THE BOTANICAL REVIEW

VOL. 67 OCTOBER–DECEMBER 2001 NO. 4

Cyanobacterial Biofertilizers in Rice Agriculture

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I. Abstract

Floodwater and the surface of soil provide the sites for aerobic phototrophic nitrogen (N) fixation by free-living cyanobacteria and the *Azolla-Anabaena* symbiotic N_2-fixing complex. Free-living cyanobacteria, the majority of which are heterocystous and nitrogen fixing, contribute an average of 20–30 kg N ha^{-1}, whereas the value is up to 600 kg ha^{-1} for the *Azolla-Anabaena* system (the most beneficial cyanobacterial symbiosis from an agronomic point of view). Synthesis and excretion of organic/growth-promoting substances by the cyanobacteria are also on record. During the last two or three decades a large number of studies have been published on the various important fundamental and applied aspects of both kinds of cyanobacterial biofertilizers (the free-living cyanobacteria and the cyanobacterium *Anabaena azollae* in symbiotic association with the water fern *Azolla*), which include strain identification, isolation, purification, and culture; laboratory analyses of their N_2-fixing activity and related physiology, biochemistry, and energetics; and identification of the structure and regulation of nitrogen-fixing (*nif*) genes and nitrogenase enzyme. The symbiotic biology of the *Azolla-Anabaena* mutualistic N_2-fixing complex has been clarified. In free-living cyanobacterial strains, improvement through mutagenesis with respect to constitutive N_2 fixation and resistance to the noncongenial agronomic factors has been achieved. By preliminary meristem mutagenesis in *Azolla,* reduced phosphate dependence was achieved, as were temperature tolerance and significant sporulation/spore germination under controlled conditions. Mass-production biofertilizer technology of free-living and symbiotic (*Azolla-Anabaena*) cyanobacteria was studied, as were the interacting and agronomic effects of both kinds of cyanobacterial biofertilizer with rice, improving the economics of rice cultivation with the cyanobacterial biofertilizers. Recent results indicate a strong potential for cyanobacterial biofertilizer technology in rice-growing countries, which opens up a vast area of more concerted basic, applied, and extension work in the future to make these self-renewable natural nitrogen resources even more promising at the field level in order to help reduce the requirement for inorganic N to the bare minimum, if not to zero.

II. Introduction

Nitrogen (N) has been established as one of the most important limiting factors in rice production. The overhead labor cost of tillage, irrigation, water control, and other operations do not vary much, whether 400 kg ha^{-1} is produced without N fertilization or 2000 kg ha^{-1} is produced through N fertilization (Patnaik & Rao, 1979). This has been found to be true for both the modest and the high-yielding rice varieties (Murayama, 1979; Stangel, 1979), since the modern rice varieties yield only little more than do the traditional varieties without an adequate supply of N fertilizer (Brady, 1979). With this came the realization of the yield potential of modern rice varieties, coupled with the practical experience that inorganic N fertilizers are becoming more expensive because of the large fossil-fuel energy requirement for their production, which necessitated a search for, surveys of, and the development of alternate low-cost, assimilable nitrogen sources; i.e., the natural nitrogen resources having the unique inherent property to biologically fix a significant amount of atmospheric nitrogen in the soil for agricultural use (Yamaguchi, 1979; Prasad & Vaishampayan, 1994b; Hegde et al., 1999).

The flooded rice soil ecosystem is characterized by aerobic and anaerobic zones in three major ecological layers: the floodwater and the surface of the soil; the anaerobic plow layer; and the rhizosphere (Venkataraman, 1993). The floodwater and the surface of the soil provide the sites for aerobic phototrophic nitrogen fixation by free-living cyanobacteria (Roger & Kulasooriya, 1980; Vaishampayan, 1996; Whitton, 2000) and the *Azolla-Anabaena* symbiotic N_2-fixing complex (Singh, 1979; Venkataraman, 1988; Singh & Singh, 1997; Vaishampayan, 1994b; Kundu & Ladha, 1995; Vaishampayan et al., 1998b, 2000a), and the reduced zone and the rhizosphere of the rice plants are the sites for heterotrophic nitrogen fixation (Matsuguchi, 1979; Hegde et al., 1999). The present review is an attempt to summarize some of the significant studies in the area of cyanobacteria-based biofertilizers.

A variety of free-living cyanobacteria are now identified as efficient components of cyanobacterial biofertilizers. In addition, a few cyanobacterial species form symbiotic associations with plants (algae, i.e., diatoms; fungi, i.e., lichens; bryophytes, i.e., liverworts, hornworts, and mosses; pteridophytes, i.e., *Azolla;* gymnosperms, i.e., cycads; and angiosperms, i.e., *Gunnera*), animals (marine sponges and achiruoid worms), nonphotosynthetic protists (belonging to the group Glaucophyta), bacteria, and hollow shafts of hairs of polar bears (Rai, 1990). The water fern *Azolla,* holding the N_2-fixing cyanobacterium *Anabaena azollae,* is another established major cyanobacterial biofertilizer. The first part of this review will deal with the free-living cyanobacteria, and the second will focus on the *Azolla-Anabaena* symbiosis, in order to present a comprehensive view of the present state of knowledge in the area of cyanobacterial biofertilizers.

III. Free-Living Cyanobacteria

Cyanobacteria are prokaryotic microorganisms that resemble gram-negative bacteria in structure but possess an O_2-evolving photosynthetic system similar to that of eukaryotic algae and higher plants (Fogg et al., 1973). They belong to ancient group of organisms that are recorded even from pre-Cambrian microfossils (Schopf, 1970) and dominate a wide range of diverse environments characterized by extremes of temperature, desiccation, pH, salinity, light intensity, and nutrients (Whitton, 2000). Rice fields in several countries—e.g., Japan, Thailand, China, the Philippines, Bangladesh, and India—have been investigated and show a dominant presence of these organisms (Roger & Kulasooriya, 1989; Venkataraman, 1981). The majority of cyanobacteria are capable of fixing the atmospheric nitrogen, and their pres-

ence in rice fields is thought to maintain the nitrogen level in the soil (Venkataraman, 1993). Ever since the importance of cyanobacteria was recognized, a considerable amount of research has been carried out to evolve methods and means to effectively utilize these organisms as a biofertilizer (Brouers et al., 1987; Shi et al., 1987, 1991; Shi & Hall, 1988; Anand, 1998b; Vaishampayan et al., 2000c).

A. NITROGEN-FIXING CYANOBACTERIA

Cyanobacteria are of morphologically diverse organization. Generally, they can be grouped into unicellular, colonial, unbranched filamentous, pseudoparenchymatous, heterocystous, and heterotrichous-heterocystous forms (Desikachary, 1959). Fogg (1949) demonstrated experimentally that heterocysts are the specialized cells that contain the nitrogen-fixing mechanism. A considerable amount of research was carried out on heterocystous forms (see Stewart, 1980; Adams & Duggan, 1999), and it was proved by immunolabeling studies that heterocysts are the location of the enzyme nitrogenase (Flemming & Haselkorn, 1973). The methodology for quantification of nitrogen fixation was also developed, through simple estimation of total nitrogen over a period of time, a mass spectrophotometric technique using isotopic nitrogen (N^{15}), and the acetylene-reduction technique (Fogg et al., 1973). The acetylene-reduction technique has been in use worldwide for assaying the activity of the enzyme nitrogenase. Based on these techniques, the N_2-fixing cyanobacteria known so far have been classified into three groups: unicellular, filamentous nonheterocystous, and filamentous heterocystous (Table I).

Taxonomic and/or floristic information on soil cyanobacteria is available from, for example, Argentina (paddy fields: de Halperin et al., 1992), Australia (cryptobiotic crusts: Eldridge & Greene, 1994), Bangladesh (rice fields: Khan et al., 1994), Czech Republic, Russia (taxonomic account: Desertova, 1974), Greece (morphology of soil species: Economou et al., 1984), India (usar/alkaline land floristics: Prasad & Srivastava, 1968; rice fields: Gupta, 1966; nonheterocystous species in rice fields: Tiwari, 1975; Nostocaceae in rice fields: Tiwari & Pandey, 1976; floristic account for rice fields near Pusa: Jha et al., 1986; floristic account for Kerala rice fields: Anand & Hopper, 1987; detailed taxonomic account of rice-field species: Anand, 1989), floristic account of Arunachal Pradesh rice fields: Singh et al., 1997a, floristic account of Nagaland rice fields: Singh et al., 1997b), Iraq (floristic account of rice fields: Al-Kaisi, 1976), the United Kingdom (illustrations and floristic account: Bristol, 1920), and the United States (soil flora of semi-desert: Anderson & Rushforth, 1976; Ashley et al., 1985; Johansen, 1993).

In majority of the cases referred to above, the soil-borne cyanobacteria have been found to be N_2 fixers (Kabli et al., 1997). In addition to light, the pertinent factors that affect cyanobacterial presence in soil are moisture, pH, mineral nutrients, and combined nitrogen (Granhall, 1975). Moisture was noted to be extremely important for their multiplication in loamy soil (Zimmerman et al., 1980). Their ability to fix atmospheric nitrogen has a competitive advantage where combined nitrogen levels are low (Howarth et al., 1988). The abundance of cyanobacteria in tropical soils is supported by their higher temperature optima (Castenholz & Waterbury, 1989). Many cyanobacteria tolerate high levels of ultraviolet irradiation (Sinha et al., 1999), permitting them to survive at the surface of the soil, whereas others photosynthesize efficiently at low photon fluence rates (van Liere & Walsby, 1982).

Figures 1a–1f show the filaments of *Anabaena* sp. and *Nostoc* sp. They are the most common nitrogen-fixing organisms in rice fields, mostly occurring as a free-floating water blooms, forming a microbial mat; the photographs show the conspicuous vegetative cells and

Table I
Important nitrogen-fixing cyanobacterial genera

Unicellular	Filamentous nonheterocystous	Filamentous heterocystous
Aphanothece	*Lyngbya*	*Anabaena*[a]
Chroococcidiopsis	LPP group	*Anabaenopsis*
Dermocarpa	*Microcoleus chthonoplastes*	*Aulosira*
Gloeocapsa (*Gloeothece*)[a]	*Myxosarcina*	*Calothrix*[a]
Myxosarcina	*Oscillatoria*	*Camptylonema*
Pleurocapsa group[a]	*Plectonema boryanum*	*Chlorogloea*
Synechococcus	*Pseudoanabaena*	*Chlorogloeopsis*
Xenococcus	*Schizothrix*	*Cylindrospermum*
	Trichodesmium	*Fischerella*[a]
		Gloeotrichia
		Haplosiphon
		Mastigocladus
		Nodularia
		Nostoc[a]
		Nostochopsis
		Rivularia
		Scytonema[a]
		Scytonematopsis
		Stigonema
		Tolypothrix
		Westiella
		Westiellopsis

[a] Some strains of these genera live symbiotically with other plants (after Venkataraman, 1993; Sinha & Häder, 1996a).

heterocysts. Other important rice-field cyanobacteria include: *Nostoc commune,* embedded in a dense matrix of mucilage and forming a ball like structure; *Scytonema* sp., showing heterocysts and characteristic typical false branching; *Calothix* sp., showing characteristic terminal heterocysts; *Nodularia* sp., with vegetative cells and heterocysts; *Gloeotrichia* sp., characterized by a ball-like circular assembly of filaments resembling radiating rays; and *Lyngbya* sp., having a typical yellow-brown coloration of the mucilage sheath due to the presence of scytonemin, a UV-absorbing compound.

The larger population of N_2-fixing cyanobacteria comprises the filamentous and heterocystous forms (Table I). *Nostoc commune* has been found to be the most important source of biologically fixed N in the Arctic Spitsbergen (Solheim et al., 1996). Activity of sheets of *Nostoc commune* assayed in situ showed linear relationships with their moisture content and assay temperature (Liengen & Olsen, 1997). Similarly, more than 100 strains of heterocystous cyanobacteria, belonging to the genera *Anabaena, Nostoc, Nodularia, Cylindrospermum, Scytonema, Calothrix, Anabaenopsis, Mastigocladus, Fischerella, Tolypothrix, Aulosira, Stigonema, Haplosiphon, Chlorogloeopsis, Camptylonema, Gloeotrichia, Nostochopsis, Rivularia, Schytonematopsis, Westiella, Westiellopsis, Wollea,* and *Chlorogloea,* have been found to be efficient N_2 fixers (Venkataraman, 1993).

Using the acetylene-reduction technique, Wyatt and Silvey (1969) were the first to report nitrogen fixation by a unicellular cyanobacterium, *Gloeocapsa.* Another report on nitrogen fixation by a nonheterocystous filamentous form, *Plectonema boryanum,* under microaerophilic conditions, was published by Stewart and Lex (1970). Carpenter and Price (1976) reported nitrogen fixation by *Trichodesmium* sp., a nonheterocystous filamentous form in the

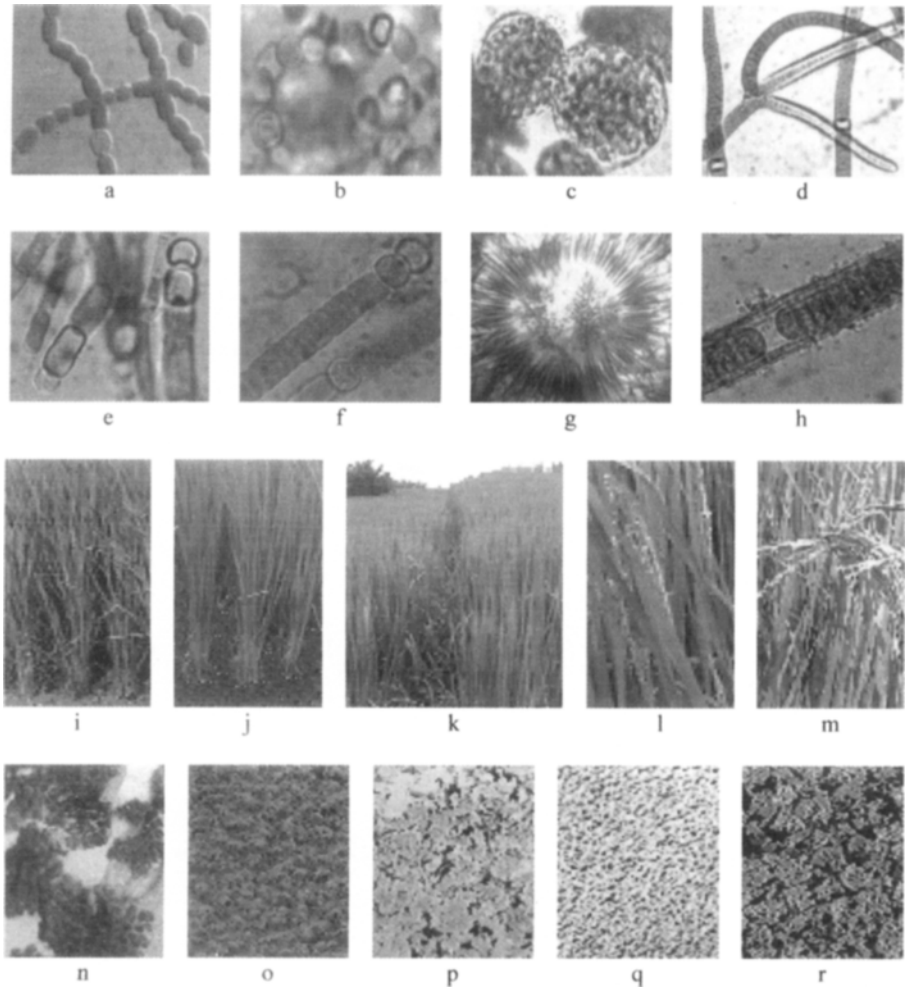

Fig. 1. Filaments of: **a.** *Anabaena* sp.; **b.** *Nostoc* sp., the most common nitrogen-fixing agents in rice fields, usually occurring as a free-floating water bloom that forms a microbial mat (note the conspicuous vegetative cells and heterocysts); **c.** *Nostoc commune,* embedded in a dense matrix of mucilage and forming a ball-like structure; **d.** *Scytonema* sp., showing heterocysts and characteristic typical false branching; **e.** *Calothix* sp., with characteristic terminal heterocysts; **f.** *Nodularia* sp., showing vegetative cells and heterocysts; **g.** *Gloeotrichia* sp., showing a globular assembly of filaments resembling radiating rays; and **h.** *Lyngbya* sp., showing a typical yellow-brown coloration of the mucilage sheath due to the presence of the UV-absorbing compound scytonemin. **i, j.** Rice plants growing without (i) and with (j) cyanobacterial inoculation. **k.** Height of rice plants without (left) and with biofertilization (right). **l.** Fruiting of rice plants without biofertilization. **m.** Early mature fruiting of rice plants with biofertilization. Different mutant strains of *Azolla-Anabaena* symbiotic N_2-fixing complex (*Azolla pinnate* var. *pinnata*): **n.** wild-type strain, whole plant (enlarged); **o.** wild-type strain, in a field nursery; **p.** low P-requiring strain, in a field nursery; **q.** temperature-tolerant strain, in a field nursery; and **r.** low P-requiring and temperature-tolerant strain, in a field nursery.

marine environment. Detailed studies were recently conducted on the distribution of the N_2-fixing enzyme, nitrogenase, and nitrogen physiology in this organism, suggesting that nitrogenase activity and synthesis exhibit an endogenous rhythm in *Trichodesmium* sp. cultures which is affected by the addition of nitrogen, and that the organism can simultaneously fix N_2 and take up other forms of nitrogen (Bergman, 1999; Mulholland & Capone, 1999, 2000; Mulholland et al., 1999). Gallon and Chaplin (1982) and Gallon (1992) performed intensive studies on the unicellular cyanobacterium *Gloeothece* and found that it fixed nitrogen in the dark phase of the light/dark cycle. Among such nonheterocystous N_2-fixing cyanobacteria (Gallon et al., 1991), most soil forms appear to be associated with conditions such as waterlogging, which lead to reduced ambient oxygen concentration (Rother et al., 1988; Rother & Whitton, 1989).

