6

BIOLOGICAL NITROGEN FIXATION BY ASYMBIOTIC AND NONLEGUMINOUS SYMBIOTIC SYSTEMS

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1. INTRODUCTION

In July 1988 the world's population touched 5 billion which, at the present rate of increase, is likely to double by early decades of the next century. The growing world population is primarily dependent on plants for food. Nitrogen is the most important plant nutrient required in large quantities for crop production. The industrial production of nitrogenous fertilisers is mainly by Haber-Bosch process where nitrogen and hydrogen are combined at high temperature (400 - 500°C) and pressure (200 - 1000 ATM). This process consumes large amounts of energy obtained mainly from fossil fuels. The increasing cost and diminishing availability of this costly input is a major concern in many countries. The continuous use of inorganic nitrogen also endangers soil health and causes problems of nitrate pollution. Search for alternative ways of increasing crop yields has obviously brought the subject of biological nitrogen fixation (BNF) to the forefront. The energy economics is also in favour of BNF because it consumes only half the energy consumed by industrial nitrogen fixation. BNF is highly attractive for developing countries which do not have enough reserves of fossil fuels and are unable to pay high prices for their purchase.

Though about 80 percent of the atmosphere is nitrogen, plants are unable to use it as nutrient since it exists in inert form. Biological nitrogen fixation is the conversion of atmospheric nitrogen by living organisms into forms that plants can use. The process is carried out by a group of bacteria and algae which 'fix' or convert the elemental nitrogen into assimilable forms either in association with plants or in free living state. The conversion of inert nitrogen is facilitated by the enzyme, nitrogenase, present in the nitrogen fixing micro-organisms. The enzyme nitrogenase is coded by a complex of genes known as *'nif'* and to fuel the process bacteria need energy which is obtained either from the organic materials in soil or from the products of photosynthesis of the associated plants.

Of the different systems of biological nitrogen fixation, the *Rhizobium* - leguminous plant association has been reviewed in detail in chapter 7 of this book. As such, this chapter covers only the other major systems.

2. NITROGEN FIXING ORGANISMS AND PLANT ASSOCIATIONS

The nitrogen fixing micro-organisms form either non-symbiotic, associative symbiotic or symbiotic relationship with plants (Table 1). Non-symbiotic or free-living organisms fix nitrogen in soil and their numbers are generally more in rhizosphere of plants. They can be categorised into obligate aerobic, facultative or anaerobic organisms. Although a number of bacteria are reported to be nitrogen fixers in this group, the benefits of inoculation have been proved only with *Azotobacter* and the blue green algae. Associative symbiosis refers to an intracellular relationship between plant roots and a dinitrogen fixing bacterium, *Azospirillum.*

They are found in cortical cells and protoxylem vessels in crops such as *Digitaria, Panicum,* maize, sorghum, wheat, barley, pearl millet and a number of others. In case of the symbiotic nitrogen fixers, the bacteria called rhizobia fix nitrogen in nodules on the roots of leguminous plants. In the symbiotic relationship, the bacteria receive the products of photosynthesis as energy source and, in return, they fix nitrogen from the air for their host. The symbiotic association of cyanobacterium, *Anabaena azollae* and the water fern *Azolla* is a potential source of nitrogen for high yielding rice crop. *Frankia,* an actinomycete form nodules called 'actinorhiza' in woody species of 18 genera, including *Casuarina.*

