forming mucilaginous colonies being less susceptible to grazing than non-colonial strains, grazing leads to the dominance of mucilaginous cyanobacteria,

especially *Nostoc* spp. (Roger *et al.*, 1987), which are often less active in biological N₂-fixation (BNF) than non-colonial forms (Grant *et al.*, 1986).

Mineral N is known to inhibit BNF by cultures of cyanobacteria. *In situ* inhibition is less marked and depends upon the method of fertilizer application. Nitrogen broadcasting, widely practiced by farmers, often strongly inhibits photodependent BNF and also causes N losses by ammonia volatilization. In contrast, deep placement reduces the inhibitory effect of N fertilizer on cyanobacteria and decreases N losses by volatilization (Roger, 1996). Phosphorus is a key nutrient for ricefield cyanobacteria, which are often P deficient (Roger *et al.*, 1986). Its application stimulates photodependent BNF and cyanobacteria growth, especially in acid soils.

Cyanobacteria are often more resistant to pesticides than eukaryotic algae, which leads to a selective effect by some algicides and insecticides, which promote cyanobacteria growth. Insecticides inhibit invertebrates that feed on algae (grazers), thus further promoting cyanobacteria and photodependent BNF. However, over the long term, insecticide use becomes detrimental to cyanobacteria by decreasing the diversity of aquatic invertebrates and causing grazers resistant to conventional pesticides to proliferate. Among pesticides other than algicides, herbicides are the most detrimental to cyanobacteria (Roger, 1996).

22.1.2 Free-living cyanobacteria as a source of N

Cyanobacteria can develop impressive blooms. Standing crops of 5–20 ton fresh weight per hectare are usually recorded for growth visible to the naked eye. But because of the low dry matter (0.2–14%) and high ash (31–71%) content, a bloom usually represents less than 10 kg N ha⁻¹ (Roger *et al.*, 1986).

Biological N₂-fixation by cyanobacteria has been mostly estimated from acetylene reducing activity measurements. Published data vary from a few to 80 kg N ha⁻¹ crop⁻¹ (mean: 27 kg). Crop cycle measurements in 180 experimental plots at International Rice Research Institute (IRRI) 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 with deep-placed N (Table 22.1), thus demonstrating the inhibitory effect of N-fertilizer broadcasting on cyanobacteria. A compilation of 211 balance studies in rice soils indicated that with no N-fertilizer applied, BNF averaged 31 kg N crop⁻¹, among which two-third was attributable to cyanobacteria. The theoretical maximum

Table 22.1. Nitrogen fixation during the crop cycle and rice yield under different urea management practices^a. (adapted from Roger & Ladha, 1992).

Urea applied (kg N ha ⁻¹)	Estimated N fixed (kg N ha ⁻¹ crop ⁻¹)	Grain yield (t ha ⁻¹)
0 (control)	20 ± 0.14	4.1 ± 0.1
38 at transplanting +17 at panicle initiation	8 ± 0.13	4.8 ± 0.1
55 deep-placed at transplanting	12 ± 0.16	5.8 ± 0.1

^a Each value is the average of 60 measurements.

BNF by cyanobacteria in ricefields can be calculated from reported productivity measurements of the photosynthetic aquatic biomass in floodwater (0.5–1.0 g C m⁻² d⁻¹). Assuming a biomass composed exclusively of N₂-fixing cyanobacteria (C/N:7) and a primary production of 0.5 t C ha⁻¹ crop⁻¹, cyanobacteria could theoretically provide up to 70 kg N ha⁻¹ crop⁻¹ (Roger & Ladha, 1992).

22.1.3 Effects on rice

Most rice-N originating from cyanobacteria is made available to the crop through mineralization after their death, because N excreted during the buildup of a bloom is either re-immobilized or lost by NH₃ volatilization. Depending on the nature of the material (fresh vs dried), the method of application, and the presence or absence of soil fauna, recovery of cyanobacteria N by rice varies from 13 to 50% (Roger, 1996). Recovery was highest with fresh cyanobacteria incorporated into a soil depleted of fauna. It was lowest with dried cyanobacteria applied on the surface of a soil rich in tubificid worms, which reduce the recovery of algal N by rice by making more soil N available through mineralization. Late decomposition of the bloom during the cycle and the resultant late N-availability to rice increases N content of the grain without increasing yield (Grant *et al.*, 1986).