B. THE N_2-FIXING ENZYME SYSTEM

The entire group of N_2-fixing cyanobacteria (including the heterocystous and nonheterocystous filamentous/unicellular forms), listed above, are able to use molecular nitrogen as a nitrogen source. Dinitrogen is reduced to ammonia by an enzyme complex known as nitrogenase, a reaction that is dependent on reduced ferredoxin and obligatorily coupled to reduction of protons, resulting in the formation of molecular hydrogen:

$$N_2 + 8\ Fd_{red} + 8\ H^+ + 16\ ATP \rightarrow NH_3 + H_2 + 8\ Fd_{ox} + 16\ ADP + 16\ P_i$$

Like nitrogenases from other prokaryotes, the cyanobacterial nitrogenase consists of dinitrogenase reductase (the homodimeric Fe protein) and dinitrogenase (a heterotetrameric MoFe protein). Electron flow coupled to ATP hydrolysis proceeds from ferredoxin to the Fe protein and finally to the MoFe protein. The genes coding for the structural subunits of cyanobacterial nitrogenase (*nif*HDK) have been cloned and sequenced (Mevarech et al., 1980; Mazur & Chui, 1982; Lammers & Haselkorn, 1983). Nitrogenase activity and synthesis are regulated at the level of both enzyme activity and gene expression. Enzyme activity may be regulated by posttranslational modification of the Fe protein (Reich et al., 1986; Smith et al., 1987; Reich & Böger, 1989).

Nitrogenase is extremely oxygen sensitive, thus requiring special conditions for enzyme activity (Fay, 1992). In heterocystous cyanobacteria the enzyme is located exclusively in the heterocysts (Wolk & Wojciuch, 1971). Differentiation of vegetative cells into heterocysts occurs in response to lack of utilizable nitrogen and involves sequential structural and physiological modifications necessary to provide an anaerobic environment for efficient nitrogen fixation (Haselkorn, 1978; Adams & Duggan, 1999). In most heterocystous species investigated so far, a genome rearrangement during the late stage of differentiation restores *nif*HDK by excision of an interrupting 11 kb element (Golden et al., 1985). The nitrogenase structural genes are subsequently transcribed from the *nif*H promoter as a polycistronic message. In nonheterocystous species the oxygen-evolving photosynthesis is separated in time from the oxygen-sensitive nitrogenase activity (Stal & Krumbein, 1985; Mitsui et al., 1986).

A special ferredoxin (FdxH) is synthesized as the specific electron donor to the nitrogenase complex in all N_2-fixing cyanobacteria so far tested (Almon & Böhme, 1997). The occurrence and function of this ferredoxin were first described in heterocysts of *Anabaena* (Schrautemeier & Böhme, 1985); meanwhile, the corresponding gene (*fdx*H) was identified and sequenced (Böhme & Haselkorn, 1988) and was crystallized in order to determine its X-ray structure (Jacobson et al., 1992). Two lysine residues were found to be essential for interaction with the nitrogenase (Schmitz et al., 1993).

Cyanobacterial nitrogenase has been purified from *Anabaena* strains (Hallenbeck et al., 1979; Reich & Böger, 1989). Preparation of the enzyme from *Anabaena variabilis* (ATCC 29413), as described by Reich and Böger (1989), was performed under strictly anaerobic conditions. After cell breakage with a French pressure cell and subsequent ultracentrifugation, the resultant supernatant was subjected to anion exchange chromatography. The MoFe protein and the Fe protein were separated by DEAE-cellulose columns using Tris/HCl buffers (pH 7.8), with increasing concentrations of $MgCl_2$ for elution of the enzyme components. For elution of the Fe protein, 80 mM $MgCl_2$ was required (in contrast to 45 mM $MgCl_2$ for elution of the MoFe protein). Further purification of the Fe protein involved preparative SDS–PAGE.

Routine assays for determination of nitrogenase activity make use of the fact that, in addition to N_2, several compounds with a triple bond are reduced by the nitrogenase. So acetylene (C_2H_2) is reduced to ethylene (C_2H_4). Both compounds have the advantage of being detected very easily by gas chromatography. Any gas chromatograph equipped with a Porapak column and a flame-ionizing detector is suitable (Almon & Böhme, 1997).

For determination of in vivo nitrogenase activity in heterocystous cyanobacterial N_2-fixing cells (cyanobacteria grown in the absence of NO_3^- or NH_4^+), the cyanobacterial filaments are suspended in the culture medium in reaction vessels that can be sealed with a gas-tight stopper. Acetylene is added to the gas phase, with a final concentration of about 10% (v/v), and aliquots from the gas phase are withdrawn at appropriate intervals and analyzed for ethylene formation. Reactions can be performed in the light or the dark; nitrogenase activity in the dark depends on the amount of carbohydrate storage compounds present in the cells at the start of the experiment. The presence of molecular nitrogen in the reaction vessel does not interfere significantly with the experiment, because acetylene is about 60 times more soluble in aqueous solution than is dinitrogen.

Induction of nitrogenase activity in nonheterocystous cyanobacteria is achieved by growth of cyanobacteria with a limiting amount of nitrate or ammonium in the culture medium (1–2 mM). After depletion of the N-source, cells degrade phycobiliproteins, and their color gradually turns yellowish green. When these cells are transferred to appropriate assay conditions, nitrogenase is induced by de novo protein synthesis within 1–3 hours. Assays are performed in an anaerobic gas phase consisting of argon + 10% (v/v) acetylene under low light conditions (about 20 $\mu E\ m^{-2}\ s^{-1}$). An equilibrium between photosynthetic oxygen evolution and respiratory oxygen uptake is achieved under these conditions, and nitrogenase activity depends on respiratory activity. If higher irradiances are used, addition of DCMU (final concentration 5×10^{-6} to 1×10^{-5}) is advantageous to prevent excess photosynthetic oxygen evolution. Alternatively, assays may also be performed in the dark, with a limiting amount of oxygen present (Almon & Böhme, 1997).

Nitrogenase activity in isolated heterocysts or cell-free preparations from cyanobacteria can also be determined by the acetylene-reduction technique. In both cases, however, a suitable electron donor has to be used. Molecular hydrogen has been proved to be the best electron donor in heterocyst preparations if the reaction is performed in the light. In cell-free preparations an ATP-regenerating system has to be used in addition, to meet the ATP requirement of the nitrogenase reaction. Sodium dithionite can be used in these systems as a direct electron donor for nitrogenase (Schrautemeir & Böhme, 1985; Böhme & Schrautemeier, 1987).

Alternatively, through the reduction of acetylene, the formation of molecular hydrogen can also be monitored to study nitrogenase activity. For these experiments it is necessary to exclude all possible substrates of nitrogenase from the assay. In the absence of suitable substrates, nitrogenase exclusively reduces protons resulting in the evolution of H_2. For determi-

nation of hydrogen formation, a gas chromatograph equipped with a thermal conductivity detector and a molecular sieve column is required (Almon & Böger, 1988). Due to counteracting hydrogenase activity that consumes molecular hydrogen, rates of nitrogenase activity in intact cyanobacteria measured as formation of H_2 have to be analyzed in detail and considering all parameters involved.

C. CYANOBACTERIAL N_2 FIXERS OF RICE FIELDS

The abundance of cyanobacteria in rice fields was first observed by Fritsch (1907). De (1939) realized their importance in maintaining the fertility of rice fields through biological nitrogen fixation, later emphasized by Singh (1950, 1961), Holm-Hanson (1968), Fogg and Stewart (1968), Horne (1971), and Alexander (1975). The relative occurrence of cyanobacteria in rice fields varies within wide limits (Singh & Singh, 1989). They constitute 86% of the total algal flora in southern Iraq (Al-Kaisi, 1976), 75% in Indian rice fields (Pandey, 1965), and 70% in Italian soil (Materasi & Balloni, 1965). It is important to mention here that the cyanobacteria are scarce in Australian rice fields, possibly due to the higher levels of copper sulfate and combined nitrogen present in the irrigation water. (Bunt, 1961). They are more prevalent in tropical and subtropical regions, as compared with the temperate belts. Their occurrence is on record in Russia (Shtima, 1972), Northern and Western Europe (Henriksson, 1971; Henriksson et al., 1972; Granhall, 1975), and North America (Shield & Durrell, 1964; Mayland & McIntosh, 1966; Jurgensen & Davey, 1968). In Sweden the cyanobacteria were present in 80% of the clay soils and more than 90% of the lime soils (Granhall & Henriksson, 1969).

Rice fields in India, being situated in the tropical belt, are quite rich in cyanobacterial flora, as shown in surveys conducted in the states of Karnataka (Bangale & Bharti, 1980), Kerala (Aiyer, 1965; Amma et al., 1966), Madhya Pradesh (Agarkar, 1967), Maharashtra (Sinha & Pandey, 1972; Kamat & Patel, 1973; Sardeshpande, 1981; Sardeshpande & Goyal, 1981a, 1981b; Kolte & Goyal, 1986), Tamil Nadu (Chacko, 1972), Uttar Pradesh (Pandey, 1965; Bendre & Kumar, 1975), Orissa (Sankaran, 1971; Singh, 1975, 1976, 1979, 1981), and West Bengal (Banerji, 1939). Singh (1978) conducted a survey in the farms of the Central Rice Research Institute, India, and reported the dominance of *Aphanothece, Anabaena, Aulosira, Cylindrospermum, Gloeotrichia,* and *Nostoc*. The species were mostly found to be area specific, as cyanobacterial inocula brought from larger distances—i.e., more than 1500 km—could not be established in the Cuttack area, due to the dominance of indigenous species (Bisoyi, 1982). Out of 2213 soil samples from rice fields, 33% were found to harbor N_2-fixing cyanobacteria in an all-India survey (Venkataraman, 1975). Upland soils in arid climates are very inhospitable to many microorganisms because of high temperatures and limited water, yet cyanobacteria are especially resistant to such adverse conditions and thus form the dominant component of the microflora in many cases (Fogg et al., 1973). Through a quantitative study of algal flora of dried soil samples from upland fields (pH 7.8–8.3) at the Indian Agricultural Research Institute, New Delhi, cyanobacteria were found to dominate in all soil samples (Dutta & Venkataraman, 1968). Among 120 soil samples collected from the Gulf of Mexico and areas in Ecuador and Colombia, 62 algal species were found. Of those species, 46 were cyanobacteria; and of the cyanobacteria, 23 were N_2-fixing species, including 21%, 13%, and 4% populations of *Nostoc muscorum, Nostoc paludosum,* and other N_2-fixing cyanophytes (Durrell, 1964).

A high density of cyanobacteria, comprising more than half the population of heterocystous species, are found growing at or floating above the surface. This is particularly evident in

wetland rice fields, which supply 86% of the world's rice requirement (Ladha & Reddy, 1995). Singh (1961) investigated the periodicity of cyanobacteria in rice fields in Uttar Pradesh and Bihar and found three prominent filamentous and heterocystous forms; i.e., *Aulosira fertilissima, Anabaena ambigua and Cylindrospermum ghorakhpurease.* Several cyanobacterial strains have been screened in laboratories for their pH tolerance, because soils of rice fields in India vary from acidic to highly alkaline. Sardeshpande and Goyal (1982) identified many strains that could adapt to a wide range of pH, grow well, and also perform nitrogen fixation efficiently. Anand et al. (1990) reported that *Nostoc calcicola* could shift the pH of an external medium from acidic or highly alkaline to support its maximum growth within six days. Anand and Revathi (1992) found that *N. calcicola* could adapt metabolically to varying pH regimes and fix nitrogen efficiently. Singh et al. (1995) reported that the pH of the habitat from which the cyanobacteria were isolated did not seem to confer pH specificity. However, critical studies are still necessary on changes in cyanobacterial species composition in the soil as the rice is growing (Whitton, 2000). Kulasooriya (1998) has stressed how this makes it difficult to compare results from different sites. However, this difficulty cannot be generalized unless we have data from a wide enough range of sites. A survey of 102 soil samples from four countries has shown an abundance of heterocystous forms, positively correlated with pH and available P content of the soils (Roger et al., 1993). In Bangladesh rice fields, too, the abundance of heterocystous species was significantly correlated with available P in the soil (Mandal et al., 1993). Although it is difficult to assess the impact of P fertilization on cyanobacteria in rice fields, because other fertilizers, particularly K, are added at the same time, the highly significant increase in cyanobacterial biomass of the cyanobacterial genera—i.e., *Aulosira, Aphanothece,* and *Gloeotrichia*—was shown specifically to be due to phosphate addition (Bisoyi & Singh, 1988a, 1988b).

Light is another decisive factor that has a significant effect on the relative abundance of dominant cyanobacterial genera, as studied in rice fields near Valencia, Spain: Quesada et al. (1998) found that the abundance of nonheterocystous forms was three times more at high than low (about 52% versus 7%) incident radiation and that the three main heterocystous components (*Anabaena, Nostoc,* and *Calothrix* spp.) responded differently to the different levels of irradiation. In this study, a higher abundance of *Nostoc* coincided with a lower abundance of the two other genera, and nitrogenase activity of all the components was greater at the higher irradiance. Most of the cyanophytes appeared to be different in rain-moistened and flooded rice fields of Bangladesh, though mats of *Scytonema mirabilis* were common under both conditions (Rother & Whitton, 1989; Whitton et al., 1989).

D. MASS CULTURE OF N_2–FIXING CYANOBACTERIAL BIOFERTILIZER MATERIAL FOR APPLICATION TO RICE

One of the biotechnological applications that has resulted from development of a cyanobacterial biofertilizer program (Patterson, 1996) has been extension work, or the preparation and distribution of biofertilizers to farmers. Earlier methods of supplying farmers with seed-based inocula of cyanobacteria received criticism, because contamination of the field by introduced cyanobacterial mixtures with soil was feared. Now, innovative methods have been developed for the distribution of cyanobacterial biofertilizer to farmers. For raising laboratory culture of cyanobacteria, use of polybag bottles, instead of expensive glass flasks, has been suggested (Sathiyamoorthy & Shanmugasundaram, 1992). The method of distribution of liquid cyanobacterial cultures in polyethylene and polypropylene sachets has also been developed (Suresh et al., 1992).

Nutrients fixed by cyanobacteria are made available, mainly in the form of ammonia, to rice plants through exudation autolysis and microbial decomposition (Roger & Kulasooriya, 1980; Venkataraman, 1986; Roger et al., 1987). However, this biofertilizer technique is still limited, chiefly because of the inability to efficiently produce good-quality inocula and the difficulty of reestablishing the inoculated strain in fields (Grant et al., 1986; Roger & Watanabe, 1986). But there have been serious attempts to introduce large-scale cyanobacterial culture and applications (Richmond, 1990). During the last decade some progress has been achieved in the production of rice-field isolates in open raceway reactors (Fontes et al., 1987; Boussiba, 1988, 1991, 1997; Querijero-Palacpac et al., 1990; Qian-Lin et al., 1991) and flat, inclined modular photobioreactors (Hu et al., 1996). In most of these cases the cultures had to be kept at a relatively low cell density to obtain a high yield. Application of the biomass harvested from the pond to the field required processing—i.e., harvesting and drying—which reduced cell viability and increased the cost of production (Watanabe, 1984; Roger et al., 1987).

One way to overcome some of the above-mentioned problems is to try to cultivate the cyanobacteria in a reactor (Borowitzka, 1996, 1999) that ensures a high yield when grown at a relatively high cell density. In such a case, direct application of the cyanobacteria to the field may be considered. Just a few years back, a new kind of reactor, one that is closed and tubular, was developed by Boussiba (1997) to overcome some of the problems of low productivity caused mainly by suboptimal temperatures in open raceway ponds (Vonshak & Richmond, 1988). The efficiency of this bioreactor for the production of a nitrogen-fixing cyanobacterium (rice-field isolate, *Anabaena siamensis*), was tested. It was shown that the rate of biomass production was significantly higher in this reactor, as compared with the open pond: 0.31 g l^{-1} vs. 0.09 g l^{-1} (Boussiba, 1993). This system was claimed to be a reliable means of supplying desired microorganisms, which are in some cases major components in a food-chain production system (Boussiba et al., 1988). Moreover, substituting nitrogen biofertilizers with nitrogen-fixing cyanobacteria produced in a closed, tubular bioreactor has been shown to have other specific advantages. Direct application of the dense cultures into the field may reduce the cost of production, because no harvesting or drying process will be required. It may also improve cell viability, which drops drastically when these algae are dried before application to the field. Using a closed, tubular bioreactor will also prevent rain from diluting the growing cultures (or even totally washing out cells), which occurs often in shallow, open ponds. Finally, the prolonged period of production possible in the closed bioreactor can provide a continuous supply of a good source of protein to be used for enterprises other than rice cultivation, such as fish or chicken production, and may help reduce the migration of farmers to urban areas when the rice season is over. The production of rice-field nitrogen-fixing cyanobacteria in this kind of tubular reactor for rice farming thus has great potential and may considerably enhance use of this fertilization technique in tropical countries where rice is considered a major staple in the human diet.

Venkataraman (1964) introduced the terminology "algalization" of rice-field soils by recommending the use of heterocystous cyanobacteria as fertilizer supplements. Several field trials have been conducted since then in India under the All-India Coordinated Research Projects sponsored by the Indian Council of Agricultural Research, and centers for multiplication of such beneficial cyanobacteria came into existence. Venkataraman (1981) also developed the technology for mass cultivation of cyanobacteria. Bisoy and Singh (1988a) reported that 6 kg N ha^{-1} could be produced in 15 days. Kannaiyan (1985) published a detailed account of developing cyanobacteria in nursery plots in the field itself. Small plots of 10 × 2 m^2 with 1.5 kg of starter culture could yield, after 15 days of growth, 15–20 kg of dry algal flakes. A systematic compilation of the practically possible and less-expensive methods of

large-scale N_2-fixing cyanobacterial production for use in rice has been provided by Venkataraman (1993). Two of these methods—trough or tank; pit—are essentially for individual farmers, and two—field; nursery-cum-algal—are for bulk production of the N_2-fixing cyanobacteria on a commercial scale (Venkataraman, 1993).

1. Trough or Tank Method

Either permanent tanks or shallow trays of galvanized iron sheet (2 m × 3 m × 23 cm) are prepared (the size can be increased depending on the amount of material to be produced). For the size specified, 8–10 kg of soil are placed per tray or tank, mixed well with 200 g superphosphate. The trays are filled with 5–15 cm of water, depending on local conditions and rate of evaporation. The soil pH is kept neutral by adding lime. In order to prevent insect-pest infestation, any suitable insecticide, preferably Carbofuran (3% granules) is added to this soil preparation. The N_2-fixing cyanobacterial clean culture (to be propagated in mass) is brought from the laboratory and sprinkled over the standing water in the tray after the soil has settled down. The unit is exposed to the sun in the open air. Water is added intermittently to make up the loss due to evaporation. During the summer (March to May) the inoculated cyanobacteria will grow vigorously and form a thick mat in 7–10 days. At this stage, watering is stopped so that the cyanobacterial mats dry in the sun and crack into flakes, which are collected and stored for field use. The flakes are removed, the troughs are again filled with water, and a handful of dried algal flakes are added as an inoculum for further propagation. The process can be repeated three or four times before fresh soil material, superphosphate, and insecticide are placed on the troughs or trays. Two harvests of cyanobacterial flakes, thus produced, give approximately 3–5 kg of active material, sufficient to biofertilize one hectare of rice field.