Table 1. List of nitrogen-fixing micro-organisms in arable soils

Type of association	Microorganisms involved
1. Asymbiotic/Associative symbiotic	
Bacteria	
aerobic	*Azotobacter, Beijerinckia, Derxia, Azomonas, Azotococcus, Methanosinus,*
Acetobacter	
microaerophilic	*Azospirillum, Herbaspirillum, Coryne-bacterium, Thiobacillus*
facultative	*Klebsiella, Bacillus, Enterobacter, Escherichia, Citrobacter, Pseudomonas*
anaerobic	*Clostridium, Propionibacterium, Desulfovibrio, Rhodospirillum, Rhodopseudomonas*
Blue green algae	*Nostoc, Anabaena, Aulosira, Calothrix, Tolypothrix, Chlorogloea, Aphanothece, Schizothrix, Scytonema, Stigonema, Anabaenopsis, Campylonema*
2. Symbiotic	
Bacteria	*Rhizobium* with legumes and *Parasponia* *Azotobacter paspali* with *Paspalum notatum*
Actinomycetes	*Frankia* with woody trees
Algae	*Anabaena azollae* with *Azolla* ,*Anabaena*/ *Nostoc* with *Cycads*, *Nostoc* with *Gunnera* *Lichens* (alga + fungus)

2.1. Azotobacter

Among the heterotrophic free-living nitrogen fixing bacteria, *Azotobacter* is the most intensively investigated genera. *Azotobacters* are present in neutral or alkaline soils and *A. chroococcum* is the most commonly occurring species in arable soils. *A. vinelandii, A. beijerinckii, A. agilis, A. insignis* and *A. macrocytogenes* are other reported species. The number of *Azotobacter* rarely exceeds 10^4 to $10^5\,g^{-1}$ of soil. The isolated cultures of *Azotobacter* fix about 10 mg nitrogen g^{-1} of carbon source under *in vitro* conditions. Apart from its ability to fix atmospheric nitrogen, *Azotobacter* is also known to synthesise biologically active growth promoting substances such as indole-acetic acid (IAA), gibberellins and B-vitamins in culture media. Many strains of *Azotobacter* also exhibited fungistatic properties against plant pathogens such as *Fusarium, Alternaria* and *Helminthosporium.*

The population of *Azotobacter* is generally low in the rhizosphere of crop plants and in uncultivated soils. The occurrence of this organism has been reported from the rhizosphere of a number of crops such as rice, wheat, maize, cotton, sugarcane, bajra, vegetables and plantation crops. A species of *Azotobacter, A. paspali* form specific association with the grass, *Paspalum notatum* which is abundant in many acidic sandy soils of Brazil (Dobereiner and Campelo, 1971).

2.2. *Beijerinckia*

The distribution of *Beijerinckia* is restricted to tropical and subtropical regions (Becking, 1961; Dobereiner, 1961). They are capable of growing on a wide range of pH from 2.9 to 10.0 and higher population of this organism is reported from soils having a pH 4.9 to 7.4 (Becking, 1961).

Beijerinckia indica is the dominant species in acidic soils. *B. fluminensis, B. mobilis* and *B. derxii* are the other species reported from tropical soils. A survey on the distribution of *Beijerinckia* in several Brazilian states demonstrated the presence of this organisms in 97 percent of the soil samples under sugarcane and 60 percent of the soil samples from other vegetation (Dobereiner, 1961). Rhizosphere occurrence of *Beijerinckia* has been reported in rice, sugarcane, forage grasses such as *Digitaria decumbens, Panicum purpurescens, Cynodon dactylon* and *Setaria sphacelata,* plantation crops such as coconut, arecanut, cashew, cocoa and pepper and pearl millet.

2.3. *Azospirillum*

Though this organism was first described as *Spirillum lipoferum* by Beijerinck in 1925, its potential to fix nitrogen was realised only in 1975 by Doberiener and Day. They isolated the organism from the roots of *Digitaria* and described the plant *Spirillum lipoferum* association (Dobereiner and Day, 1976). Tarrand *et al.*, (1978) renamed the organism as *Azospirillum* and described two species, *A. lipoferum* and *A. brasilense*. Though three more species, *A. amazonense, A. halopraeferens, A .irakense* were added to the genus, the world wide distribution and benefits of inoculation have been proved mainly with the first two species. Of the two physiological types within the genus *Azospirillum,* one group has an oxidative metabolism and the other has the ability to ferment certain sugars, producing acid. In addition to their nitrogen fixing ability, certain strains denitrify under anaerobic conditions and could also assimilate NH_4, No_3 or No_2.