Other possible beneficial effects of cyanobacteria on rice include (1) competition with weeds, (2) increased soil organic matter content, (3) excretion of organic acids that increase P availability to rice, (4) inhibition of sulfide injury in sulfate-reduction-prone soils by O₂ production, (5) increased water temperature, and (6) possible production of plant growth regulators (PGR) (Roger, 1996). However, despite many claims that cyanobacteria can benefit rice plants by producing PGRs, this aspect is still controversial (Pedurand & Reynaud, 1987) and no study shows the isolation and characterization of a cyanobacterial PGR (Metting & Pyne, 1986).

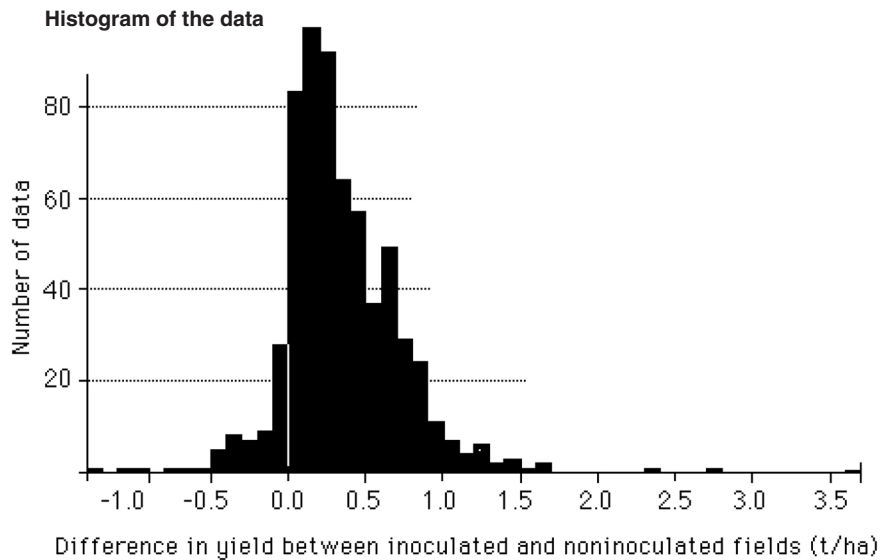
22.1.4 Inoculation of ricefields with cyanobacteria

Applied research on cyanobacteria inoculation has been conducted mostly in India, where the All-India Coordinated Project on Algae was initiated in 1977 and, to a lesser extent, in Burma, Egypt, and China. Inocula are usually produced from a multistrain starter inoculum produced from laboratory cultures that is propagated on the spot, in open-air trays or microplots with 5–15 cm water, about 4 kg soil m⁻², 100 g superphosphate m⁻², and insecticide. When necessary, lime is added to adjust pH to 7.0–7.5. In one to three weeks, an algal mat develops, which is allowed to dry. Algal flakes are then collected and stored for use at 10 kg ha⁻¹ (Venkataraman, 1981).

A compilation of 634 field experiments reported in the literature and in technical reports (Table 22.2) showed a very large variability of the yield difference between inoculated and non-inoculated plots (CV: >100%) (Roger, 1996). Because of the strong asymmetrical data distribution, the median (257 kg ha⁻¹) was considered a better index of the average effect of inoculation than the mean (337 kg ha⁻¹). While the difference in average yield

Table 22.2. Bibliographic study of the effect of cyanobacterial inoculation on rice yield* (adapted from Roger, 1996).