2. Pit Method

This technique includes the same method as described above, but it is extremely easy and least expensive for small farmers, who can operate it by themselves in their backyards or farms. The difference is that instead of troughs or tanks, shallow pits are dug in the ground and lined with a thick polyethylene sheet to hold the water. The rest of the procedure is the same as that used in the trough or tank method.

3. Field Method

This method is a large-scale version of the trough or tank method, used to generate bulk quantities of cyanobacterial biofertilizer material. The production field is divided into 40 m^2 plots. The soil has to be well puddled, if loamy, to facilitate waterlogging. The plots are flooded to have a thin layer of water above the soil. Superphosphate at 12 kg per plot and an insecticide (either Carbofuran 3% granules or Ekalux 5% granules) at 250 g per plot are applied. BHC or Furadon may also be used as an alternative insecticide. The cyanobacterial inoculant is sprinkled as in the trough or tank method. The water level is always maintained intermittently. In clear, sunny weather, if the soil is clayey, cyanobacterial growth will be optimal within two weeks; in loamy soil it takes almost double the time to achieve such growth. After reaching the thick, floating mat stage, watering is stopped and sun-dried cyanobacterial flakes are collected and stored in sacks for use at the farmer's field. By reflooding the plots and adding superphosphate and insecticide, the process of cyanobacterial propagation and harvest can be repeated many times without further cyanobacterial inoculation. An average

yield of cyanobacterial biofertilizer through this method is 16–30 kg per plot in each harvest. Adoption of this method has provided farmers with a record production of 15.6 tons ha^{-1} of fresh N_2-fixing cyanobacterial biofertilizer material between March and May in clear sunny weather in some Indian regions.

4. Nursery-cum-Cyanobacterial Method

In this method, farmers can produce cyanobacterial biofertilizers along with rice-seedling generation in nurseries. In a 360 m^2 allotted land area, a rice nursery can be prepared in a 320 m^2 plot, with the adjoining 40 m^2 plot to be used for cyanobacterial production using the aforementioned field method. Through this concurrent nursery method, 15–20 kg of efficient cyanobacterial material are easily made available to biofertilize 1.5 ha of rice field. Moreover, transplantation is made in the entire 360 m^2 nursery, so the land is neither wasted nor locked up exclusively for cyanobacterial biofertilizer production (Meeting, 1988). As early as 1977, the Chinese Agricultural Research Institute, in Nanjing, used a mixture of *Anabaena* sp. and *Nostoc* sp. as inoculants at 750 kg ha^{-1} to produce, within 10–15 days, very efficient cyanobacterial biofertilizer material amounting to 7.5 tons ha^{-1} in normal cool weather and 15 tons ha^{-1} when the temperature rose above 30°C (FAO, 1977).

E. BENEFITS OF CYANOBACTERIAL BIOFERTILIZER IN RICE CULTIVATION

Enhanced grain and straw yields resulting from the use of cyanobacterial strains as biofertilizers were reported by De and Mandal (1956). Further evidence came from the work of Subramanyan (1972), based on experiments conducted at the Central Rice Research Institute, in Cuttack. Singh (1961) reported that alkaline usar soils in Uttar Pradesh could be reclaimed by using cyanobacteria to neutralize the pH of the soil. The cyanobacteria are also known to increase soil fertility by enhancing the available N and P levels (Singh & Bisoyi, 1989). The beneficial effects of cyanobacterial biofertilization may be represented under the following headings:

1. Effect of Cyanobacterial Biofertilization on Rice Plants

Although the most frequently used criterion for assessing the effects of cyanobacterial biofertilization has been enhanced grain yield in rice (Singh, 1984; Singh & Singh, 1986, 1987), beneficial effects have also been noted on plant size, on nitrogen content, and on the number of tillers, ears, spikelets, and filled grains per panicle (Roger & Kulasooriya, 1980; Singh & Singh, 1989; Venkataraman, 1993). The relative increase in grain yield over the control has been an average of 28% in pot experiments (Roger & Kulasooriya, 1980) and 15% in field experiments in India (Venkataraman, 1993; Vaishampayan et al., 1996). The latter are presented in Table II.

Figures 1i–1m are photographs of rice variety HUR–36, grown in experimental fields without biofertilization and with cyanobacterial biofertilization, respectively. Obviously, plants growing with cyanobacterial flakes are more robust than are those without biofertilization. Figure 1k gives a comparative account of the height of this variety without (left) and with cyanobacterial biofertilization (right). Fruiting without biofertilization, shown in Figure 1l, and early mature fruiting with biofertilization, shown in Figure 1m, clearly suggest the additive effect of cyanobacterial biofertilizer (a mixture of *Anabaena* sp., *Nostoc* sp., and *Scy-*

Table II

Average rice-grain yield with 10 kg ha^{-1} cyanobacterial biofertilizer applied in experimental fields supplemented or unsupplemented with inorganic nitrogen fertilizer

	Rice grain yield (kg ha^{-1})			
Nitrogen level	With biofertilizer	Without biofertilizer	Increase (%)	Reference
0	2,541	2,079	22.2	Venkataraman, 1981
50 kg ha^{-1} N	4,834	4,176	15.75	Vaishampayan et al., 1996
100 kg ha^{-1} N	5,485	5,112	7.29	Venkataraman, 1981
100 kg ha^{-1} N	5,654	5,079	11.32	Vaishampayan et al., 1996

tonema sp. isolated from the local rice fields in Varanasi and cultured in the laboratory of A. Vaishampayan for testing the responsiveness of rice varieties to cyanobacterial biofertilization under the All-India Coordinated Biological Nitrogen Fixation Research Project, operative at this center).

Cyanobacterial application to rice fields has been found to result in increased grain yield not only in India (Venkataraman & Goyal, 1968; Subrahmanyan, 1972; Kannaiyan, 1985; Singh, 1985; Bisoyi & Singh, 1988a, 1988b; Mohapatra & Jee, 1991; Tiwari et al., 1991; Dubey & Rai, 1995) but also in China (Ley, 1959), Egypt (El-Nawawy & Hamdi, 1975), Japan (Watanabe, 1965), the Philippines (Pantastico & Gonzales, 1976), and other rice-growing tropical countries. Watanabe (1962) used the cyanobacterium *Tolypothrix tenuis* as a source of green manure in about 40 rice varieties in various parts of Japan and noted an average increase in rice yield by 2% in the first year, 8% in the second year, 15% in the third year, and 19.5% in the fourth year. Using the same organism, Aboul-Fadi et al. (1967) obtained a 16.6% increase in paddy yield in the United Arab Republic. Sharma et al. (1986) observed that plots biofertilized with cyanobacteria (with or without chemical N) supported increases in rice yield of 12.7%, 12.9%, and 13.2% during 1982, 1983, and 1984, respectively. Remarkably, *Aulosira fertilissima* was found to support an increase in rice yield of 368% in pots and 114% in fields (Singh, 1961). In Tamil Nadu, beneficial effects of cyanobacterial biofertilization were obtained with supplements of only 0 and 25 kg N ha^{-1}, helping to save 25 kg N ha^{-1} (Subramani et al., 1980). Gaur and Singh (1982) reported that the beneficial effect of cyanobacteria was not obtained beyond 20 kg N ha^{-1} level at one out of three experimental locations in Uttar Pradesh. Contrary to this, Venkataraman (1972), in field experiments conducted in different parts of India, showed that beneficial effects of cyanobacteria on rice yield were evident even up to 150 kg N ha^{-1}. Under field conditions, cyanobacterial biofertilizer applied in combination with 50 kg N ha^{-1} supported a grain yield as good as that with 120 kg N ha^{-1} (Singh et al., 1981). Srinivasan and Ponnaya (1978) reported an increase in rice yield of 0.2 tons ha^{-1} at two locations and of 0.6 tons ha^{-1} at a third location with cyanobacterial inoculation in the farmer's field.

In addition to increasing the grain and straw yields (Sharma & Gupta, 1983; Ahmed & Ahmedunnisa, 1984; Singh & Singh, 1989), cyanobacterial inoculation has also been reported to support an increase in the N content of grain and straw (Singh, 1984). It was also reported to support an increase in plant height (Singh, 1961), leaf length (Watanabe, 1956), number of tillers (Aiyer et al., 1972), ears (Singh, 1961), number of spikelets per panicle (Rao et al., 1977), number of filled grains per panicle (Rao et al., 1977), and amount of dry matter (Bisoy, 1982). The cyanobacterial inoculation program is generally more successful in the dry season, dur-

ing which a saving of 20–30 kg N ha^{-1} is generally reported (Venkataraman, 1993). The effect of cyanobacterial biofertilization was reported to be statistically erratic and limited (Roger, 1991), and decaying rice straw was found to inhibit cyanobacterial growth and N_2 fixation (Rice et al., 1980). The best results were obtained where mixed inocula were produced from local stocks (Venkataraman, 1981; Reddy & Roger, 1988; Vaishampayan et al., 1996) and applied with reduced or no inorganic nitrogenous fertilizers (Kaushik, 1998).

2. Availability of Cyanobacterial Nitrogen to Rice Crops

Cyanobacteria release the fixed nitrogen mainly in the form of polypeptides, with lesser amounts of free amino acids, vitamins, and auxinlike substances (see Venkataraman, 1993), either by exudation or by microbial degradation after cell death (Subramanian & Shanmugasundaram, 1986a, 1986b). Under field conditions, part of the fixed nitrogen is made available to the rice plants, and the rest is reincorporated into the soil (see Singh & Singh, 1989). The transfer of fixed N from the cyanobacteria to the crop and other organisms has been investigated using the ^{15}N tracer technique (Mayland & McIntosh, 1966; Stewart, 1967, 1970), where the uptake could be both by active assimilation and passive processes (see Venkataraman, 1993). Wilson et al. (1980) reported that 37% and 51% of the nitrogen was recorded in rice from ^{15}N labeled *Aulosira* sp. spread on the soil and incorporated into the soil, respectively. Watanabe et al. (1977) found 40% of the fixed N at maturity in the rice portion not directly exposed to $^{15}N_2$. However, using the $^{15}N/^{14}N$ tracer technique, 40% of the cyanobacterial nitrogen was found to be recovered by the rice plants, and it has been shown that fixed nitrogen from cyanobacteria is directly transferred to rice plants; addition of ammonium chloride equivalent to 100 kg N ha^{-1} had no adverse effect on the recovery of cyanobacterial nitrogen (Venkataraman, 1981; Mian & Stewart, 1985; Ladha et al., 1987). Using similar techniques, release of dissolved organic nitrogen—50% of N_2 fixation—has been shown in a marine cyanobacterium (Glibert & Bronk, 1994). Tirol et al. (1982) reported that the availability of ^{15}N from cyanobacteria incorporated into the soil ranged from 23% to 28% for the first crop and 27% to 36% for the second crop. These authors further demonstrated in pot experiments that for the first crop, cyanobacterial ^{15}N was less available than was $(NH_4)_2SO_4$ but that for the second crop its availability was very similar. After two crops, 57% ^{15}N from cyanobacteria and 30–40% from $(NH_4)_2SO_4$ remained in the soil, which indicates the slow-release nature of cyanobacterial nitrogen and thus a cumulative effect of cyanobacterial inoculation (Roger & Kulasooriya, 1980). The ^{15}N balance in plants and soil after two crops—a result of pot experiments with dried cyanobacterial flakes—showed that losses of ^{15}N from $(NH_4)_2SO_4$ were more than those from cyanobacteria irrespective of the mode of application (see Singh & Singh, 1989). These observations suggest that cyanobacterial material, because of its organic nature, is less susceptible to N losses, as compared with inorganic N fertilizers and that its low C/N ratio (5–7) provides it with a better N availability than does an organic fertilizer; e.g., green manure. The relative availability of cyanobacterial nitrogen to rice depends on the susceptibility to decomposition of the cyanobacterial material, which varies not only with the strain but also with the physiological state, as demonstrated by the discrepancy between the values (Wilson et al., 1980; Tirol et al., 1982). Using similar ^{15}N tracer techniques, release of dissolved organic nitrogen (50% of N_2 fixation) has been shown in a marine cyanobacterium (Glibert & Bronk, 1994). Cyanobacteria are sometimes conspicuous on the surface of aquatic roots of deepwater rice (Whitton et al., 1989), and there is evidence from $^{15}N_2$ studies (Kulasooriya, 1998) that part of this nitrogen reaches the rice plant either as the result of release of extracellular combined N or indirectly following grazing and parasitism.

3. Cyanobacterial Influence on Soil Properties and Roots

Chauhan and Gupta (1984) suggested that, in addition to supporting an increase in grain yield, cyanobacteria secrete growth-promoting substances, such as cytokinins. Rice seeds presoaked with cyanobacterial culture extracts showed enhanced germination, promotion of the growth of roots and shoots, and an increase in the weight and protein content of grains (Jacq & Roger, 1977). Such effects, also reported by many other authors, were ascribed to growth-regulating substances; i.e., hormones and vitamins (Roger & Kulasooriya, 1980). However, a cyanobacterial regulator has not yet been isolated and characterized. Pedurand and Reynaud (1987) screened 135 nonaxenic cyanobacterial isolates on the germination and growth of rice and found that 30% strains had no effect on germination, that 30% caused inhibition, and that 8 strains of *Anabaena* stimulated growth of which only one remained effective after it had been made axenic, suggesting thereby that probably only the positive results in this respect were considered for reporting. Further critical studies are, therefore, required on the hormone and vitamin-forming potentials of cyanobacteria to be substantiated with their well-proved roles in improving soil organic content, water-holding capacity, N enrichment, and formation of extracellular polysaccharides leading to improved soil aggregation and solubilization of phosphates (Roger & Kulasooriya, 1980).

Rao and Burns (1990) demonstrated that changes in the properties of the upper 0.7 cm of experimental columns of a brown earth silt loam incubated in light were possibly due to the increase in the cyanobacterial population. The cyanobacterial counts eventually reached 10^6 g^{-1} dry soil in this experiment, whether or not the columns were inoculated deliberately. Improvements measured in soil-aggregation properties, together with an increase in dehydrogenase, urease, and phosphatase activities, were significant. The activities of these three enzymes were lower than in arable soils, which suggests that waterlogged conditions may limit these enzyme activities to some extent.

The rice-grain-yield measurements suggest that cyanobacterial inoculation produces both cumulative and residual effects, evident through the buildup of both the organic content and the number of cyanobacterial propagules in the soil, facilitating the reestablishment of the cyanobacterial biomass (Ghosh & Saha, 1997). Several reports indicate an increase in organic matter and nitrogen content of soils inoculated with cyanobacteria in pot and field conditions (see Singh & Singh, 1989; Venkataraman, 1993; Vaishampayan, 1998). Singh (1961) indicated the possible role of cyanobacteria in reclaiming the "usar soils," and since then there have been increasingly large numbers of reports on cyanobacteria in general and on N_2-fixing cyanobacteria in particular, in relation to soil fertility (see Whitton, 2000). It seems to have little effect on the physical properties of the soil. However, the improvement of soil-aggregation properties by extracellular polysaccharides released by cyanobacteria has been well studied (Bailey et al., 1973; Roychoudhury et al., 1980; de Winder et al., 1989; de Caire et al., 1997). This is important, because soil aggregation and arrangement of the soil aggregates affect aeration, temperature, and infiltration rates of the soil, which in turn improve the physical environment of the crop. The effects of *Nostoc* strains on two clay soils from Tuscany were studied to show not only aggregation initiation but also protection of soil porosity due to reduction of the damaging effect of water addition (Falchini et al., 1996).

The addition of N by cyanobacterial inoculation was found to increase in the presence of phosphate fertilizers, and both total and organic N levels were maintained beyond the tilling stage (Chopra & Dube, 1971). The cyanobacterial ability to mobilize insoluble forms of inorganic phosphate is evident from the findings of Kleiner and Harper (1977), who reported more extractable P in soils with cyanobacterial cover than in nearby soils without cover. Of

the 18 strains tested, 17 solubilized tricalcium phosphate (Bose et al., 1971). The cyanobacterial P-solubilizing activity also acts on hydroxyapatite (Cameron & Julian, 1988) and Mussorie rock phosphate (Roychoudhury & Kaushik, 1989). Whitton et al. (1991) assayed 50 cyanobacterial strains, including some soil isolates, for their ability to grow with organic phosphates as their sole P source and found that all strains used the monoesters, that almost all used the diesters, and that just a few used phytic acid. Of these, most strains released part of their phosphomonoesterase extracellularly, but not the phosphodiesterase. Some evidence exists, awaiting necessary scientific interpretation, about cyanobacterial effectiveness at increasing P availability in saline soils (Kaushik & Subhashini, 1985).

In a long-term experiment there was a gradual increase in organic carbon due to cyanobacterial inoculation, but the amount remained steady at the end of three years (Sankaran, 1971). Bisoyi (1982) also reported that the influence of cyanobacterial inoculation on soil properties was not significant in the first and second rice-cropping seasons but responded well in the third season, showing 2–11% increases in soil organic carbon as a result of cyanobacterial inoculation. Cyanobacterial growth has been noted to initially cause an increase in the soil pH that later declined to the original value (Saha & Mandal, 1979). It was also shown in these experiments that P content decreased up to 90 days of cyanobacterial growth and began to increase toward the later period of incubation. A significant reduction in pH, electrical conductivity, exchangeable sodium, hydrolic conductivity, and aggregation status of the soil, as well as a significant increase in the total N content of the soil concomitant with cyanobacterial growth, were noted by Subhashini and Kaushik (1981), who also reported that cyanobacterial inoculation in combination with gypsum had an appreciable reclamation property applicable to sodic soils. In a soil rendered saline due to bad farm management, a 25–30% decrease in salinity was observed through the repeated cultivation of *Anabaena torulosa* (Thomas, 1977). Cyanobacteria from saline rice fields were studied, and a halotolerant *Anabaena* sp. was found to be a good nitrogen fixer (Thomas & Apte, 1984).