Azospirillum is a common soil inhabitant of tropics. The organisms form associative symbiosis with many plants particularly with those having the C_4 - dicarboxylic acid pathway of photosynthesis (Hatch and Slack pathway). They grow and fix nitrogen on salts of organic acids such as malate, succinate, pyruvate or lactate.

The development of white, dense and undulating fine pellicle on semi-solid malate medium is the characteristic of *Azospirillum*.

2.4. Other Bacteria

A number of bacteria capable of fixing nitrogen have been isolated from the rhizosphere and roots of tropical grasses and cereals. They include *Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, Enterobacter agglomerans, Citrobacter freundi, Alcaligenes faecalis, Pseudomonas* sp. *Escherichia intermedia, Bacillus polymyxa, Bacillus macerans* and *Bacillus circulans.* Wani (1985) isolated *Derxia gummosa, Erwinia herbicola, Enterobacter* sp. and *Bacillus polymyxa* from a survey in 200 sites in the traditional millet growing areas in Northwestern India. Kundu and Tauro (1986) found the association of *Bacillus, Pseudomonas* and *Klebsiella* with the roots of wheat, barley and pearl millet. A new bacterium, *Acetobacter diazotrophicus* was recently isolated from certain sugarcane varieties which were capable of obtaining large quantities of nitrogen through biological fixation. The bacterium behaved as an endophyte and possessed unique physiological properties for a diazotroph such as tolerance to low pH, high salt and sugar concentrations, lack of nitrate reductase activity and nitrogenase activity which tolerates short term exposure to ammonia (Boddey *et al.* 1991).

Mishustin and Yemtsev (1982) reported variation in the distribution of different species of anaerobic *Clostridium* such as *C. pasteurianum, C. butyricum* and *C. acetobutylicum* in different agroclimatic zones in USSR. Nitrogen fixing ability of different species varied from 2.6 to 9.5 mg nitrogen g^{-1} of glucose. Application of carbon containing compounds, particularly plant residues, stimulated anaerobic nitrogen fixation in flooded soils sown to rice.

2.5. Asymbiotic Blue Green Algae

The rice fields in India and South East Asia provide a congenial environment for the growth of blue green algae (BGA) comprising of unicellular, colonial and filamentous forms. The nitrogen fixing BGA mainly belong to the orders Nostocales and Stigonematales. Most of the nitrogen fixing BGA possess heterocysts which are large, thick walled empty cells. Evidences suggest that heterocysts are the sites of nitrogen fixation. There are also reports of nitrogen fixation by non-heterocystous forms. The results of an All India Survey which covered 2213 soil samples from rice fields revealed the occurrence of nitrogen fixing BGA in 33 percent samples (Kaushik, 1985). The distribution pattern of algae in different states were : Uttar Pradesh (87%), West Bengal (60%), Orissa (43%), Tamil Nadu (10%), Kerala (9%) and Kashmir (7%).

2.6. Symbiotic Blue Green Algae

Blue green algae form symbiotic association capable of fixing atmospheric nitrogen with fungi, liverworts, ferns and flowering plants. The association between heterocyst forming nitrogen fixing blue green alga- *Anabaena azollae* and the aquatic fern *Azolla* can be a potential source of organic manure and nitrogen in rice production. The fern forms a green mat over water with a branched stem, deeply bilobed leaves and roots. The dorsal fleshy lobe of the leaf contains the algal symbiont within a central cavity. *Azolla* can be applied as green manure by incorporating in fields prior to rice planting. It can also be grown simultaneously with rice seedling after transplanting and incorporated into soil by hand. *Azolla pinnata* is indigenous in Asia, but recently other species such as *A. caroliniana, A. microphylla, A. filiculoides* and *A. mexicana* have been introduced for agricultural use because of higher biomass production.