	Difference between control and inoculated plots	
	Absolute	Relative
Major statistics of the data		
Number of observations (n)	634	634
Maximum	3700 kg ha ⁻¹	+168.2%
Minimum	-1280 kg ha ⁻¹	-19.3%
Average	337 kg ha ⁻¹	11.3%
Median	257 kg ha ⁻¹	7.9%
Standard deviation	398 kg ha ⁻¹	16.0%
Coefficient of variation	118%	141%



between inoculated and non-inoculated 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 algal 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 were often not reported as indicated in some reports and confirmed by the asymmetrical distribution of the data. No published study reporting a significant increase in yield after algal inoculation includes BNF or cyanobacteria biomass estimates.

Reports on the adoption of algal inoculation are controversial, but even with the most optimistic evaluations, adoption seems to have been restricted to a limited area in a few states in India, in Egypt, and possibly in Burma. Currently, inoculation of ricefields with cyanobacteria is still not a technology adopted by farmers, which probably reflects the low and erratic increase in yield obtained.

22.1.5 Reconsidering cyanobacteria as a biofertilizer for rice

Methods for utilizing cyanobacteria in rice cultivation need to be reconsidered in view of studies showing that: (1) N_2 -fixing forms are ubiquitous; (2) their growth is most commonly limited by low pH, P deficiency, grazing, and broadcasting of N fertilizer; (3) non-indigenous strains inoculated in various soils rarely establish themselves (Reddy & Roger, 1988); and (4) indigenous N_2 -fixing cyanobacteria are frequently more numerous than cyanobacteria contained in the recommended dose of 10 kg of soil-based inoculum (Roger *et al.*, 1987). More attention should be paid to cultural practices alleviating factors that limit growth and BNF by indigenous strains (liming of acidic soils, P split application, grazer control, and deep placement of N-fertilizer). These practices might suffice to realize more of the potential of indigenous cyanobacteria and are a prerequisite for establishing inoculated strains, if and when inoculation is needed.

Inoculation might be useful because the accumulation of P by the propagules of the inoculum (produced with high levels of P) gives them an initial advantage over the propagules of the indigenous cyanobacteria, which are usually P-deficient. Because spore germination is photodependent, inoculated propagules applied on the soil surface should germinate more readily than the indigenous propagules mixed with the soil. The effect of inoculation is likely to be more significant when indigenous populations have been decreased by an upland crop grown before rice or a long dry fallow. Inoculation might also permit the early establishment of a N_2 -fixing bloom and thus the availability of more fixed N to rice. Available data clearly suggest that use of an inoculum produced from the soil to be inoculated should be tested whenever experiments are conducted. Local strain inoculation seems especially advisable after a long dry fallow which often results in partial sterilization in very warm climates. In that case, inoculation should be combined with either deep placement or delayed application of N-fertilizer.

One can speculate on the possibility of selecting for or designing efficient strains for inoculation. Several authors have screened for high N_2 -fixing activity, but there is little reason *per se* why high N_2 -fixing activity should correlate with *in situ* colonization potential. In fact, most fast-growing strains (doubling time of 5–12 h) belong to the genus *Anabaena*, have short filaments, and are therefore susceptible to grazing. Another approach has been the selection of nitrogenase-derepressed mutants which excrete NH_4^+ into the medium. Such an *Anabaena variabilis* mutant was found to provide N to rice in a N-free gnotobiotic culture more efficiently than the parent strain (Latorre *et al.*, 1986). However, studies at IRRI showed that the strain was not competitive and did not multiply when inoculated in soil (Roger *et al.*, unpublished).

Biological engineering of cyanobacteria is currently limited to unicellular strains that are morphologically, physiologically and ecologically very different from the N_2 -fixing strains considered for inoculating ricefields. Probably *super N₂-fixing cyanobacteria* can be selected or designed and grown in test tubes, but the characteristics that will enable them to develop in ricefields are still largely unknown.