The forms in which Fe, Mn, and also possibly Zn occur in soil are reported to be influenced by cyanobacterial growth in flooded rice fields (Das et al., 1991). Their presence led to a decrease in ammonium acetate–extractable forms of Fe and Mn and an increase in other forms of these elements. These changes were considered to result from the release of oxygen and the addition of organic matter, especially extracellular material. Further changes in the various fractions were noted as a result of the decomposition of the cyanobacterial biomass, which were ascribed to the development of reducing conditions and the formation of organic acids. A decreased content of readily available Fe may help to minimize Zn deficiency in rice. In many cyanobacterial species, the gelatinous sheath was able to chelate Fe, Cu, Mo, Zn, Co, Mn, and other elements essential for their growth (Lange, 1976). The sheath was also thought to influence the availability of elements to other organisms (Belnap & Harper, 1995). These authors examined whether N, P, K, Mg, Ca, and Fe were present in significantly larger concentrations in *Festuca octoflora* growing on soils heavily encrusted with cyanobacteria/cynolichens than in plants on the same soil where the crust had been destroyed. In the case of *Mentzelia multiflora,* N, Mg, and Fe were present at significantly higher concentrations, but P was present at a significantly lower concentration. In fact, cyanobacterial N_2 fixation is likely to increase the availability of N to adjacent plants, and the reduced P uptake may have been due to direct competition of its near-surface root layer with the surface crust. A cyanobacterial sheath reduces particle erosion and may adsorb charged nutrient cations (see Whitton, 2000). Chelating compounds present in the sheath may also be responsible for the enhanced uptake of Fe. The inorganic and organic nutritional aspects of cyanobacteria are described by Vaishampayan (1981, 1982a, 1982c, 1984d, 1984f, 1995, 1996).

The higher plants also stimulate or inhibit cyanobacterial growth in soil (Parks & Rice, 1969). Intracellularly occurring cyanobacteria have also been shown in rice (Kozyrovskaya, 1990), wheat (Gantar et al., 1991a, 1991b, 1993; Dodds et al., 1995), and maize, beans, sugar beets, and, again, rice (Svircev et al., 1997). In addition to loose associations of *Anabaena* with root hairs of wheat, *Nostoc* colonies were found to be in tighter associations, penetrating the root epidermis and cortex. The beneficial effects in these cases, if any, were greater in liquid than in a sand culture (see Sinha et al., 1998). Various other such observations open up a wide scope of further studies on the qualitative and quantitative importance of intracellular cyanobacteria in the roots of plants at field sites where cyanobacteria are conspicuous at the soil surface.

4. Economics of Cyanobacterial Biofertilizer Production and Use

A cyanobacterial biofertilizer technology easily adaptable by the farmers has been studied well in many countries. Positive results have mostly been achieved with respect to grain yield and economics through this technology tested in field trials and experimental farms. A detailed budget sheet on the economics of inoculum production was first presented by Venkataraman (1979) through results analyzed in a state farm in Tamil Nadu. The calculation was in terms of chemical N input: 25 kg N ha^{-1} then cost about U.S.$12.20, whereas commercially produced cyanobacterial material required for field inoculation to provide this amount of N cost about U.S.$3.65. However, if the same amount of cyanobacterial biofertilizer is produced by the farmers themselves, the costs are negligible. In trials conducted at five stations in India as early as 1978, it was noted that by adding 10 kg ha^{-1} of cyanobacterial flakes (costing less than U.S.$1.00), an extra grain yield of rice worth U.S.$10.00–$15.00 was obtained on average (Rao, 1978). The payoff technology was discussed in the 1979 All-India Coordinated Research Project on Algae (Singh & Singh, 1989). It was concluded that in terms of N input, an amount of 35 kg N ha^{-1} cost around U.S.$2.00. The cyanobacterial material required for field inoculation (10 kg ha^{-1}) to provide this amount of N cost less than U.S.$1.00. It was proposed that if cyanobacterial technology were introduced to even 50% of the rice-growing areas in India, it would result in a saving of 3.8×10^5 tons of N, worth about U.S.$30,400. Accordingly, in a large number of trials where the recommended high levels of inorganic nitrogenous fertilizers were complemented with cyanobacterial applications (at 10 kg ha^{-1}), an average increase of about 300 kg was observed in the grain yield. Clearly, this meant an additional income of U.S.$6.00 for an investment of less than U.S.$1.00 spent on cyanobacterial material. The cost-benefit ratio was thus calculated to be 1:10. However, this is workable under Indian conditions only, and no such estimate is available from other countries.

F. GENETIC IMPROVEMENT OF N_2–FIXING CYANOBACTERIA

Research on cyanobacteria under laboratory conditions has been in progress parallel to field experiments. Refinement of technology for mass multiplication of cyanobacteria for effectively using them as biofertilizers has always been under consideration. Choice of suitable strains that may not only survive in the adverse and extreme ecological conditions in rice fields but also be a good nitrogen fixer under such conditions has been one of the concerns. In this context, some of the very important reports on selection of the beneficial natural isolates of N_2-fixing cyanobacteria, as well as those developed through genetic improvement, are described below.

The cyanobacteria have been critically examined to form the N_2-fixing heterocysts and synthesize active nitrogenase specifically in zero or least-combined N-supplied condition in the laboratory (Stewart & Rowell, 1975; Singh et al., 1978a, 1978b; Vaishampayan & Singh, 1981a, 1981b; Vaishampayan, 1982b) and in fields (Mikheeva et al., 1990; Singh, 1990; Singh et al., 1990), except in *Anabaena* strain CA (ATCC 33047), in which a covalent modification mechanism of nitrogenase during inhibition by combined N is nonoperative (Bottomley et al., 1979), and in nitrate reductase-deficient mutants (Singh & Sonie, 1977; Vaishampayan & Prasad, 1982) of *Nostoc muscorum*. Reportedly, a highly suppressed in situ cyanobacterial nitrogenase activity is operative in wet fields at the recommended field dose of nitrogenous fertilizers between 20 and 100 days after rice transplantation (Singh, 1975). Nevertheless, fertilizer needs of the crops may be reduced when weeds are controlled with herbicides, because these exert a more perfect weed control than do physical methods. However, the oxygen-evolving photosynthetic apparatus of the cyanobacteria (as nontarget species) may be damaged by the herbicides, for the latter are designed to be either photosynthetic inhibitors or uncouplers (see Vaishampayan & Prasad, 1984b; Prasad & Vaishampayan, 1994a; Vaishampayan, 1998; Vaishampayan et al., 1998d). Thus, photosynthesis turns out to be a disadvantage with respect to the viability of the N_2-fixing cyanobacteria in pesticide-treated modern agricultural fields. This paradoxical situation raises an essential need to use large doses of synthetic N fertilizers (Singh et al., 1988), which do repress the N_2-fixing activity of the few surviving cyanobacterial cells (see Vaishampayan et al., 1998d). In fact, this is one of the prime reasons why the rate of cyanobacterial contribution of soil N is supposed to be less than that of *Azolla-Anabaena* symbiosis or the other heterotrophic N_2 fixers (Prasad & Vaishampayan, 1994b). Rather, under the situation of repressed nitrogenase, the surviving cyanobacterial cells are forced to consume a part of the applied synthetic nitrogenous fertilizers to maintain their own growth. Because the practice of agrochemicalization has to continue to maintain the high-yielding crop varieties, a unified concept has evolved during the last few decades toward developing ammonia-derepressible, pesticide-resistant cyanobacterial mutants as a viable and efficient cyanobacterial biofertilizer in modern agrochemicalized rice fields (Meeting, 1988; Reddy & Roger, 1988; Venkataraman, 1993; Anand, 1998b; Vaishampayan et al., 1998d). Some of the N_2-fixing cyanobacterial taxa have a high genetic elasticity (Ladha & Kumar, 1978; Prasad & Vaishampayan, 1984, 1987; Vaishampayan, 1984e, 1994a; Vaishampayan & Prasad, 1984a; Haselkorn, 1995; Vaishampayan et al., 1998a, 2000c) and are known to respond well to chemical stressors and/or mutagens (Singh & Vaishampayan, 1978; Singh et al., 1979; Vaishampayan, 1984g, 1984h, 1985b, 1985c, 1985d; Sinha & Häder, 1996b, 1997), apart from their suitability in physiological and biochemical studies (Vaishampayan, 1981, 1982a, 1984d, 1995, 1996; Badger & Price, 1992; Bothe et al., 1995) and adaptability to different ecophysiological conditions (Hori et al., 1994; Sinha & Häder, 1996a). Such fundamentally important, versatile properties of many N_2-fixing cyanobacteria have helped their genetic improvement during the recent past in relation to nitrogenase derepression and pesticide resistance for their more promising and prospective use as an efficient solar-energy-dependent, economical biofertilizer in modern rice fields.

1. Derepressed and Ammonia-Releasing N_2-Fixing Cyanobacterial Strains

The effects of nitrogen and non-nitrogen commercial fertilizers were studied on 12 cyanobacterial strains (Singh, 1975), and in all cases the higher concentrations of fertilizers were found to be detrimental to the growth of these microorganisms. Later, Rodgers (1982) found

that ammonium N fertilizers at concentrations as low as 0.2 μM markedly repressed cyanobacterial N_2 fixation in fields. The non-nitrogen fertilizers did not affect heterocyst differentiation. Anand and Karuppuswamy (1987) found that lower fertilizer concentrations supported all morphological types of cyanobacteria. However, Fernandez-Valentine et al. (1997) found that the highest level of ammonium fertilizer (140 kg N ha^{-1}) in rice fields near Valencia, Spain, led to a significant reduction in cyanobacterial nitrogenase activity and that the partial reduction in activity increased over the cultivation cycle, being highest at the end. With the increasing practice of cultivating high-yield rice varieties, the input of inorganic N fertilizer, which is injurious to the N_2-fixing machinery of cyanobacteria, is also increasing; hence the need to develop derepressed cyanobacterial mutants with the capacity of uninterrupted N_2 fixation even in the presence of high concentrations of inorganic N fertilizer (Singh & Singh, 1978; Mishra et al., 1991b). Anand (1992) reported that certain cyanophycean members could continue to fix nitrogen even in the presence of commercial nitrogen fertilizers. Suseela and Goyal (1995) screened several strains for their capacity to fix nitrogen in the presence of ammonium nitrogen and found that nitrogenase was active even at 100 mg ammonium ml^{-1}. Certain strains of *Anabaena* were found to excrete the fixed ammonium (Subramanian & Shanmugasundaram, 1986a, 1986b). Anand and Parmeswaran (1992) reported that there were strains which released ammonium as they grew in a normal medium.

Although some cyanobacterial strains that thrive in rice fields (Venkataraman, 1975) release small quantities of the major fertilizer product, ammonia, during their active growth phase, most of the fixed products are made available mainly through autolysis and microbial decomposition (Martinez, 1984). Under these circumstances, it is difficult to control the flow of nitrogen compounds needed for the development of rice plants. A possible solution for this problem is to develop strains of cyanobacteria that release ammonium continuously. However, in this effort, some classes of cyanobacterial mutants either had a totally defunct glutamine synthetase (GS), leading to an exogenous glutamine requirement (Kerby et al., 1985; Verma et al., 1990), or had too low an expression of nitrogenase with the exogenous NH_4^+ to serve any practical purpose (Prasad et al., 1991). But a number of mutants of nitrogen-fixing strains that release ammonia continuously have been isolated. The most common features of these mutants are reduced GS activity together with increased nitrogenase activity (Polukhina et al., 1982; Boussiba et al., 1984; Lattore et al., 1986; Spiller et al., 1986; Subramanian & Shanmugasundaram, 1986a, 1986b). The main strategy to induce extracellular release of ammonia is to supply a specific inhibitor of GS activity; e.g., -methionine-DL-sulfoximine (MSX), a glutamate analogue (Stewart & Rowell, 1975). The continuous presence of the inhibitor was a limitation for long-term ammonia production and thus for practical applications; this is because MSX leads rapidly to a deficiency of glutamine and other nitrogenous compounds that are necessary for metabolism of the cells with cessation of ammonia production and, finally, lysis of the cells. It was shown that it is possible to overcome this limitation at least partly and to lengthen the production period by adding small amounts of glutamine (Ramos et al., 1984) or by allowing the cells to recover in the absence of the inhibitor (Brouers, 1986). Another approach for increasing the long-term productivity is the use of immobilized resting cells (Musgrave et al., 1982; Hall et al., 1985). Substantial ammonia production from atmospheric nitrogen has also been observed in the presence of MSX (Musgrave et al., 1982; Ramos et al., 1983, 1987; Hall et al., 1985; Newton & Cavins, 1985).

In contrast to the mutants of enteric bacteria that lack the *amt* system (Castorph & Kleiner, 1984; Jayakumar et al., 1989) and release ammonia, the nitrogen-fixing cyanobacterial mutant (Boussiba, 1997) releases ammonia continuously while fixing nitrogen but still possesses an ammonium uptake system, as evidenced by the ability to accumulate $^{14}CH_3NH_3^+$.

Mutants of *Anabaena variabilis* resistant to the ammonium analogue, ethylene diamine (EDA), and to the glutamine analogue, MSX, have been reported to release ammonium (Kerby et al., 1986, 1987; Spiller et al., 1986). These, however, exhibit a slower growth than do their parents. Another major problem of using cyanobacteria as biofertilizer is the competition between indigenous and introduced strains, the former generally dominant. It was assumed that ammonium-excreting mutants, isolated from strains indigenous to the rice field, would overcome the constraints of the rice-field environment better than strains derived from other habitats would. The reinoculation and establishment of these mutants in rice fields would thus be comparatively more successful.

In this respect, *Anabaena siamensis,* a rice-field isolate from Thailand, proved promising and is already marketed as an algal biofertilizer for rice fields. Its efficiency in increasing the growth and yield of rice plants is apparently due to its high nitrogen-fixing capacity (Antarikanonda, 1982a, 1982b). It was reported to release a variety of amino acids during its active growth phase (Antarikanonda, 1984), but not ammonium, as observed for other nitrogen fixers found in rice fields. Selection of mutants resistant to MSX was found to lead to reduction in GS activity, and, consequently the unassimilated ammonium is released into the medium without the induction of MSX (Spiller et al., 1986). It is assumed that a strain that released ammonium continuously would be a better biofertilizer (Lattore et al., 1986). The MSX-resistant mutant of *A. siamensis,* SS_1 (Boussiba, 1997), seems to conform with this expectation (Thomas et al., 1990), for it releases ammonium due to the high activity of nitrogenase, both of which are controlled by cell density of the culture. The direct effect is apparently light availability to each cell which becomes progressively limited as cell density increases. The rate of ammonium release is consequently maximum only during the early log phase of growth in batch cultures. A similar pattern is also seen under immobilized conditions. Based on the above observations, SS_1 growing in continuous culture at low cell density (Chlorophyll value of 5–7μ g ml^{-1}) seems an ideal system for sustained ammonium release. The rate of ammonium release by SS_1 is lower than the rates obtained for other mutants of *A. variabilis;* i.e., 35–50 μmol mg Chl-a^{-1} h^{-1} in batch and immobilized cultures (Kerby et al., 1986; Spiller et al., 1986). Although the parent and the SS_1 strains have a similar pattern of nitrogenase activity, the activity of SS_1 enzyme is 30% higher than that of the parent during steady-state growth and 50% higher under immobilized conditions. In the presence of ammonium, SS_1 nitrogenase activity is about five times greater than that of the parent, due to a stronger repression by the end product in the parent. GS also seems defective in SS_1, exhibiting less than 50% of the parent's enzyme activity. Hence it appears that the decreased susceptibility of nitrogenase activity to ammonia repression and the defective GS are the main factors controlling ammonium excretion in the SS_1 mutant. The other ammonium-excreting mutants of *A. variabilis,* SA_1, ED_{81}, and ED_{92}, were found to have derepressed nitrogenase activity and lower GS activity (Hien et al., 1988). Analysis of GS and its mRNA into EDA-resistant mutants suggested that ED92 is a regulatory mutant containing less GS mRNA and consequently less GS protein, as found for *Anabaena azollae* growing in symbiosis (Nierzwicki-Bauer & Haselkorn, 1986). On the other hand, ED_{81} is a structural mutant with a catalytically deficient GS, resulting in reduced activity of GS, which synthesizes protein at the same rate as its parent, as found for *Nostoc* sp. 7801 growing in symbiosis (Kerby et al., 1987).

Fertilization of rice plants under laboratory conditions by application of another MSX-resistant mutant of *A. variabilis,* SA_1, was found to be successful (Lattore et al., 1986). The shorter doubling time of SS_1 and the lack of a lag period at the beginning of the growth cycle as compared with SA_1 (Spiller et al., 1986), which exhibits a lag period, are obvious assets for mass cultivation of the former. These characteristics make possible the production of consid-

erable amounts of inoculum material within a short period. The usefulness of SS_1 as a biofertilizer to rice plants in the actual isolate location, Thailand, should be studied in order to scale up the technology transfer from the laboratory to the rice field.

2. Pesticide-Resistant N_2-Fixing Cyanobacterial Strains

Most earlier studies on the response of cyanobacteria to pesticides, fungicides, insecticides, and herbicides were confined to toxicity levels (Anand, 1989), but later the possible mode(s) of action of many of these chemicals were studied in the different cyanobacterial systems (Vaishampayan et al., 1998d). Although some of the pesticides at doses recommended for field application were not found to affect the cyanobacteria (Goyal, 1989), others proved to be extremely damaging, causing inhibition of N_2 fixation/fixed N assimilation either directly or indirectly through inhibiting chlorophyll biosynthesis, photochemical generation of reducing power, photophosphorylation, or fixed N assimilation (references cited in Table III).