The alga-fungus association, known as lichen occurs on soils, rocks and tree tops. *Nostoc, Calothrix* and some other genera of BGA are involved in the symbiosis. Nitrogen fixation has been proved by ^{15}N technique in genera of lichens such as *Collema, Sterecaulon, Leptogium, Lichina* and *Peltigera.* Some species of mosses and liverworts have the lower surface of the thallus inhabited by species of *Nostoc.* Yet another example of symbiosis is the occurrence of *Anabaena* and *Nostoc* in distinct zones in the cortex of coralloid nodules in roots of higher plants belonging to *Cycadaceae.*

2.7. *Frankia*

Angiosperms belonging to the several genera such as *Casuarina, Alnus, Myrica, Dryas, Coriaria, Ceanothus, Discaria, Elaeagnus, Hippophae, Shepherdia,* etc. form symbiotic association with actinomycete, *Frankia.* Among the nodulated non-legumes, those species which belong to the genus *Casuarina* have the potential to stabilize eroding land surfaces and to improve the nitrogen status of impoverished soils in addition to providing timber, firewood or charcoal. The nodules first appear as small lateral swellings on the roots but later develop into new lobes at their apices, so that a clustered coralloid structure is formed. In the host cells, the endophyte is seen in the form of hyphae, vesicles and bacteria like cells. Convincing evidence of nitrogen fixation has been obtained in the species of *Alnus, Hippophae, Casuarina, Shepherdia* and *Ceanothus* when studies were conducted with nodulated plants using ^{15}N labelled gas.

3. ESTIMATES OF NITROGEN FIXATION

Both direct and indirect methods have been employed to estimate nitrogen fixation associated with various non-legumes in pot culture and field studies. Direct methods include nitrogen balance studies and ^{15}N based techniques such as ^{15}N isotope dilution and ^{15}N incorporation while acetylene reduction assay (ARA) is the widely used indirect technique.

3.1. ^{15}N Based Methods

^{15}N isotope dilution technique provides the most accurate estimates of the contribution of biologically fixed nitrogen to the nitrogen nutrition of legumes and non-legumes alike. Nitrogen fixation associated with sugarcane, cereals and actinorhizal plants has been estimated by this technique with and without addition of inoculum (Table 2).

Ventura and Watanabe (1983) reported that a substantial proportion of plant nitrogen (32 - 35 %) was derived from associative fixation in uninoculated rice plants under flooded condition in the Philippines. Yoo *et al.*, (1986) observed contribution of 11-19 percent nitrogen by biological fixation when rice was inoculated with *Klebsiella oxytoca*. de Freitas *et al.*, (1984) found that biological fixation contributed 22-26 percent of plant nitrogen in sugarcane. Miranda and Boddey (1987) reported that 24 -39 percent of plant nitrogen was derived from biological fixation in forage grasses. Biological fixation contributed 53 to 65 percent of plant nitrogen when *Casuarina equisetifolia* was inoculated with *Frankia* (Sougoufura *et al.* 1990). Boddey *et al.* (1991) reported that certain sugarcane varieties were capable of obtaining upto 80 percent plant nitrogen from biological fixation.

Nitrogen fixation associated with tropical grasses, sugarcane, rice and sorghum has also been estimated by ^{15}N incorporation technique.

3.2. Nitrogen Balance Studies

Non-symbiotic N_2 fixation to the extent of 18-20 kg N ha^{-1} $year^{-1}$ was reported from long term nitrogen balance studies at Rothamsted, England (Jenkinson, 1977). Substantial positive balances for nitrogen have been reported in pot experiments with sorghum, pearl millet, finger millet and napier bajra at ICRISAT, Hyderabad (Dart and Wani, 1982). App *et al.* (1980) reported positive N_2 balance for flooded soils in pots planted to rice. There was relatively higher level of nitrogen fixation associated with grasses in the tropics when compared to that in temperate countries. Dobereiner *et al.* (1972) reported fixation of 90 kg N ha^{-1} $year^{-1}$ by *Paspalum notatum*. Substantial nitrogen contribution by *Azolla-Anabaena* associations, blue green algae and photosynthetic bacteria to the total nitrogen input of rice has also been reported (Venkataraman, 1975; Watanabe, 1981).