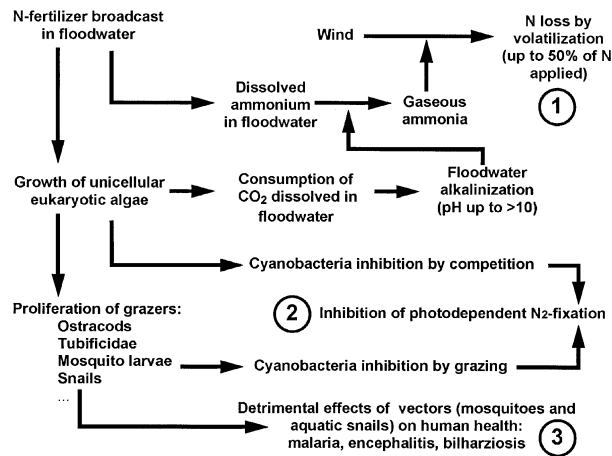


Fig. 22.1. The three major effects of N fertilizer broadcasting in ricefield floodwater.

It can be concluded that after several decades of experiments, inoculation is not a proven technology. As cyanobacteria are ubiquitous in ricefields, a specific management may insure the expression of their potential ($10\text{--}30\text{ kg N crop cycle}^{-1}$) through grazer control combined with P application and deep placement of N-fertilizer, but this is rarely economically viable. N-fertilizer deep-placement on its own is definitely the best technology to take advantage of the potential of indigenous cyanobacteria. It avoids the proliferation of unicellular eukaryotic algae, which causes floodwater alkalization and N losses by ammonia volatilization. It avoids the early proliferation of predators of microalgae and vectors of human diseases (ostracods, mosquito larvae, etc.). It allows N_2 -fixing cyanobacteria growth, otherwise inhibited by competition and grazing (Fig. 22.1). A large set of experiments in non-inoculated ricefields have demonstrated the efficiency of this technology in increasing rice yield by promoting photodependent BNF and improving N-fertilizer efficiency (Table 22.1). A better understanding of the factors that could allow inoculated non-indigenous strains to establish *in situ* is needed before cyanobacterial inoculation could be reconsidered as an agricultural practice in rice cultivation.

22.2 Symbiotic cyanobacteria: *Azolla*

The only symbiosis between cyanobacteria and plants of agronomic significance is that between *Anabaena azollae* and the freshwater fern *Azolla*. The endophytic *Anabaena* resides within a basal cavity of each leaf of *Azolla*. The association may proliferate without mineral N.

Azolla has been used as green manure in ricefields for centuries, in North Vietnam and South China, because of its ability to grow rapidly together with wetland rice and its high N content. It prefers areas and seasons with a cool temperature (daily average around 25°C), because at higher temperatures *Azolla* growth is reduced and damage by insect pests is severe. Usually, *Azolla* is grown for one month and incorporated before transplanting.

Sometimes, *Azolla* is grown before and after transplanting. To a lesser extent, wide-row transplanting permits *Azolla* cultivation with rice and several incorporations during the crop cycle. This technology requires a regional network for providing farmers with fresh inoculum because *Azolla* is maintained and multiplied only vegetatively. *Azolla* biomass production by farmers then requires labor-intensive agricultural practices, including repetitive fertilization with P, K, farm-yard manure, or ash; insect control; protection against washing-out by rains; one or several incorporations, with sometimes pre-composting of part of the *Azolla* crop (Roger & Watanabe, 1986).

22.2.1 Ecology in ricefields

Azolla is widely distributed throughout the world (Lumpkin & Plucknett, 1982), but to develop significant biomass in ricefields it needs to be introduced and cultivated.

The optimum temperature for most species (20–30°C) is below the average temperature in the tropics. Cool weather is a key to successful *Azolla* cultivation in Vietnam and China. *Azolla* grows optimally at pH 4.5–7.0 and survives within a range of 3.5–10. The effect of pH is probably mostly related to nutrient deficiencies (Fe, Zn, etc.) occurring at high values, or toxicities (Al, Fe, etc.) occurring at low values (Lumpkin & Plucknett, 1982).

Phosphorus is the major limiting nutrient for *Azolla* growth *in situ*. Reported threshold values of P deficiency are 0.4% in *Azolla* (dw basis), 0.15 ppm in floodwater, and 20 ppm available Olsen P in soil (Watanabe & Ramirez, 1984), therefore P fertilization of *Azolla* is required in most soils.