In attempts to genetically improve the N_2-fixing cyanobacteria for developing pesticide resistance, substantial work has been carried out during the last two decades. Astier et al. (1980) were the first to show that *Aphanocapsa* sp. could resist up to 10 ppm concentration of the herbicide diuron. Following this, Tiwari et al. (1981) reported a 100 ppm 2–4,D-resistant mutant of *Anacystis nidulans*. But in both cases the cyanobacteria used were not N_2-fixing. *Nostoc muscorum* is a thoroughly studied filamentous and heterocystous N_2-fixing cyanobacterium in which mutagenesis for resistance to a number of pesticides has been successfully induced: e.g., dithane (Vaishampayan & Prasad, 1981), blitox (Vaishampayan & Prasad, 1982), monuron (Vaishampayan, 1984a, 1984b; Vaishampayan et al., 1992a), diuron (Vaishampayan, 1984c, 1985a), mercury fungicide (Prasad et al., 1986), atrazine (Mishra et al., 1991a), amitrole (Mishra et al., 1991b), carbendazine (Prasad et al., 1991), and sandoz 6706 (Vaishampayan et al., 2000b). In another unicellular N_2-fixing cyanobacterium, *Gloeocapsa* sp., mutants resistant to 10–100 ppm concentrations of the herbicides glyphosate, machete, PCP, and swep were reported (Singh et al., 1987). Genetic recombinations for many characters are now established in N_2-fixing cyanobacteria (Vaishampayan et al., 2000c), which include both interspecific (Vaishampayan, 1984e; Vaishampayan & Prasad, 1984a) and intergeneric (Singh et al., 1987) transfer of herbicide and fungicide resistance markers in the N_2-fixing cyanobacterium *Nostoc muscorum* (involving the *Gloeocapsa* sp. as donor in case of the latter), suggesting that herbicide resistance may be a plasmid-born characteristic (Vaishampayan et al., 1998d), similar to the plasmid-born resistance to the herbicide paraquat reported in the bacterium *Pseudomonas* sp. (Saleh et al., 1989).

Two of the *Nostoc muscorum* mutants, resistant to the herbicide amitrole (3-amino-1,3,4-triazole) and the fungicide carbendazine (2-methoxycarbamoyl-benzamidazole), isolated by Mishra et al. (1991b) and Prasad et al. (1991), respectively, have been tested with rice (Vaishampayan et al., 1996) and found to support an increase in grain yield by 28–32% in three traditional rice varieties—Saket–4, Lanjhee, and Adamchini—and 15–20% in three high-yielding rice varieties—Pant-4, HUR–36, and Saryu-52 (Table IV). These two cyanobacterial mutants were found important not only with respect to their stable, high resistance to the respective agrochemicals but also in view of their nitrogenase-derepressed nature, because they had a higher heterocyst frequency and uninterrupted nitrogen fixation even in the presence of nitrogen fertilizer (see Vaishampayan et al., 1998d). A mutation for carbendazine or amitrole resistance is suspected to have adversely affected the NH_4^+-metabolizing enzyme system enough to inactivate the control factor for the repression of heterocysts (the N_2-fixing

Table III
Biological effects of various pesticides studied in cyanobacteria

Pesticide	Organism	Effect	Sensitivity level (ppm)	Reference
Atrazine	*Nostoc muscorum*	Photosynthetic inhibitor	10.00	Mishra et al., 1991a
BHC	*Anabaena* sp.	Inhibitory to N_2 fixation	50.00	Das, 1977
Blitox	*Nostoc muscorum*	Inhibitory to N_2 fixation	10.00	Vaishampayan & Prasad, 1982
Carbaryl	*Nostoc muscorum*	Mutagenic	10.00	Vaishampayan, 1985d
2,4–D	*Nostoc muscorum*	Inhibitory to N_2 fixation	11.00	Lundkvist, 1970
2,4–D	*Cylindrospermum* sp.	Inhibitory to N_2 fixation	11.00	Lundkvist, 1970
2,4–D	*Nostoc punctiforme*	Inhibitory to N_2 fixation	11.00	Lundkvist, 1970
2,4–D	*Tolypothrix tenuis*	Inhibitory to N_2 fixation	4.50	Hamdi et al., 1970
Diquat	*Nostoc muscorum*	Mutagenic	10.0	Vaishampayan, 1984g, 1984h, 1985b, 1985c
Dithane	*Nostoc muscorum*	Photosynthetic inhibitor	10.00	Vaishampayan & Prasad, 1981
Diuron	*Nostoc muscorum*	Inhibitory to CO_2/N_2 fixation	2.34	Vaishampayan, 1984c, 1985a
Eptam	*Calothrix brevissima*	Inhibitory to N_2 fixation	0.10	Ibrahim, 1972
Hg-fungicide	*Nostoc muscorum*	Inhibitory to CO_2/N_2 fixation	10.00	Prasad et al., 1986
Lasso	*Nostoc muscorum*	Mutagenic	10.00	Singh et al., 1979
Linuron	*Anabaena* sp.	Inhibitory to N_2 fixation	10.00	Das, 1977
Machete	*Nostoc muscorum*	Mutagenic	10.00	Singh & Vaishampayan, 1978
Malathion	Many species	Inhibitory to N_2 fixation	100.0	Da Silva et al., 1975
MCPA	*Nostoc muscorum*	Inhibitory to N_2 fixation	19.00	Lundkvist, 1970
MCPA	*Nostoc punctiforme*	Inhibitory to N_2 fixation	19.00	Lundkvist, 1970
MCPA	*Cylindrospermum* sp.	Inhibitory to N_2 fixation	19.00	Lundkvist, 1970
Molinate	*Tolypothrix tenuis*	Inhibitory to N_2 fixation	25.00	Hamdi et al., 1970
Monuron	*Nostoc muscorum*	Inhibitory to CO_2/N_2 fixation	0.34	Vaishampayan, 1984a, 1984b
Monuron	*Anabaena* sp.	Inhibitory to CO_2/N_2 fixation	0.34	Das, 1977
Paraquat	*Nostoc muscorum*	Mutagenic	10.0	Vaishampayan, 1984g, 1984h, 1985b, 1985c
Propanil	*Tolypothrix tenuis*	Growth stimulatory	0.010	Ibrahim, 1972
Propanil	*Calothrix brevissima*	Growth stimulatory	0.010	Ibrahim, 1972
Propanil	*Anabaena cylindrica*	Growth stimulatory	0.030	Wright et al., 1977
Propanil	*Nostoc entophytum*	Growth stimulatory	0.030	Wright et al., 1977
Propanil	*Gloeocapsa alpicola*	Growth inhibitory	0.005	Wright et al., 1977
Propanil	*Tolypothrix tenuis*	Growth inhibitory	0.100	Hamdi et al., 1970
Propanil	*Calothrix brevissima*	Growth inhibitory	0.100	Ibrahim, 1972
Propanil	*Nostoc entophytum*	Growth inhibitory	0.170	Wright et al., 1977
Propanil	*Anabaena variabilis*	Growth inhibitory	5.000	Wright et al., 1977
Propanil	*Tolypothrix tenuis*	Chl. biosynthesis inhibitor	1.800	Hamdi et al., 1970
Propanil	*Anabaena cylindrica*	Photosynthetic inhibitor	8.000	Wright et al., 1977
Propanil	*Tolypothrix tenuis*	Photosynthetic inhibitor	8.000	Wright et al., 1977
Propanil	*Nostoc entophytum*	Photosynthetic inhibitor	8.000	Wright et al., 1977
Propanil	*Nostoc muscorum*	Photosynthetic inhibitor	50.00	Vaishampayan et al., 1978
Sandoz 6706	*Nostoc muscorum*	Phosphorylation inhibitor	10.00	Vaishampayan et al., 2000b
Trifluralin	*Tolypothrix tenuis*	Inhibitory to N_2 fixation	25.00	Hamdi et al., 1970

compartments) and N_2 fixation (Mishra et al., 1991b; Prasad et al., 1991). These mutants have shown a higher heterocyst frequency and nitrogenase activity than the wild-type parent *Nostoc muscorum*. Furthermore, compared with the wild-type parent, the mutants have shown release of unassimilated ammonium in the exogenous medium at a nearly doubled rate. The latter activity has been shown to be due to a combination of ammonium produced as a result of N_2 fixation and metabolic conversion of nitrate and nitrite to ammonia and accumulation of the latter due to the weak ammonia-assimilating system. Biochemically, apart from N_2 fixation, the nitrogen metabolic pathways leading to the reduction of nitrate and nitrite (through nitrate reductase) is operating well in the derepressed mutants, but, because of the weakening of its ammonia-assimilating pathway, the excess of ammonium is being discharged into the exogenous medium; hence their significant supportive effects on increased grain yield in rice. These results emphasize the acceptability and efficient use of cyanobacterial mutants for economical rice cultivation. At this stage, resistance of at least one representative member from all major chemical groups of pesticides needs to be introduced, one after the other, to the efficient N_2-fixing cyanobacterial species. A systematic approach in this direction will have to be made before this phycotechnology is practically and more vigorously transferred to the rice fields, for some of these reconstructed N_2-fixing cyanobacterial strains are expected to develop cross-resistance to a large number of related agrochemicals that would have wider applicability as viable bio-N fertilizers in wet, agrochemicalized fields.

3. Possibility of Establishing an Efficient Artificial N_2-Fixing Cyanobacterial Symbiosis

Many natural and mutant isolates of N_2-fixing cyanobacteria (NH_4^+ liberating, pesticide resistant, etc.) are now available that would theoretically maximize the combined N available for rice (see Vaishampayan et al., 1998d). Of particular importance are the strains with improved traits of agronomic importance; e.g., pesticide resistance with especially high rates of nitrogenase activity in laboratory studies (see Vaishampayan et al., 1998d) and those that release much of the dinitrogen fixed extracellularly (Spiller et al., 1986). Although the induced mutants (or the rare natural isolates) may be resistant to many abiotic stresses and may release enough ammonia leading to transfer of ^{15}N and hence improved growth of rice in pot experiments (Kamuru et al., 1998), their capacity to compete with the indigenous strains in soil is still questionable under field conditions. As a possible approach, Kannaiyan et al. (1997) have tried to give such special strains a competitive advantage under flood conditions by immobilizing them on polyurethane foam, with the idea that foam-immobilized cyanobacteria could excrete considerable amounts of ammonia into the floodwater, leading to an increase in rice grains and straw yields. But in these experiments, it is questionable whether the strain used, *Anabaena azollae,* was an endosymbiont of the N_2-fixing water fern *Azolla* (Whitton, 2000). Thus, in spite of the fact that some of the genetically improved N_2-fixing cyanobacterial strains perform well under in situ conditions, these do need further improvement to adapt to the various biotic and abiotic stress factors operative in the fields, particularly with respect to strain competition during changes in climatic conditions (Häder et al., 1995; Häder & Worrest, 1997). Obviously, it is a difficult task to develop strains resistant to a host of known (as well as many unknown) biotic and abiotic stress factors that hamper their thorough exploitation as an improved bio-N fertilizer in agricultural fields. Nevertheless, it is suggested, under the circumstances, that such ecologically restricted strains be used as efficient N_2-fixing microsymbionts in artificial symbiosis with wet-field cereal crops; i.e., to develop plant-cyanobacterial artificial symbiosis to shelter genetically improved cyanobacterial strains for

Table IV

Grain yield (kg ha^{-1}) of Saket–4 (after Bhan, 2000) and HUR–36 (after Dey, 1999) rice varieties grown with or without wild-type / mutant *Azolla* application at graded nitrogen levels (N_0 = 0 kg N ha^{-1}; N_1 = 40 kg N ha^{-1}; N_2 = 60 kg N ha^{-1}; N_3 = 80 kg N ha^{-1}; A_0 = without *Azolla*; A_1 = wild-type *Azolla*; A_2 = mutant *Azolla*)

	Rice variety	
Treatment	Saket–4	HUR–36
$N_0 A_0$	337.6	500.0
$N_0 A_1$	510.8	695.2
$N_0 A_2$	586.0	804.4
$N_1 A_0$	659.6	1282.8
$N_1 A_1$	820.4	1434.0
$N_1 A_2$	876.8	1537.2
$N_2 A_0$	932.4	1590.8
$N_2 A_1$	1188.0	1804.0
$N_2 A_2$	1288.8	1574.0
$N_3 A_0$	1240.8	1962.8
$N_3 A_1$	1315.6	2013.2
$N_3 A_2$	1404.8	2073.6
C.D. 5% (*Azolla*)	87.6	130.8
C.D. 5% (nitrogen)	90.0	135.2

constitutive N_2 fixation in close proximity to and for the maximum benefit of the host plant (Sinha et al., 1998; Vaishampayan et al., 1998d).

Construction of several artificial plant-cyanobacterial symbioses is on record (Gusev & Korzhenevskaya, 1990; Sinha et al., 1998), including those of *Nicotiana tabacum* with *Anacystis nidulans, Anabaena variabilis,* and *Nostoc* sp.; *Panex ginseng* with *Chlorogloeopsis fritschii, Anabaena variabilis, Calothrix elenkinii,* and *Nostoc muscorum; Solanum laciniatum* with *Anabaena variabilis* and *Chlorogloeopsis fritschii; Papaver somniferum* with *Anabaena variabilis; Dioscorea deltoidea* with *Anacystis nidulans;* and *Daucus carota* with *Anabaena flos-aquae, Plectonema boryanum,* and *Gloeothece* sp. Attempts have been made in the successful cases to establish interactions for nitrogen metabolites of cyanobionts (cyanobacterial microsymbionts), with the complete exclusion of combined nitrogen from the medium for facilitating gain of fixed nitrogen by plant cells in the presence of artificially introduced cyanobionts (Gorelova et al., 1985).

Still, due to inadequate introduction techniques, many attempts to introduce cyanobacteria into isolated plant protoplasts, including cereals, e.g., rice, the wet-field crop, failed to produce viable systems (Vitousek et al., 1997; Ladha & Pareek, 2000). In fact, while attempting to construct an artificial plant-cyanobacterial symbiosis at the intracellular level, it has to be ascertained first whether it is possible to introduce a N_2-fixing cyanobacterial cell into a plant cell and what the fate of both the partners after fusion is. At the same time, it is important to assess whether the cyanobacterial cell introduced into a plant protoplast will supply fixed N to the plant cell and, if so, whether it is possible to regenerate a stable N_2-fixing plant from the cell. The latter is to be considered even in the case of intercellular interactions in which cyanobacterial cells mixed with plant callus can occupy intercellular space inside the plant tissues.

In case of intercellular interactions the plant rarely contains a microsymbiont in its cells, and thus its association with an N_2 fixer will take place only in individual zones or organs of the plants (Vasil et al., 1977), like the natural symbiotic systems where the cyanobacterial biomass constitutes only 1–2% of the total biomass (Stewart et al., 1983). However, there are both ease and limitations in developing an artificial plant-cyanobacterial symbiosis.

a. Ease in Establishing an Artificial Plant–Cyanobacterial N_2-Fixing Symbiosis

The benefits of introducing the intact cells of N_2-fixing cyanobacteria into plant cells and tissues are immense in the case of intracellular symbiosis. The most pronounced advantage is that the oxyphobic N_2-fixing enzyme, nitrogenase, will remain protected from the irreversible damaging effect of the oxygen generated by the plant cells. Moreover, cyanobacterial photosynthetic energy will be available for performing the function of N_2 fixation, as well as photoassimilation of fixed nitrogen with subsequent transfer into organic compounds of the cells. In view of a variety of specific properties that they possess, cyanobacteria are treated as a suitable partner of plant cells in artificial associations (Sinha et al., 1998). Margulis (1981) discussed the possible role played by the various cyanophytes in the establishment of eukaryotic cells through forming symbiosis with nonphotosynthetic organisms in the evolutionary process. Evidently, with the exception of green algae, cyanobacteria are the most prevalent phototrophs to enter into symbiotic relations with other organisms in nature, establishing exosymbiotic or endosymbiotic associations, a fact that presents valid grounds for their introduction into both isolated protoplasts (Reisser, 1984) and cultured cells (Fogg et al., 1973). In addition to acting as a N_2-fixing component, the cyanobacteria perform a number of metabolic functions in symbiosis with plants that involve excretion of carbohydrates, polysaccharides, peptides, amino acids, organic acids, glycerol, gibbereline-like growth hormones, and vitamins (Wolk, 1973; Shah & Vaidya, 1977; Gibson & Smith, 1982) to help plant-cell culture. The oxygen generated during cyanobacterial photosynthesis is useful for the respiration of the host plant in the symbiosis (Pearl, 1982). Furthermore, cyanobacterial growth rates are comparable with those of plant cells in batch cultures (Street, 1977; Suleimanova & Mineyeva, 1981), which is extremely important, considering the necessary synchrony in growth of the two partners during the establishment of symbiosis (Sinha et al., 1998).

b. Limitations on Establishing an Artificial Plant–Cyanobacterial N_2-Fixing Symbiosis

Despite the points in favor of establishing an artificial plant-cyanobacterial N_2-fixing symbiosis, certain still-unresolved limitations hamper developing all sorts of desired associations. The first serious limitation in this context is the fact that certain cyanobacterial species, belonging to the genera *Nostoc* and *Anabaena,* occur in natural associations with only a limited number of higher plants, displaying a high degree of specificity (Stewart et al., 1983; Gusev & Korzhenevskaya, 1990). In fact, toxins produced by most of the cyanobacteria may affect the higher plant partner (Gibson & Smith, 1982), so selection of a host plant capable of avoiding the effects of such toxins is important (Sinha et al., 1998). The optimum pH values are acidic (5.0–5.5) in plant cells and neutral to alkaline (7–10) in cyanobacteria. The optimum temperature for the growth of plant cells is 30–40°C, which is higher than that of cyanobacteria (24–27°C). The concentration of mineral salts in the medium for growing plant cells is higher than that used for culturing cyanobacteria (Vaishampayan, 1995, 1996). Furthermore, cyanobacteria do not require an organic source in the medium, whereas plant cells re-

quire sucrose and other organic compounds (Stanier et al., 1971; Yeomann & Macleod, 1977). Where there are no such limitations, isolated plant protoplasts have been used to introduce various cyanobacterial species, including *Anacystis nidulans* (Davey & Power, 1975), *Gloeocapsa* sp. (Burgoon & Bottino, 1976, 1977; Hughes et al., 1978), *Anabaena variabilis* (Venkataraman, 1981), and *Nostoc muscorum* (Hughes et al., 1978), of which the latter three are N_2 fixers. Of these, *Anabaena variabilis,* though a phototroph, is capable of chemoheterotrophic growth in the dark (Wolk & Shaffer, 1976). Possibly this ensures the growth of this cyanobiont in the dark, such as during germination of seeds (Meeks et al., 1978). For forming an artificial association in this case, *A. variabilis* filaments are fragmented, and cells are treated with lysozyme to obtain spheroplasts (Agafodorova et al., 1982; Semenova et al., 1982) for their introduction in the preisolated plant protoplasts. Rather following this technique, even an auxotrophic mutant of this N_2-fixing cyanobacterium has been successfully introduced into plant protoplasts to establish a possible dependence of the cyanobiont on the growth and metabolism of the host plant (see Sinha et al., 1998). This clearly indicates that it is possible to combine not only the N_2-fixing natural cyanobacterial isolates but also their rare mutant strains (resistant to various stress factors and capable of constitutive N_2 fixation), which otherwise fail to compete with the native species on inoculation to the fields, in artificial symbiosis with a variety of fruit, vegetable, and cereal crop plants. It would be a significant benefit at the applied level to maintain viability and effective use of the improved cyanobacterial fixed N suppliers through establishing such functional symbiosis.