3.3. Acetylene Reduction Assay

This is a simple and sensitive assay for studying the nitrogenase activity of microbial cultures and plants in pot culture and field experiments. Nitrogenase activity has ben reported in many grasses by the excised root assay when the roots were exposed to acetylene after pre-incubation for 8-18 h under reduced oxygen tension (Dobereiner and Day, 1975). Soil root cores removed from the field at harvest have also been used for measuring nitrogenase activity of both grasses and grain crops (Day *et al.* 1975). Wani *et al.* (1984) developed a non-destructive intact plant assay for pot grown plants and it can also be used for screening tube-grown seedlings or bacterial strains in association with plants for their nitrogenase activity. The method has been well adapted for agronomic studies and plant breeding programmes on N_2 fixation in the field. Observations recorded in the IRRI have established that a quantity of nitrogen equal to 50 percent of the nitrogen removed by the crop was fixed in the standard pot, planted with rice where blue-green algae

Table 2. ^{15}N dilution estimates of N_2 fixation associated with various crops

Test Plant		Reference plant	Soil	Percentage	Reference
Species/cultivar	Inoculum	Species/cultivar	treatment	of plant N derived from fixation	
Oryza sativa L. IR 42	Nil	*Oryza sativa* L., IR 42	U, C	32 - 35	Ventura and Watanabe, 1983
Oryza sativa C 5444	*Klebsiella oxytoca*	*Oryza sativa* C 5444	H, C	11 - 19	Yoo *et al.* 1986
Triticum aestivum L. Fielder	*Azospirillum brasilense*	*Triticum aestivum* L. Fielder	U	5 - 11	Kucey, 1988
Saccharum sp. NA 5679, CB 4176	Nil	*Glycine max* (non-nodulating)	--	22 - 26	de Freitas *et al.* 1984
Sorghum sudanense	Nil	*Triticum aestivum* L. (Ticena 2)	--	16 - 39	Vose *et al.* 1982
Panicum maximum (11 ecotypes)	*A. lipoferum* + *A. brasilense* + *A. amazonense*	*Brachiaria radicans* (IRI 442)	U	24 - 39	Miranda and Boddey, 1987.

Reference plants are not inoculated
U - unsterilised, H - heat sterilised, C - covered with black lid
(Adapted from Chalk, 1991)

were spontaneously growing. Alimango and Yoshida (1977) reported nitrogenase activity showing a fixation of 18 - 33 kg N_2 ha^{-1} in Alby province in the Philippines when assayed by ARA method. The ARA was attributed mostly to blue green algae and *Azolla* and no activity was observed when soil was covered with black cloth.

4. CROP INOCULATION

Convincing evidences are available on the contribution of significant amounts of nitrogen to the nutrition of many agricultural plants by biological fixation. Considering the practical significance of such additions, scientific studies have been directed towards augmenting the biological source of nitrogen in agriculture through the manipulation of the organisms, associated host plants and the environmental factors. The most popular approach has been through inoculation, but the responses have been quite variable.

4.1. Inoculum Production

Bacteria

Carrier based microbial inoculants known by the name biofertilisers or bioinoculants are being used for field scale inoculation of seeds. Materials such as powdered peat soil, lignite or powdered farm yard manure are used as carriers to produce *Azotobacter* and *Azospirillum* inoculants. The powdered carrier is neutralised with calcium carbonate, autoclaved and mixed with a broth culture of the bacterium. The mixture is cured in trays for 2-5 days and then packed in polythene bags. The broth culture for inoculum production can be prepared either by growing the cultures in large flasks on a rotary shaker or in batch fermenters depending on the quantity of inoculum needed. The viability of the organisms in carriers is determined by plating the inoculants on agar medium after different periods of incubation. Tilak *et al.* (1979) found farm yard manure: soil (1:1) mixture as the most suitable carrier for *Azospirillum*.