Recorded *Azolla* pests include 30 insects, two Arachnoidea, and nine Mollusca. Yield losses of field-grown *Azolla* due to insects may reach 60% (Mochida *et al.*, 1985). Pathogenic fungi developing on plant wounds magnify the effects of insect attacks. *In vitro*, BNF by *Azolla* is more tolerant of combined N than BNF by free-living cyanobacteria. *Azolla caroliniana* grown in various nutrient solutions at 35 ppm N, derived more than 60% of its N from BNF (Peters *et al.*, 1981). *In situ*, however, N favors the growth of competing aquatic plants that may hinder *Azolla* growth.

Herbicides are toxic to *Azolla*. A beneficial effect of *Azolla* is its weedicide effect, therefore there is little interest in combining *Azolla* use with that of herbicides. Insecticides usually favor *Azolla* growth by decreasing pest incidence.

22.2.2 *Azolla* as a source of N

Biological N₂-fixation (BNF) by *Azolla* has usually been estimated by measuring biomass and assuming that *Azolla* N mostly originates from BNF, which was confirmed by the ¹⁵N dilution and the δ¹⁵N methods. In experimental plots, N in maximum standing crops ranged from 20 to 146 kg ha⁻¹ and averaged 70 kg ha⁻¹ (*n*:17; cv: 58%). N₂-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; cv: 47%) (Roger & Watanabe, 1986). In four-year field trials at 37 sites in ten countries,

productivity was lower than in experimental plots: 10–50 kg N ha⁻¹ (average 30 kg N ha⁻¹) (Watanabe, 1987).

22.2.3 Effects on rice

Azolla N becomes available to rice upon decomposition and mineralization. Nitrogen recovered by rice from ¹⁵N-labeled *Azolla* ranged from 20 to 34% and was higher when *Azolla* was incorporated into the soil (see Roger, 1996). *Azolla* has other beneficial effects. The threshold concentration of K for absorption from floodwater is lower for *Azolla* (0.85 ppm K₂O) than for rice (8 ppm) (Liu, 1984). As irrigation water usually contains 1–5 ppm K, *Azolla* absorbs and concentrates K thus becoming a source of K for rice when incorporated. *Azolla* decreases weed incidence and water evaporation (Lumpkin & Plucknett, 1982). It improves soil structure as do most organic manures; that becomes important when an upland crop is grown after rice. Field trials for four years at 37 sites in ten countries (Watanabe, 1987) showed that incorporating one crop of *Azolla* grown before or after transplanting was equivalent to application of 30 kg N ha⁻¹, and incorporating two *Azolla* crops grown before and after transplanting was equivalent to split application of 60 kg N ha⁻¹.

22.2.4 Current use

In the late 1970s *Azolla* was probably used on more than 2 000 000 ha of ricefields in China and Vietnam (Lumpkin & Plucknett 1982). In the 1980s, studies were conducted to improve the technology and trials were established in Brazil, India, Pakistan, Senegal, Sri Lanka, and Thailand to study the feasibility of *Azolla* adoption by rice farmers (IRRI, 1987). However, during the 1980s, the area devoted to *Azolla* in Vietnam and China decreased dramatically and *Azolla* use did not spread to other countries. In Vietnam and China this was partly attributed to (1) the advent of cheap sources of urea and potash, (2) the changing governmental economic policies, and (3) a change from transplanted rice – favorable to *Azolla* growth – to direct seeded rice – where early growth of *Azolla* is detrimental. Now, *Azolla* use has become incidental in China and Vietnam.

22.2.5 Factors limiting *Azolla* use and possible methods for alleviation

Major constraints limiting *Azolla* use include: difficulties in maintaining inocula throughout the year, P deficiency, low tolerance to high temperature, damage by pests, poor water control, and economics in relation to changing agricultural practices and policies.

Phosphorus application is needed in most soils for growing *Azolla*. To be economically feasible, it requires a ratio of N fixed to P applied greater than the ratio of the prices of the N- and P-fertilizers (four in most Asian countries). As P-fertilizer is rapidly immobilized in the soil, basal application is often uneconomical while split application has an efficiency of

5–10 g N₂ fixed · g⁻¹ · P applied (Watanabe *et al.*, 1988), but requires more work.