IV. Agronomics and Biology of the *Azolla-Anabaena* Symbiotic N_2-Fixing Complex

For centuries, *Azolla* has been used successfully to increase rice yields in Vietnam and southern China (Fogg et al., 1973; Watanabe & Liu, 1992). The earliest written record of *Azolla* is in an ancient Chinese dictionary, *Er Ya,* which appeared about 2000 years ago. A Chinese book on agricultural techniques, published by Jia si Xue in A.D. 540, mentioned *Azolla* in relation to applied plant cultivation (Shi & Hall, 1988). Nowadays, *Azolla* is widely accepted as a green-manure biofertilizer in Vietnam and southeastern China (Watanabe et al., 1977; Becking, 1979; Lumpkin & Plucknett, 1982). Recent field studies regarding the use of *Azolla* as a biofertilizer for rice cultivation was given importance only 22 years ago, at the international symposium Nitrogen and Rice, held at International Rice Research Institute in Manila in September 1978. Considering the potential positive impacts of growing rice in association with *Azolla,* the first workshop on *Azolla* for Rice Production was organized by the University of Puerto Rico in November, 1982. Since then a number of investigations have been carried out by the global scientific community on the use of *Azolla* as a potential bio-N fertilizer (Singh et al., 1982; Watanabe, 1982; Bozzini et al., 1984; Diara et al., 1987; Mahapatra & Sharma, 1989; Nierzwicki-Bauer, 1990; Salawar, 1992; Singh, 1994; Vaishampayan, 1994a, 1994b; Vaishampayan et al., 1998b).

A. THE ENDOSYMBIOTIC CYANOBACTERIUM, ANABAENA AZOLLAE, IN AZOLLA

The ability of the endosymbiont *Anabaena azollae* to fix substantial atmospheric nitrogen inside *Azolla* has made the association useful in rice culture in countries like Thailand (Moore, 1969), Indonesia (Brotonogero et al., 1982), India (Singh, 1979), China (Lumpkin, 1982), the Philippines (Watanabe et al., 1977), Vietnam (Tuan & Thuyet, 1979), Taiwan (Lee & Lin, 1981), Brazil (Fiore & Ruschel, 1982), Italy (Espinosa-Abarca et al., 1985), and

Mexico (Ferrera-Cerrato & Romero, 1982) and in West Africa (Reynaud, 1982). The *Azolla-Anabaena* symbiotic N_2-fixing complex has attracted a great deal of attention in order to understand the nature of obligate endosymbiotic association of the cyanobiont, *Anabaena azollae,* with its eukaryotic partner, the macrosymbiont *Azolla.* Obviously, a free-living form of *A. azollae* becomes a prerequisite for a comprehensive study of this kind. Although possibilities of its occurrence in a free-living condition exist, it has not been possible to reassociate it with the fern (see Peters & Meeks, 1989; Vaishampayan et al., 2000c). Efforts to isolate *A. azollae* from the various *Azolla* species have also been well documented (Huneke, 1933; Venkataraman, 1962; Ashton & Walmsley, 1976; Becking, 1979; Newton & Herman, 1979; Tel-Or et al., 1983; Berliner & Fisher, 1987; Meeks et al., 1988), but none of these has been reconstituted with an endophyte-free *Azolla.* In this context, the report of Lin et al. (1988) on an artificial reconstitution of *Azolla-Anabaena* symbiosis from heterologous *Azolla* megasporocarps is worth mentioning. Actually, when the apical cap and indusium were excised from a fertile megaspore apparatus, the plants emerging from such megaspores were cyanobiont free. However, if an apical cap from a different fertile megasporocarp was placed on top of the excised megasporocarp, reinfection of the cyanobacterium associated with the transplanted cap could be demonstrated and verified through SEM, monoclonal antibodies, and nitrogen-fixation assay (Lin et al., 1988). However, using this technique, when free-living *Anabaena azollae* or any other filamentous, heterocystous and N_2-fixing cyanobacterium was allowed to infect an endophyte-free *Azolla* megaspore apparatus, the difference in growth rate eventually resulted in one partner outgrowing the other (Peters & Meeks, 1989; Vaishampayan, 1997). Therefore, efforts to reconstitute functional symbiosis by replacing the cyanobiont chamber with a cultured cyanobacterial isolate have yet to be strengthened for a conclusive answer.

As a stable marker for *Anabaena azollae,* the use of DNA endonuclease RFLP detected by southern hybridization, primarily with cloned nitrogenase (*nif*) structural genes from the free-living *Anabaena* sp. PCC7120, was introduced (Franche & Cohen-Bazire, 1985). A detailed analysis of the sequence divergence based on such hybridization studies with leaf-cavity cyanobionts (microsymbionts) of four species from the subsection Euazolla and five strains of *Azolla pinnata* (belonging to the subsection Rhizosperma) indicated that *A. azollae* associated with the different *Azolla* species belong to a common ancestor owing to a great internal similarity, yet these show slightly divergent evolutionary lines in relation to the Euazolla and Rhizosperma subsections (Franche & Cohen-Bazire, 1987). Other investigations of *Anabaena azollae* isolated from *Azolla filiculoides* (Tel-Or et al., 1983) and two independent isolates from *Azolla caroliniana* (Newton & Herman, 1979; Meeks et al., 1988) indicated that these isolates were identical neither to the associated *Anabaena azollae* in any of the *Azolla* species nor to each other (Nierzwicki-Bauer & Haselkorn, 1986). Meeks et al. (1988) showed how the cyanobiont *Anabaena azollae* from *Azolla caroliniana* has a uniformly contiguous organization of *nif*HDK genes in all the cells. This is quite distinctive if one compares the *nif*HDK genetic pattern of the other *Anabaena azollae* isolates with the free-living heterocyst-forming cyanobacteria (Franche & Cohen-Bazire, 1985, 1987; Saville et al., 1987), because in all these cases the *nif*D gene (coding for the *a* subunit of nitrogenase) in vegetative cells is nearly 11 kbp apart from *nif*K (Golden et al., 1985; Meeks et al., 1988). However, like the situation in free-living cyanobacteria, this gap is excised with the formation of transcribed *nif*HDK operon in the later stages of heterocyst maturation (Haselkorn, 1995).

In an exponentially growing culture of *Azolla* whole plants under controlled conditions, *Anabaena azollae* accounts for nearly 16% of the total chlorophyll and protein of the association (Ray et al., 1978), as well as the optimum contents of phycobiliproteins, phycocyanin,

phycoerythrin, and allophycocyanin (Tyagi et al., 1980; Kaplan et al., 1986). In ex planta conditions, *A. azollae* exhibits a rate of photosynthesis close to that of the free-living cyanobacterial cultures, along with an action spectrum in which the optimum quantum yield occurs in the region of phycobiliprotein absorption (Ray et al., 1979; Kaplan & Peters, 1988). The action spectra of *Azolla* whole plant and endophyte-free macrosymbiont with respect to photosynthesis are so close to green plants that the cyanobiont's contribution is hardly detectable (Ray et al., 1979), for the latter fixes no more than 5% of the total CO_2 fixed by the whole plant (Kaplan & Peters, 1988). However, an alteration in photosynthetic assimilation of inorganic carbon by *Anabaena azollae* in symbiosis has been shown to occur by hitherto unknown mechanism(s) that affect the noncyclic electron transport and/or activity of ribulose-1,5-biphosphate carboxylase. Intermediates of the reductive pentose phosphate cycle during CO_2 photoassimilation are manufactured by both microsymbionts and macrosymbionts (Ray et al., 1979). Sucrose is the primary photosynthetic end product in the whole plant as well as the endophyte-free macrosymbiont but not in the microsymbiont, although it is present as an important component of the soluble carbohydrate pool extracted with boiling water from freshly isolated *Anabaena azollae* (Peters et al., 1985), thereby suggesting that the cyanobiont may be having a photoheterotrophic or mixotrophic metabolism in the mature leaf cavities (see Vaishampayan et al., 1998b). Although the biochemical mechanism(s) of transport/uptake of the photosynthate from *Azolla* to *Anabaena azollae* is not known, transfer of photosynthetically fixed carbon former to the latter has been demonstrated (Kaplan & Peters, 1988).

Only at the cost of photosynthetically generated energy and reductant does the nitrogen-fixing (nitrogenase) activity proceed inside the *Azolla-Anabaena* system. Even the aerobic nitrogenase activity in the dark is fully dependent on the endogenous photosynthetic reserve (Peters & Calvert, 1983). The ultimate dependence of nitrogen fixation on photosynthesis has also been demonstrated by the action spectrum for nitrogenase-mediated acetylene reduction where the rate per incident quantum in both the association and ex planta *Anabaena azollae* is as high in the region of phycobiliprotein absorption as it is in the region of chlorophyll absorption (Tyagi et al., 1981). Thus, nitrogenase activity of the cyanobiont in the mature leaf cavities of the macrosymbiont is presumably supported by photosystem I and cyclic photophosphorylation-mediated energy generated inside the N_2-fixing compartments of *Anabaena azollae;* i.e., the heterocysts (up to 30% of vegetative cells develop into heterocysts during N_2 fixation, as compared with only 5–6% in the case of free-living cyanobacteria) that absorb light energy least effectively harvested by *Azolla* pigments.

Regarding assimilation of fixed nitrogen, it has been established, by the use of N isotopes in pulse-chase experiments, that the freshly separated *Anabaena azollae* releases around 40% of fixed nitrogen in the form of ammonia that is translocated from the mature cavities to the stem apices of *Azolla* macrosymbiont to support the undifferentiated filaments devoid of nitrogenase activity (Kaplan et al., 1986). Ray et al. (1978) demonstrated the activity of three N-assimilating enzymes—glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH)—in crude extracts of the *Azolla* whole plant as well as the endophyte-free microsymbiont. Apparently, *Azolla* has the capacity to assimilate N_2-derived NH_4^+ into glutamate either by the GS–GOGAT pathway (Rhodes et al., 1980) or by the concurrent GS–GOGAT–GDH activity. However, it is now known, based on the kinetics of the incorporation of radioisotopic N into glutamine and glutamate and by the effects of GS/GOGAT inhibitor(s) on this process, that *Azolla* can assimilate well both dinitrogen-derived and exogenous ammonium via GS–GOGAT, with little or no contribution from biosynthetic GDH (Meeks et al., 1987; Peters & Meeks, 1989). It is important to mention here that the catalytic activities of all three N-assimilating enzymes have been detected in crude extracts

of *Anabaena azollae* (Stewart et al., 1980; Uheda, 1986). Using radioisotopic N, it has been established that GS–GOGAT activity is operative inside the microsymbiont, whereas GDH activity is localized in the leaf-cavity trichomes of the macrosymbiont (Meeks et al., 1985). In general, the specific catalytic activity of GS in *Anabaena azollae* preparations has been relatively low, compared with the diverse cultures of free-living *Nostoc* or *Anabaena,* which obviously is an index of lower amount of GS protein in the cyanobiont (Peters & Meeks, 1989).

B. USE OF THE AZOLLA–ANABAENA SYMBIOTIC N_2–FIXING COMPLEX AS A BIOFERTILIZER WITH RICE

The *Azolla-Anabaena* complex is a thoroughly exploited phototrophic N_2-fixing symbiont in waterlogged rice fields (Singh, 1979), and the nitrogen fixed by this most efficient symbiotic cyanobacterial biofertilizer is a readily assimilable source of organic N in the rice-crop system (Kannaiyan, 1993). This is the only water fern (plant-cyanobacterial symbiosis) used in agriculture, supplying 40–60 kg N ha^{-1} $crop^{-1}$ (Plazinski, 1990). In addition to its substantial rates of N_2 fixation, *Azolla* grows rapidly. A shallow, freshwater habitat, such as in a flooded rice field, is the ideal environment for its growth. Moreover, because *Azolla* floats on the water surface, it does not compete with rice for light and space. In a moist climate, *Azolla* grows best under a partial shade of vegetation, which a rice canopy in its early and intermediate stages of growth can easily provide. When rice approaches maturity, *Azolla* begins to die and decompose due to low irradiance under its canopy and to depletion of nutrients, resulting in the nutrient's release in the medium. *Azolla* decomposes rapidly, so the nitrogen it has fixed and the phosphorus and other nutrients it may have absorbed from the water, perhaps in competition with rice, are rapidly released back into the medium and made available for uptake by the rice during grain development (Dey, 1999).

1. Development of the Azolla-Anabaena *Symbiotic N_2-Fixing Biofertilizer in Field Nurseries*

A simple nursery method for vegetative multiplication of *Azolla* has been developed for an easy, large-scale production of this form of efficient N_2-fixing cyanobacterial biofertilizer to be adopted by the farmers (see Kannaiyan, 1993). The field to be used for this purpose is thoroughly prepared and leveled. It is divided into small plots (20 m × 2 m) by providing suitable dams and irrigation channels. Water is maintained at a depth of 10 cm. Ten kg of fresh cattle dung mixed with 20 liters of water is sprinkled over each plot. The cow dung is used to enhance the growth and N_2-fixing activity of the fern-cyanobacterial complex (see Kannaiyan, 1993). Fresh (exponential-phase surface-sterilized laboratory grown) cultures of *Azolla-Anabaena* are introduced into each plot at 8 kg inoculum $plot^{-1}$. Superphosphate (100 g) is applied in three split doses at regular intervals of 4 days as the top-dressing fertilizer for *Azolla*. Granules of the insecticide furadone are applied at 100 g $plot^{-1}$ after a week of *Azolla* inoculation, to protect the nursery against insect-pest infestation, which is rampant particularly in the summer season, coinciding the rice sowing/planting time (Dey, 1999). Nearly 40–55 kg of fresh fronds of *Azolla-Anabaena* symbiotic N_2-fixing biofertilizer is obtainable every week from the nurseries thus prepared and maintained in a cyclic order. Four small nursery plots are required for raising enough biofertilizer material for application to one hectare of rice field.

2. *Noted Gains in Rice through Biofertilization with the* Azolla-Anabaena *Symbiotic N_2 Fixer*

An increase in rice-grain yield of 38–40% has been reported through *Azolla* N incorporation (Reynaud, 1982). At Davis, California, the use of *Azolla* supported an increase in rice yield by 112% over the unfertilized control when applied as intercrop with rice and by 216% when applied both as monocrop and intercrop (Peters, 1978). Tung and Shen (1985) reported 42–55% higher grain yields with *Azolla* over the control (where no *Azolla* was applied). *Azolla* was found to compensate for urea equivalent to 40–50 kg N ha^{-1} (Vlek et al., 1995) and to 30–60 kg N ha^{-1} (Lakshmanan et al., 1997). Supporting evidence also shows increases in rice-grain yield due to the addition of *Azolla* (Singh, 1980; Mandal & Bharti, 1983; Roy, 1984; Mahapatra & Sharma, 1989).

N gain using *Azolla pinnata* in India is around 840 kg ha^{-1} $year^{-1}$ (Singh, 1979). This value differs from species to species of *Azolla* and between locations. Numerous studies have indicated that growth of *Azolla* before or after rice planting is equivalent to the application of 30–40 kg N fertilizer ha^{-1} (Singh, 1977a, 1977b; Rains & Talley, 1979; Watanabe, 1982; Kikuchi et al., 1984; Sisworo et al., 1990; Mandal et al., 1993), 40–60 kg N ha^{-1} (Kannaiyan, 1982, 1992), 30–60 kg N ha^{-1} (Lakshmanan et al., 1997), 70–110 kg N ha^{-1} (Singh & Singh, 1995), 70 kg N ha^{-1} (Watanabe et al., 1981), and 72 kg N ha^{-1} (Yanni, 1992a, 1992b), in the form of urea or ammonium sulfate. Average N_2-fixing rates by various *Azolla* species were in the range of 0.4–3.4 kg N ha^{-1} day^{-1} (Kannaiyan, 1993). According to Watanabe (1985), in rice fields in the Philippines 25 tons of fresh weight *Azolla* ha^{-1} correspond to 45 kg of fixed N ha^{-1}.

Studies demonstrated that of all the *Azolla* nitrogen used by rice, 45–50% was incorporated into rice straw, 30–45% into rice grain, and 10–20% into rice roots (Ito & Watanabe, 1983). According to Mian and Stewart (1984), *Azolla* N applied was released in 60 days, of which 71% was assimilated by the rice plants, 2% remained in the soil as ammoniacal N/nitrate N, and 27% was denitrified/released as N_2.

The use of *Azolla* as a biofertilizer not only increases grain and straw yields of rice but also improves the quality of grain by increasing its protein content (Singh, 1977b; Liu, 1979) and by inducing better growth and early flowering (Singh, 1979). It also increases plant height, number of panicles, number of grains per panicle, weight of panicle, 1000 grain weight, and total chlorophyll content of leaves (Kalita & Sarma, 1994). In addition to having a positive influence on the rice plant, *Azolla* has the potential to curb volatilization of NH_3 following application of urea to a mixed *Azolla*-rice culture. A full cover of *Azolla* can significantly reduce losses of applied urea N from 45% and 50% to 20% and 13% for 30 and 60 kg N ha^{-1} treatments, respectively (Vlek et al., 1995).

According to Mandal et al. (1992), water-holding capacity, porosity, and cation exchange capacity (CEC) of soil increased by 19.9%, 22%, and 8.6%, respectively, at the end of four successive rice crops with the application of *Azolla* biomass at 20 tons ha^{-1} $season^{-1}$. Singh and Singh (1995) found that *Azolla* application improved soil fertility by increasing total nitrogen, organic carbon, and available phosphorus in the soil. Other studies (Satpathy, 1993; Thangaraju & Kannaiyan, 1993) supported these findings. In a low potassium environment, *Azolla* has a greater ability to accumulate potassium than does rice; thus, when the fern decomposes, it acts indirectly as a potassium fertilizer (Van Hove, 1989).