Blue green algae

The inoculum of BGA can be raised by farmers using natural sunlight under conditions simulating rice fields in open air troughs or tanks made of galvanized iron sheet or brick and mortar or pits lined with polythene (Venkataraman, 1972). For a tank of 2m x 1m x 22 cm size, it is necessary to add a mixture of 10 kg soil and 200 g superphosphate and water to a level of 5 cm - 15 cm. Liming is recommended in acid conditions to keep the pH of soil to neutral. The contents of the tank are thoroughly mixed and starter culture of algae can be sprinkled on the surface after a day. A thick algal scum is formed on the surface within 2-3 weeks. Then the contents can be dried and the dried flakes stored in polythene bags.

4.2. Inoculation Methods

A slurry of the carrier based bacterial culture is made in 5 percent aqueous solution of jaggery or sugar and the seeds are inoculated by thoroughly mixing with the slurry. The seeds are dried in shade and sown. In case of transplanted crops, the root system of seedlings are dipped in the slurry before planting. The second and subsequent inoculations, if necessary, are done by pouring slurry in root zone. The inoculants can also be mixed with farm yard manure and applied near the root zone. Subba Rao (1988) suggested the use of 0.5 kg ha^{-1} of *Azospirillum* biofertiliser for seed inoculation of pearl millet and sorghum.

In case of blue green algae, the powdered flakes can be applied as broadcast at the rate of 10 kg ha^{-1} one week after rice transplantation (Venkataraman, 1972).

5. RESPONSE OF CROPS TO INOCULATION WITH BIOFERTILISERS

5.1. *Azospirillum* and *Azotobacter*

Most of the field inoculation studies with non-symbiotic nitrogen fixers have been conducted in the former USSR, Israel, India and Egypt. *Azotobacter chroococcum, Azospirillum brasilense* and *A. lipoferum* were the common species used and the crops covered were mostly cereals such as rice, wheat, sorghum, pearl millet, maize, barley etc. Significant response has been reported in the growth and yield parameters such as plant biomass, nitrogen uptake, grain nitrogen content, number of tillers and grain yield. Negative response has also been reported in few cases. The response to inoculation varied with crops, locations, seasons, agronomic practices, bacterial strains and other soil factors.

The first attempt to test *Azotobacter* as a crop inoculant was reported from the former USSR in 1902, using oats grown in pot cultures. Several experiments conducted there subsequently showed positive benefits of inoculation in pot culture and field conditions. Results of field experiments with *Azotobacter* in that country revealed an average increase in yield of 13.7 % in spring wheat, 15.3 % in winter wheat, 12 % in barley, 15.1 % in oats, 18.7 % in rye, 22.3 % in millet and 14 % in corn (Rubenchik, 1963). Positive response of wheat to *Azotobacter* inoculation was reported in 28 out of 71 field trials in Australia while 4 trials yielded negative results and 39 trials showed no effect on grain yield (Ridge, 1968). One of the problems with *Azotobacter* is its poor performance in colonizing the rhizosphere. In general, only less than 50 percent of the field trials produced positive results. Apart from nitrogen fixation, the benefits from inoculation were also attributed to the production of growth regulators, protection from root pathogens and modification of nutrient uptake by the plant.

Many experiments were conducted in which *Azotobacter* inoculation was done at different levels of application of inorganic nitrogen (Table 3). Crops which received *Azotobacter* inoculation and moderate levels of fertiliser nitrogen gave similar grain yields as that of crops grown under higher levels of fertiliser, but uninoculated. This indicated the possibility of reducing fertiliser nitrogen application also by inoculating with appropriate biofertilisers.