Although commercial pesticides control *Azolla* pests, no method of field application is economical (IRRI, 1987). The possible use of insecticides is limited to inoculum production.

Azolla strains exhibit a wide range of behavior with regard to environmental factors, P requirement, BNF, productivity, etc. The ability to combine favorable characters such as resistance to high temperature and insects, low P requirement, and erect growth (permitting higher productivity) would allow strains to be designed for specific agroecological conditions. For this purpose, recombination of different cyanobacterial and plant symbionts (Lin *et al.*, 1988) and sexual hybridization between species (Wei *et al.*, 1986) proved feasible. However, producing *Azolla* hybrids requires macrosporangia and microsporangia and no satisfactory method is yet available to induce sporocarp formation and germination at will. This also prevents using sporocarps for inoculum maintenance and distribution to farmers. A method for *Azolla* conservation as sporocarps was developed in China, but their growth was too slow to meet inoculum requirements *in situ* (Lu, 1987).

The major limiting factor remains economics, because *Azolla* technology is labor intensive. Economic studies in the Philippines concluded that under favorable conditions (soil exceptionally rich in available P) the labor cost becomes critical where wage rates approach \$2 per day (Kikuchi *et al.*, 1984) whereas in other areas *Azolla* use was uneconomical (Rosegrant & Roumasset, 1988).

22.2.6 What future for *Azolla*?

Azolla still has a future as green manure in areas where it can spread spontaneously and where pest incidence is low because of a dry climate, such as some sahelian and sub-tropical areas.

Azolla has also potential as a multipurpose biofertilizer, feed for pigs and chickens, and primary producer in rice–fish cultivation. In a rice–fish–*Azolla* system in China, average rice yield increased by 5% over the traditional system and fish production averaged 625 Kg ha⁻¹. Weed biomass and pest incidence decreased (Liu, 1988). The economics of integrated *Azolla* culture might be more favorable than green manuring alone.

The recent interest of European and American consumers in *organic* agriculture, offers another opportunity to use *Azolla* to produce *bio* or *organic* rice (produced without agrochemicals), a label that gives it a higher commercial value than rice produced with agrochemicals.

22.3 Conclusion

Among N₂-fixing micro organisms found in ricefields, cyanobacteria play a major role. Free-living indigenous forms have spontaneously achieved modest but constant yields (1–2 t ha⁻¹) to be obtained for centuries without N-fertilizer. Symbiotic forms were also used for centuries in China and Vietnam, allowing significant yields (2–5 t ha⁻¹) to be obtained through a labor-intensive and quite sophisticated green manuring technology.

Research on cyanobacteria as biofertilizers for rice reached intensive development in the 1970s and the 1980s. Since then, the number of research papers dealing with these topics has strongly declined.

Free-living cyanobacteria have a modest potential of about $30 \text{ kg N ha}^{-1} \text{ crop cycle}^{-1}$ which may translate to a yield increase of $300\text{--}450 \text{ kg ha}^{-1}$. Inoculation of ricefields with selected or transformed strains is not a proven technology. On the other hand, the knowledge acquired on the general ecology of the ricefield floodwater has allowed practical recommendations to optimize its management in a way that favors BNF by cyanobacteria, decreases N-fertilizer losses, and helps controlling detrimental invertebrates. Over the long term, biological engineering may design *super N₂-fixing cyanobacteria* but the characteristics that will enable them to survive, develop, and fix N₂ in ricefields are still unknown.

Azolla has proven useful as a biofertilizer in China and Vietnam. Currently *Azolla* use has drastically diminished in these countries and has not been adopted in the countries where it was tested. Progress in strain improvement has opened ways to alleviate many environmental and nutritional limitations of *Azolla*. However, socio-economic limitations are the most important and are increasing in relation to changes in agricultural policies and practices. Some studies have shown that *Azolla* also has potential as a multipurpose biofertilizer, an animal feed, and a primary producer in rice–fish–*Azolla* cultivation. The potential of *Azolla* as a multipurpose crop, may revive interest in its use.

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