When using *Azolla* as a nitrogen fertilizer, it is important to determine the appropriate inoculum density and time of inoculation for efficient growth and production. If the inoculum density is too low, it can be overgrown by algae and weeds (Watanabe, 1982); if it is too high,

both growth rate and N_2-fixation rates are reduced (Ashton, 1974). In India, the density normally ranges from 0.10 to 0.20 kg m^{-2} of fresh weight of *Azolla* (Singh, 1979). Lumpkin (1987) recommended 500–800 g m^{-2}, followed by subdivision and partial incorporation every 2–4 days. When used as a dual crop, 200 kg fresh weight *Azolla* ha^{-1} has been recommended (Kannaiyan, 1982). Singh and Mandal (1997) reported the highest grain yield, along with uptake of N, P, and K, with the application of *Azolla* at 10 tons ha^{-1} + N_{30} + N_{30} + N_{30} during the dry season and at 10 tons ha^{-1} + N_{15} + N_{15} + N_{15} in the wet season. According to Wagner (1997), there are three primary methods for applying *Azolla* to crops. First, it can be grown as a monocrop during the fallow season and incorporated into the soil before planting the target crop. Second, *Azolla* may be grown as an intercrop among the target crop. Third, natural or deliberately cultured growth of *Azolla* may be harvested from plants, swamps, and so forth and applied to target crops, by incorporation into the soil, as a top dressing, or as combination of both. Reports on time of *Azolla* inoculation vary widely. Watanabe et al. (1989a, 1989b) suggested that the best time to inoculate *Azolla* is 30 days after transplanting for short- and medium-duration rice varieties. Satpathy (1993) recommended *Azolla* inoculation 5 days after transplanting for the highest grain yield at 5.06 tons ha^{-1}. Solaiman et al. (1994) concluded that inoculating it on three dates—7 days before and 30 days and 60 days after transplanting—could reduce urea application rates by 50%. According to Adhikary et al. (1997), the rice yield was highest (3.68 tons ha^{-1}) when *Azolla* was grown together with 37.5 kg N ha^{-1} each of urea and P_2O_5. Mian (1985) stated that the potentially serious losses of nitrogen from *Azolla* could be avoided if their incorporated residues in flooded soils were left no longer than 3 weeks before planting a rice crop. Flooded conditions, high humidity, moderate temperature, and shade extended by the crop canopy provide ideal conditions for multiplication of *Azolla* (Kannaiyan, 1993). The factors that determine decomposability and availability of *Azolla* to rice include species, N and lignin contents, handling before incorporation, and climatic conditions; drying and freezing of *Azolla* reduce decomposability (Watanabe et al., 1977; Kumarsinghe et al., 1986). Doubling time of *Azolla*—a measure of its growth—varies from 2 to 3 days for *Azolla pinnata* (Kannaiyan, 1993) and from 2 to 9 days in other *Azolla* species (Barone-Lumage et al., 1988; Kaplan & Peters, 1988).

C. MUTAGENESIS OF THE AZOLLA–ANABAENA SYMBIOTIC N_2–FIXING COMPLEX TO SERVE AS AN EFFICIENT BIOFERTILIZER

It is important to point out that the combined application of free-living and symbiotic (*Azolla-Anabaena*) cyanobacterial biofertilizers on rice variety CO–41 has been noted to result in a tremendous increase in grain yield of rice, significantly better than the individual application of either of the two forms of cyanobacterial biofertilizer, and the values in each case were further enhanced by the addition of inorganic N fertilizers (see Kannaiyan, 1993). The *Azolla-Anabaena* system is, nevertheless, a better organic N supplier than are free-living cyanobacteria (Singh & Singh, 1989). Yet genetic improvement through mutagenesis, which is now widespread with respect to the free-living cyanobacteria (see Vaishampayan et al., 1998c), is presently in a state of infancy in relation to the symbiotic cyanobacterial biofertilizer; i.e., the *Azolla-Anabaena* symbiotic N_2-fixing complex. The main problem in case of the latter is the selection of an appropriate starting material for clonal mutagenesis, because spores of *Azolla,* harboring the N_2-fixing cyanobacterial germ plasma, seldom germinate under the control conditions (Vaishampayan et al., 1998b). Some success has been achieved in this direction through the use of shoot/frond meristem mutagenesis in the *Azolla-Anabaena* symbiotic N_2-fixing complex (Vaishampayan et al., 2000c).

In fact, despite the well-documented role of the *Azolla-Anabaena* N_2-fixing complex as a biofertilizer, it is strange that cultivation and mass culture are still confined to coastal regions, such as Orissa and Tamil Nadu (Manna & Singh, 1989), and to some other Southeast Asian regions because of its requirement for high relative humidity for vegetative propagation, prevalent specifically at those places. In addition, there are other limitations with respect to the wider adoption of this fern-cyanobacterial N_2-fixing symbiotic complex as a biofertilizer, particularly in Brazil, India, and Pakistan (IRRI, 1987). Mutagenesis of this fern-cyanobacterial N_2-fixing complex is a relatively new area of work; some success has been achieved to make possible the versatile use of this symbiotic cyanobacterial biofertilizer in areas not compatible for growth of the wild material due to one or the other reason described below:

1. Mineral Requirements

Growth of *Azolla* depends on the presence of sufficient concentrations of key nutrients. The most important macronutrients are K, Ca, Mg, and P (Watanabe, 1982). The effect of nutrient deficiencies on growth and nitrogen-fixation rates of *Azolla pinnata* were reported by Yatazawa et al. (1980). The threshold concentration of P for growth is 0.5 mM. The reported minimum P requirement of *Azolla* is 0.4% of its dry weight (Ali & Watanabe, 1986). Sah et al. (1989) reported medium P concentrations between 0 and 0.5 μM and about 1.0 g tissue P kg^{-1} dry weight for optimum growth of *Azolla*. Such P-rich soils are uncommon, and P fertilization limits the growth of *Azolla*.

The P% and N% of *Azolla* increased with the increase of floodwater P until it reached about 0.4 mg l^{-1} P. At a floodwater P content below 0.15 mg l^{-1}, *Azolla* suffered severely from P deficiency (Watanabe et al., 1989a, 1989b). Rakotonaivo and Schramm (1988) observed the requirement of phosphate for *Azolla* growth and deduced that accumulated P in *Azolla pinnata* could be the source of available P. The phosphorus requirement of *A. pinnata* is lower than that of the other *Azolla* species (Kushari & Watanabe, 1991). Although interest in *Azolla* research is expanding in different sectors, use of this source of bio-N is severely limited by its high P requirement (Watanabe & Ramirez, 1984; Kushari & Watanabe, 1991). Vaishampayan et al. (1992a, 1992b) conducted laboratory experiments to culture *Azolla* populations with reduced P requirement in trays, following N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) mutagenesis on excised leaf meristems of the fern. The resulting isolate not only was remarkable for its normal rates of multiplication with a 50% reduced P supplement under N_2-fixing conditions but also proved useful in view of its ability to respond equally well to the various P sources used.

2. Thermosensitivity of Azolla

Temperature is one of the most important environmental factors governing the adaptability of *Azolla,* its growth, and N_2 fixation. The problem encountered with the *Azolla-Anabaena* N_2-fixing complex is primarily that of high temperature (above 40°C) during summer, which coincides with rice cultivation in northern India and other rice-growing countries. The relative humidity also drops below 60%, which causes a severe desiccation problem. Standing water is required throughout the growth cycle, and because the plants have to be propagated vegetatively, the inoculum has to be maintained in nurseries all year long and multiplied for distribution before field cultivation. Thus, in summer the mass culture of *Azolla* and its use with rice as a biofertilizer becomes a difficult problem.

Numerous studies have been conducted on the effect of temperature on the growth and/or nitrogen-fixation rates of *Azolla*. Differences in temperature responses of *Azolla* species and their ecophysiological strains have been reported (Peters et al., 1980; Li et al., 1982; Tung & Watanabe, 1983; Watanabe & Berja, 1983; Zimmermann, 1985a, 1985b). The optimum temperature requirement for *Azolla* is 20–30°C, which is below the average temperature in Tropics (Lumpkin, 1987). Singh (1977a, 1977b, 1979, 1981) suggested that the most favorable temperature for growth and N_2 fixation of *Azolla pinnata* is between 20°C and 30°C. Hechler and Dawson (1995) found that the peak of N_2-fixing activity in *Azolla* species was at 25°C, which sharply decreased at higher and lower temperatures.

Peters et al. (1980) found that tolerance of an elevated temperature was maximal in *Azolla mexicana,* followed by *A. pinnata, A. caroliniana,* and *A. filiculoides.* In another report, by Tung and Watanabe (1983), the relative tolerance of *Azolla* strains to a 37/29°C thermoperiod was *A. pinnata* > *A. microphylla* > *A. mexicana* > *A. caroliniana* > *A. filiculoides.* Watanabe et al. (1989a) reported that *A. microphylla* and *A. pinnata* from Paraguay were most tolerant of high temperatures and had highest the N content.

In northern India, *Azolla pinnata* grows from July to December. In February sporulation starts. It disappears from the ponds in the hot summer months (April and June) at a temperature above 38°C (Gopal, 1967; Vaishampayan et al., 1998c, 1998d). Kannaiyan and Somporn (1989) studied the effect of high temperatures on growth, N_2 fixation, and chlorophyll content of five species of the *Azolla-Anabaena* complex and reported species-dependent variations in chlorophyll content, N content, and growth rate at higher temperatures.

The *Azolla-Anabaena* symbiotic diversity and relatedness of Neotropical host taxa has been stated (Zimmermann et al., 1991). Lin (1992) reported that *Anabaena azollae* plays some role in *Azolla*'s tolerance of higher temperatures, whereas Watanabe et al. (1989b) stated that heat tolerance is determined by both host fern and symbiont *Anabaena.* Tung and Watanabe (1983) reported that exposure of *Azolla* to 37/29°C progressively decreased the heterocyst frequency (nitrogen-fixing sites) in *Anabaena* and hence the N_2-fixing ability of the association, as compared with the situation at 28/20°C, suggesting thereby that high temperature directly affects the microsymbiont. Lai et al. (1988) stated that protein synthesis in the test organism eventually stopped at higher temperatures.

In order to solve these problems with *Azolla,* mutagenesis work has been initiated in this symbiotic complex, not only by genetically improving them to require less phosphorus (Vaishampayan et al., 1992a, 1992b) but also for making them resistant to higher temperatures (Vaishampayan et al., 1998b, 1998c). In laboratory tests these novel mutants have proved their enhanced efficiency as a better N_2-fixing biofertilizer even in the presence of several stresses. Figures 1n–1r are photographs of different mutant strains of *Azolla-Anabaena* symbiotic N_2-fixing complex (*Azolla pinnate* var. *pinnata*).

An *Azolla pinnata* mutant grows well in the laboratory with the 50% reduced P supplement (10 mg l^{-1}), whereas the wild-type strain grows strictly with the normal (20 mg l^{-1}) P supplement (Vaishampayan et al., 1992a, 1992b). P levels are not so absolute for plants (Tisdale & Nelson, 1975), but they appear to be very specific *for Azolla,* for the reduction in P concentration caused the typical P-deficiency morphology (elongation of rhizoids and browning of the material, followed by death of the organism) in the wild-type material. However, no such P-deficiency symptoms were noted in the mutant *Azolla* with the reduced P supplement (Vaishampayan & Banerjee, 1995; Vaishampayan, 1997). The mutant, therefore, appeared to be a definitely improved material with respect to its P requirement. Whether the mutant has a high P utilization efficiency or higher P uptake has not yet been worked out, and

thus it seems to be a fertile area for future biochemical work on the *Azolla-Anabaena* symbiotic N_2-fixing complex.

The mutant strain of *Azolla* was found to grow well up to a temperature of 40°C, whereas the wild-type strain showed a sharp decline in growth when the temperature rose above 25°C (Vaishampayan et al., 1998b, 1998c, 2000a). It appears, therefore, that like in the thermophilic forms of bacteria and cyanobacteria, the mutant *Azolla* has possibly had a considerable degree of intracellular physiological and biochemical specializations as a mutational event, enabling it to tolerate higher temperatures. Thermostability has been reported to be brought about by a high stability of cellular proteins and enzymes (Koike & Katoh, 1979) and membrane systems (Fork & Murata, 1977; Yamaoka et al., 1978) for the proper functioning of the vital life processes necessary for survival, particularly photosynthesis and nitrogen fixation (Watanabe et al., 1989a, 1989b). A large variety of such stable enzymes—e.g., glyceraldehydes-3-phosphate dehydrogenase, alcohol dehydrogenase, phosphofructokinase, superoxide dismutase, and a restriction endonuclease—have been isolated from *Bacillus stearothermophillus* growing at up to 60–65°C, the functional and molecular properties of which are quite similar to those of the respective counterparts from mesophillic microbes, the only difference being that the former are more resistant to heat denaturation (Fontana, 1984). However, qualitative and quantitative estimations of the possible existence of such a heat-tolerant system still needs to be worked out in the mutant *Azolla* at the molecular and biochemical levels—an important area for future studies.

The improved features of mutant *Azolla* enabled it to multiply in field nurseries with a 50% reduced P input at the normal rate just prior to and during rice sowing and planting, when summer temperatures in this belt (Varanasi and adjoining areas) rise much above 40°C (with water temperatures reaching 40 ± 1°C). Rather, the rate of multiplication of this mutant strain of *Azolla* with 7 kg P ha^{-1} was much better than was that of wild-type *Azolla* cultured in field nurseries with the P input of 14 kg ha^{-1} (Dey, 1999). The reason was the temperature sensitivity of the latter, which, unlike the low P-requiring temperature-tolerant mutant, could not tolerate the summer temperature of North India just prior to and during the rice sowing/planting season. This is why wild-type *Azolla* nurseries require a higher labor input for cool watering of the *Azolla* plants twice a day to safeguard their viability and multiplication even at a lower rate: This is essential to running parallel experiments with the mutant *Azolla* in order to assess possible biofertilization activities in the two different rice genotypes, tested recently by Bhan (2000).

3. Use of the Mutant Azolla-Anabaena *Symbiotic N_2-Fixing Complex as an Efficient Biofertilizer*

The comparative results obtained on biofertilization of the rice varieties Saket–4 (a traditional variety) and HUR–36 (a high-yield variety) and wild-type and thermotolerant mutant *Azolla* strains at graded N levels are interesting, for they have shown the supporting effects of biofertilization on grain yields and related physiological traits in both rice varieties (Dey, 1999; Bhan, 2000). Table IV provides data on increases in grain yield through biofertilization with wild-type and mutant *Azolla-Anabaena* bio-N fertilizer. Table V demonstrates the biofertilization effect in terms of supporting percentage increase in rice-grain yield, whereas Table VI represents the N economy achievable as a result of wild-type or mutant *Azolla* application.

A significant increase in rice-grain yield as a result of biofertilization with *Azolla* may be attributed to the higher biomass buildup of *Azolla* inoculated to the experimental rice field

Table V

Increase in grain yield of Saket–4 (after Bhan, 2000) and HUR–36 (after Dey, 1999) rice varieties over respective controls on the application of wild-type or mutant *Azolla*

Variety of rice	Inorganic N level (kg ha^{-1})	Percentage increase in grain yield	
		With wild-type *Azolla*	With mutant *Azolla*
Saket–4	0	51.30	73.57
	40	24.37	32.92
	60[a]	27.41	38.22
	80	6.02	13.21
HUR–36	0	39.04	60.88
	40	11.78	19.83
	60[a]	13.40	24.08
	80	2.56	5.64

[a] Both of the rice varieties responded to *Azolla* maximally at the 60 kg N ha^{-1} level. Although HUR–36 produces a greater yield than does Saket–4, the percentage increase in grain yield of Saket–4 through the application of *Azolla* was higher than that of HUR–36 by 12.26% with wild-type *Azolla* and 12.69% with mutant *Azolla* at the 0 N level; by 12.59% with wild-type *Azolla* and 13.09% with mutant *Azolla* at the 40 N level; by 14.01% with wild-type *Azolla* and 14.14% with mutant *Azolla* at the 60 N level; and by 3.46% with wild-type *Azolla* and 7.57% with mutant *Azolla* at the 80 N level.

that, on mineralization, may have added sufficient organic matter and N to the soil to improve the C:N ratio of the soil. The diazotrophic (N_2-fixing) character of the *Azolla-Anabaena* symbiosis is the other important feature by which *Azolla* adds to soil N fertility, thereby facilitating a better availability of assimilable N to the rice crop for improving yield. Moreover, the *Azolla* canopy prevents light from penetrating to the floodwaters; thus the growth of other phototrophs is inhibited, and photodependent CO_2 uptake is depressed. *Azolla* may be expected to reduce N losses from inorganic sources by lowering ammonia volatilization (Singh, 1977a, 1977b; Hamdi et al., 1980; Varghese, 1990; Vlek et al., 1995). Organic nitrogen from *Azolla,* due to its slow mineralization, may have supplied additional amounts of available nitrogen, which is reflected in higher grain yields. Evidence supporting increases in grain yield through *Azolla* application are on record (Singh, 1980; Barthakur & Talukdar, 1983; Mandal & Bharti, 1983; Roy, 1984; Watanabe, 1985; Mahapatra & Sharma, 1989; Kannaiyan, 1993; Rathore et al., 1995). Furthermore, *Azolla* has been reported to have a lower K absorption threshold than has rice in floodwater (Liu, 1987), which enables it to become a source of K for rice when incorporated, and there have been indications that K is generally a direct contributor to grain formation (Liu, 1987). All of these features may collectively add to an enhanced grain yield in the two rice varieties tested for biofertilization with two strains of *Azolla.*

The mutant *Azolla* has given a statistically significant much better mean performance than has the wild type in both rice genotypes (Dey, 1999; Bhan, 2000). This enhanced yield with mutant *Azolla* may be due to its reduced susceptibility to higher temperature (prevalent during rice cultivation in this area), resulting in larger organic matter supply/N-contribution. In N-unfertilized conditions, gains in percentagegrain yield were quite substantial by biofertilization with wild-type *Azolla* (51.30% for Saket–4 and 39.04% for HUR–36) and even higher with mutant *Azolla* (73.57% for Saket–4 and 60.88% for HUR–36). Such a substantial increase in grain yield with *Azolla* under N-unfertilized condition may be due to the fact that the N released by *Azolla* has been the only available external N source that contributes directly to yield enhancement. Similar results were reported earlier on grain-yield enhancement in rice with

Table VI

Saving of inorganic nitrogen fertilizer due to the application of mutant *Azolla* to obtain the optimum grain yield in Saket–4 (after Bhan, 2000) and HUR–36 (after Dey, 1999) rice varieties

(N_0 = 0 kg N ha^{-1}; N_1 = 40 kg N ha^{-1}; N_2 = 60 kg N ha^{-1}; N_3 = 80 kg N ha^{-1}; A_0 = without *Azolla;* A_1 = wild-type *Azolla;* A_2 = mutant *Azolla*)

Variety of rice	Treatment	Grain yield (kg ha^{-1})	Saving of fertilizer N (kg ha^{-1})	Additional gain due to *Azolla* (kg ha^{-1})
Saket–4	N_{40} A_0	659.6		–
	N_{40} A_1	820.4		160.8
	N_{40} A_2	876.8		217.2
	N_{60} A_0	932.4		–
	N_{60} A_1	1188.0		255.6
	N_{60} A_2	1288.8)	20 kg ha^{-1}	356.4
	N_{80} A_0	1280.8[a])		
	N_{80} A_1	1315.6		74.8
	N_{80} A_2	1404.8		164.0
HUR–36	N_{40} A_0	1281.6		–
	N_{40} A_1	1434.0		151.2
	N_{40} A_2	1537.2		254.4
	N_{60} A_0	1590.8		–
	N_{60} A_1	1804.0		253.2
	N_{60} A_2	1974.0)	20 kg ha^{-1}	383.2
	N_{80} A_0	1962.8[a])		
	N_{80} A_1	2013.2		50.4
	N_{80} A_2	2073.6		110.8

[a] The optimum grain yield obtainable with 80 kg of inorganic fertilizer N ha^{-1} (without biofertilization); the same is obtainable with only 60 kg N ha^{-1} N when biofertilized with mutant *Azolla,* thus making possible a saving of 20 kg N ha^{-1} in both of the rice varieties through the application of mutant *Azolla.*

Azolla by 36.6–38.0% (Barthakur & Talukdar, 1983) and 32.30% (Singh & Mandal, 1997). Under N-supplemented conditions, however, the percentagegains in grain yield with *Azolla* are different. At the 40 N (40 kg ha^{-1}) level, the gains in percentagegrain yield were 24.37% (Saket–4) and 11.78% (HUR–36) with wild-type *Azolla* and 32.92% (Saket–4) and 19.83% (HUR–36) with mutant *Azolla.* The maximum percentagegain in grain yield with *Azolla* under N-fertilized condition was noted at the 60 N (60 kg ha^{-1}) level (27.41% and 13.40% with wild-type *Azolla* and 38.22% and 24.08% with mutant *Azolla* in Saket–4 and HUR–36, respectively). Apparently, this was the result of an appropriate combination of inorganic and organic N that contributed sufficiently to grain-yield increases in both rice genotypes. Accordingly, at the 80 N (80 kg ha^{-1}) level, considerably lower gains in percentageyield were obtained (6.02% and 2.56% with wild-type *Azolla* and 13.21% and 5.64% with the mutant *Azolla* in Saket–4 and HUR–36, respectively). Such a narrow range of increase in percentagegrain yield at the 80 N level may be due to physiological yield stagnation at this N level in the two rice genotypes tested and to a considerable repression of the N_2-fixing machinery of the *Azolla-Anabaena* symbiotic complex, for the enzyme responsible for N_2 fixation is sensitive to higher concentrations of assimilable N (Braun-Howland & Nierzwicki-Bauer, 1990). Evidently, a combination of 60 kg inorganic N ha^{-1} and mutant *Azolla* was sufficient to support the maximum possible gain in additional grain yield of both rice varieties, along with a saving of 20 kg N ha^{-1}.

Azolla was also found to support an increase in total plant biomass at all N levels in the two rice varieties, and the corresponding values were even higher with mutant *Azolla,* suggesting thereby that biofertilization with *Azolla* has a supporting effect on an increase in straw yield as well as grain yield, given that crop biomass includes both (Dey, 1999). This is possible, for *Azolla* contributes a very high quantity of N, together with a large amount of organic matter, to the soil and ultimately to the rice crop, which may not only improve grain yield in rice but also contribute to dry-matter production needed for the nutrition of rice plants (Vlek et al., 1995; Paramanik & Mahapatra, 1997; Sarkar et al., 1997). Evidently, the chlorophyll content and protein, as well as other metabolic processes, depend on the supply of N, which ultimately influences growth and yield. Reports in support of such valid interpretations are on record (Liu, 1987; Singh, 1989). Nevertheless, greater activity of roots, faster division of cells, and rapid accumulation of protein up to the panicle primordial stage may also be responsible for greater biomass buildup and efficient N absorption at the early stages of plant growth. In Saket–4, increases in the number of effective tillers and in panicle length (Bhan, 2000) through biofertilization with wild-type and mutant *Azolla* at all N levels (mutant *Azolla* supporting better than the wild-type *Azolla* at most of the places) suggest an enhanced level of available N to rice through both inorganic and organic (*Azolla*) N. This correlate to some extent with the increase in percentagegrain yield of both rice varieties (Table V). This finding disproves the earlier general belief of farmers that *Azolla* mat during the initial stages of crop growth may have hindered tiller formation. However, in support of the results, increases in the number of effective tillers through *Azolla* application have been demonstrated (Mandal et al., 1993).

Regardless of their genotypic differences, both Saket–4 and HUR–36 responded qualitatively similarly to *Azolla* biofertilization (showing yield increases that were small but significant with wild-type *Azolla* and larger with mutant *Azolla*). A scientific question is, When grain yield increases in both rice varieties after the application of wild-type *Azolla,* what necessitates the use of mutant *Azolla?* Is it only because percentageyield increases supported by mutant *Azolla* are significantly higher than those induced by wild-type *Azolla* in both rice genotypes? The answer to this is easily drawn when we take a comprehensive look at the overall results of this work. First, to acquire the vegetative mass culture of the requisite population size of wild-type *Azolla* (2 tons ha^{-1} for application to the rice field) in the rice-sowing (hot) season involves twice as much labor (daily cool watering of the nursery, etc.) as does the mutant *Azolla,* due to the wild-type high-temperature sensitivity. Second, even after the inoculation of identical population size of wild-type and mutant *Azolla* in rice fields (in parallel sets of experiments), sustenance of the former may not be as smooth as that of the latter, because persistent higher temperatures are injurious to growth and N_2 fixation by wild-type *Azolla.* Clearly, raising the requisite population size of wild-type *Azolla* for field use in summer (coinciding the rice sowing/planting season) is apparently uneconomic/less economic.

Consequently, the recorded saving of N fertilizer and additional gains in rice-grain yield through the use of wild-type *Azolla* may not be that attractive because of the high labor input needed to raise the vegetative population for this purpose. On the other hand, the recorded data on the saving of N fertilizer and additional gains in rice-grain yield through biofertilization with mutant *Azolla* are doubly attractive in view of the low labor input involved in raising the required vegetative population of mutant *Azolla* and the significantly higher gains in grain yield, along with a saving of 20 kg N ha^{-1} for the culture of both rice genotypes with the mutant *Azolla* strain. Concerted efforts in this direction will help not only to screen out the various high-yield and traditional rice varieties responsive to biofertilization with the natural as well as the genetically improved *Azolla-Anabaena* symbiotic N_2-fixing strains but also the

wider use of this extremely potent symbiotic cyanobacterial biofertilizer in areas where its cultivation is limited due to stress factors, particularly P-deficiency and temperature extreme.

4. Efforts to Raise Spore-Mediated Cultures of Azolla-Anabaena *Symbiotic N_2-Fixing Biofertilizer*

Spores of *Azolla* do not readily germinate that could have been the easiest starting material for mass propagation, i.e., a handful of spores (bearing the germplasm of the N_2-fixing cyanobacterial endophyte *Anabaena azollae*) thrown onto the waterlogged fields could have germinated and developed into the necessary quantity of vegetative *Azolla* for inoculation as a bio-N fertilizer with rice without any problem of labor-intensive mass plant propagation and transportation (Braun-Howland & Nierzwicki-Bauer, 1990; Nierzwicki-Bauer, 1990; Vaishampayan, 1994b). Fortunately, the *Azolla pinnata* mutants described above, induced by alkylation mutations, have helped improve the strain, not only with respect to increased temperature tolerance and decreased P-dependence but also, remarkably, in inducing a significant enhancement of sporulation and germination (Banerjee, 1994; Dey, 1999), as compared with the wild-type strain (see Table VII).

In nature, sporulation has been shown to be induced by the interacting effects of a variety of environmental and cultural factors, such as plant density (Kannaiyan, 1978), nutrients (Lales & Murte, 1986), light intensity (Becking, 1979), temperature (Kannaiyan et al., 1988), mat formation (Talley et al., 1977), and strain specificity (Dovan, 1985). The positive effect of some growth hormones in inducing sporulation/spore germination has also been shown (Banerjee, 1994; Kannaiyan, 1994; Singh et al., 1996). In the mutant described above, a proliferation in the number of sporocarps, coupled with the higher incidence of germination, was noted not only at the higher temperatures but also at the 50% reduced P level (Table VII). This indicates that an alkylation mutation in the *Azolla-Anabaena* symbiotic N_2-fixing complex favorably interacts with the factor responsible for either removing the repressor or producing an inducer related to the emergence of sporocarps in this symbiotic cyanobacterial biofertilizer—but this must be substantiated by future sophisticated molecular genetic studies. Moreover, a detailed biochemical analysis of the nature of the repressor or inducer in this natural nitrogen resource would ascertain the exact site and molecular basis of the mutation in relation to sporulation and spore germination as the life-cycle phase of choice in this fern-cyanobiont N_2-fixing mutualistic complex.

Earlier, laboratory experiments on the physiology of spore germination were confined to *Azolla mexicana* (Kannaiyan, 1985), *A. microphylla* (Kannaiyan et al., 1988), and *A. caroliniana* (Singh et al., 1990). Zhang et al. (1990) examined the process of establishing a symbiotic relationship between *Anabaena azollae* and its host during megaspore germination and sporeling development. Most of the *Anabaena* spores adhere to the hair cells arising from the sporelings. Germinating *Anabaena* spores are found in the shoot region and the cavities of the sporeling (92% of them being on or near the hair cells that exhibit the ultrastructural characteristics of transfer cells), suggesting that *Anabaena* spores may receive the chemical signal that stimulates germination or a substance needed to support cell multiplication from the host. Some of the vegetative cells derived from the *Anabaena* spores were differentiated into N_2-fixing heterocysts within the cavity. Hybrids of *Azolla microphylla* (obtained by crossing the male and female sporocarps of different strains within the species) produced significantly higher biomass and chlorophyll content, along with cyanobiont's increased heterocyst frequency, nitrogenase activity, nutrient content, and ammonia-assimilating enzymes (Gopalaswamy & Kannaiyan, 1998). However, work on *Azolla pinnata* was found to be difficult

Table VII

Percentage of sporulation (sporocarps produced per 25 g fresh fronds [after Banerjee, 1994]) and spore germination (per 100 megasporocarps [after Dey, 1999]) in wild-type and mutant *Azolla pinnata* whole plants (bearing the N_2-fixing endosymbiotic cyanobacterium, *Anabaena azollae*) at varying temperatures and graded phosphate levels.

The temperature values presented are the means of 30 random samples ± standard errors. (P–0 = phosphate unsupplemented; P–10 = 10 mg l^{-1} phosphate; P–20 = 20 mg l^{-1} phosphate)

Character	Temperature (°C)	Wild type			Mutant		
		P–0	P–10	P–20	P–0	P–10	P–20
Sporulation	25.0	0	65 ± 1.80	104 ± 0.70	0	116 ± 1.67	120 ± 1.70
	30.0	0	38 ± 0.74	77 ± 1.70	0	111 ± 0.70	116 ± 2.13
	35.0	0	12 ± 0.63	48 ± 0.95	0	106 ± 0.70	110 ± 1.67
	37.5	0	0	0	0	98 ± 1.12	105 ± 0.40
	40.0	0	0	0	0	71 ± 1.00	84 ± 1.58
Spore germination	25.0	0	10.0 ± 0.81	30.0 ± 0.70	0	38.0 ± 1.38	39.6 ± 0.67
	30.0	0	2.0 ± 0.70	17 ± 0.83	0	25.8 ± 0.66	35.8 ± 1.28
	35.0	0	0	2 ± 0.66	0	19.6 ± 0.74	31.6 ± 0.74
	37.5	0	0	0	0	14.2 ± 0.79	22.4 ± 0.86
	40.0	0	0	0	0	5.2 ± 0.37	8.0 ± 0.32

because if the complicated internal structure (glochidia and massullae) of its sporocarps (Braun-Howland & Nierzwicki-Bauer, 1990). But, of course, the increased incidence of sporulation and spore germination in case of the latter, achieved as a result of alkylation mutation (Banerjee, 1994; Dey, 1999), widens the horizon and scope of fundamental and applied studies in *Azolla pinnata*–based cyanobacterial biofertilizer, and it seems promising that sporocarps of this widely distributed, prevalent species of the *Azolla-Anabaena* symbiotic N_2-fixing complex may be used to culture sporophytes as an instant symbiotic cyanobacterial biofertilizer under field conditions, like *Azolla filiculoides,* which is limited to China (Shuying, 1987; Quing-Yuan et al., 1987).

V. Concluding Remarks

The production of nitrogen-fixing cyanobacteria for N-fertilization in rice fields in relatively simple tubular reactors may be a much more attractive way to utilize these agriculturally beneficial organisms. The selection of mutants that continuously release ammonia may also help to overcome some of the difficulties involved in this technology. Strain improvement by genetic engineering/manipulation and tissue culture (for establishing an artificial plant-cyanobacterial symbiosis) of a wide array of luxuriantly available, beneficial N_2-fixing cyanobacteria in tropical wet fields is an area of considerable scope when we think of advanced cyanobacterial types as the biofertilizer components. Strain improvement is needed not only with respect to rapid growth, constitutive nitrogen fixation, and pesticide tolerance under photoautotrophic, photoheterotrophic, and chemoautotrophic conditions but also for the ability to fix atmospheric nitrogen equally well under a wide range of oxygen tension found in rice fields, from aerobic and microaerobic to anaerobic conditions. The nonheterocystous N_2-fixing cyanobacteria need to be critically screened to determine whether they can be included as a cyanobacterial biofertilizer component. An analysis of nitrification and deni-

trification as a result of cyanobacterial biofertilization of agricultural fields can be helpful in obtaining a realistic picture of the actual contribution of available cyanobacterial N to the crop. Furthermore, concurrent with N_2 fixation, the cyanobacteria synthesize and excrete a variety of organic/growth-promoting substances, a detailed systematic characterization of which is still needed to explore their possible role in breaking the physiological yield barrier in rice on application of cyanobacterial biofertilizer at recommended inorganic N levels.

Despite significant progress in fundamental and applied research on the *Azolla-Anabaena* symbiotic N_2-fixing complex—e.g., the molecular biology of symbiosis, the interchange of endosymbiotic N_2-fixing partners, sexual hybridization and mutagenesis for beneficial agronomic traits, and application of this mutualistic association as an efficient biofertilizer with rice—the recognition processes between *Azolla* and *Anabaena* need to be established. Inability to maintain a pure and confirmed ex planta culture of *Anabaena azollae* has probably been a major limitation in this regard, one that needs to be resolved for a clear understanding of the symbiotic process as well as a routine genetic improvement at the microsymbiotic level. Apart from this, constraints persist in relation to ecophysiological research on this form of cyanobacterial symbiosis in the rice soil-water ecosystem. This often causes an inconsistency in the response of rice crops to *Azolla* inoculation. A number of management problems are also associated with the use of *Azolla* in rice culture, and the successful management scheme will vary in accordance with the availability of labor, water, P, propagation space, and rice-propagation schedules. A systematic approach needs to be adopted at smaller levels to manageably implement *Azolla-Anabaena* biofertilizer technology. *Azolla* germ plasma containing various species and worldwide collections are now available at the International Rice Research Institute. On a priority basis, these need to be tested at different locations for their possible inherent ability to resist the various biotic and abiotic stress factors, such as high temperatures in the Tropics (which also indirectly induce greater damage of *Azolla* fronds because of vigorous insect-pest infestation, which worsens with heat), desiccation, salinity, and low phosphorus level for sustenance of their biofertilizer properties in each area. This is particularly important because mutants, resistant to some of these stress factors, may have a competition problem of adaptability on soil inoculation. Numerous reports are now available on *Azolla* spore technology for its potentially more economic, easy, and direct mass propagation in transplanted rice fields, but raising spore-mediated full-fledged *Azolla* sporophytes from the application point of view still lies ahead. More concerted efforts are necessary to make the concept of *Azolla* spore technology practicable for significantly reducing the cost of mass vegetative *Azolla* production and transportation. This would also contribute to easy clonal mutagenesis and interspecific crosses, using potentially germinating spores as the starting material, for developing new *Azolla* with specifically designed traits.

Screening the area-specific rice genotypes that are responsive to the free-living or symbiotic cyanobacterial biofertilizer is essential for good results, because more of the inorganic N can be saved by cyanobacterial biofertilization. Quality-control procedures need to be developed and specified. It has to be kept in mind that the effects of these microbial biofertilizer inoculants on crop growth and yield are seldom dramatically visible in one or two trials. But, of course, what is not visible even after the first year of biofertilization is the fact that the soil is conserved; i.e., for achieving the optimum grain yield, less of the soil N will be lost after cyanobacterial biofertilization. The farmers need to be educated in this regard by close interactions with extension personnel at the users' level, followed by efforts to create a consumer demand for such economic, self-renewable, and soil-healthy biofertilizer by field-level extension workers and the mass media. Our ultimate aim is to supply farmers with inexpensive indigenous biofertilizers and to lessen their need for expensive chemical fertilizers. Thus, any

practice aimed at increasing biological nitrogen fixation through cyanobacterial biofertilization must be acceptable to a large spectrum of farmers. The present status is conservation by supplementation, and the future hope is total replacement of chemical fertilizers with biofertilizers—probably still a long way down the road.

VI. Acknowledgments

A portion of unpublished work incorporated in this article was supported by the University Grants Commission, Government of India, in the form of a career award research project awarded to AV, by the Council of Scientific and Industrial Research, Government of India, in the form of a postdoctoral research fellowship awarded to RPS, and by the Indian Council of Agricultural Research, New Delhi, in the form of an all-India coordinated research project on biological nitrogen fixation awarded to the Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University.

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