Table 3. Yield response of cereals to *Azotobacter* inoculation at different levels of nitrogen application in the field

Crop	Nitrogen applied (kg ha^{-1})	Grain yield (t ha^{-1})		Increase (t ha^{-1})	Reference
		Control	Inoculated		
Rice	0	4.14	3.91	-0.23	
	60	5.21	5.78	0.57	Shinde, 1965
	120	5.63	6.57	0.94	
Wheat	12	1.58	1.66	0.08	Konde and
	24	2.07	2.40	0.33	Shinde, 1986
	36	2.53	2.97	0.44	
	48	2.91	3.47	0.56	
	60	3.41	4.01	0.60	
Sorghum	0	2.27	2.78	0.51	
	30	2.90	3.44	0.54	Shinde *et al.* 1986
	60	3.24	3.76	0.52	
	90	4.10	4.36	0.26	
Maize	0	3.43	3.60	0.17	Konde and
	33	3.95	5.60	1.65	Shinde, 1986
	66	3.98	6.98	3.00	
	100	4.15	7.07	2.92	
Pearl millet	0	1.79	1.92	0.13	Wani *et al.* 1989
	20	2.43	2.58	0.15	
	100	2.62	2.84	0.22	

In the last two decades, more field trials have been conducted with *Azospirillum* as inoculant. Positive benefits of inoculation with *Azospirillum* have been reported from Egypt, Israel, Belgium, USA, Germany, Pakistan and India. The crops which responded to inoculation include fodder grasses, sorghum, rice, wheat, maize, sugarcane, pearl millet, minor millets, cotton, banana and several vegetable crops. Experimental work in countries other than Australia has shown that inoculation of wheat with *A. brasilense* and *A. lipoferum* can increase significantly yields of foliage and grain. In India, Subba Rao (1979) reported grain yield increase due to *Azospirillum* inoculation in field grown rice at five sites. *A. brasilense* inoculation increased grain yields of pearl millet significantly in six out of nine locations in a multilocational trial in different agroclimatic regions in India. In a similar trial with sorghum, *A. brasilense* inoculation increased grain yields in four out of nine locations (Subba Rao, 1986). Of the 24 experiments with millets using *Azospirillum lipoferum* as inoculant, significant increase in grain yield was obtained in 11 experiments while significantly higher yields were obtained only in 8 of the 24 trials with *Azotobacter chroococcum*. (Wani, 1990). In Israel, significant response to *Azospirillum* inoculation has been reported in C_4 plants such as corn, sorghum, *Panicum* and *Sitaria* (Okon, 1988).

The effect of *Azospirillum* inoculation on the grain yield of cereals has also been tested in the field at different levels of fertiliser nitrogen application (Table 4).

Table 4. Yield response of cereals to *Azospirillum* inoculation at different levels of nitrogen application in the field

Crop	Nitrogen applied (kg ha^{-1})	Grain yield (t ha^{-1}) Control	Grain yield (t ha^{-1}) Inoculated	Increase (t ha^{-1})	Reference
Rice	0	2.93	3.08	0.15	
	30	3.52	3.98	0.46	Rao *et al.* (1983)
	45	3.93	4.54	0.61	
	60	4.45	4.85	0.40	
Wheat	0	3.78	3.90	0.12	Subba Rao
	40	4.54	4.58	0.04	(1979)
	80	4.94	5.75	0.81	
	120	4.95	6.29	1.34	
Pearl millet	0	1.79	1.91	0.12	Wani *et al.*
	20	2.43	2.48	0.05	(1989)
	100	2.62	2.79	0.17	
Sorghum	0	3.26	3.92	0.66	Subba Rao, *et al*
	20	3.92	4.79	0.87	(1986)
	40	4.46	5.38	0.92	
	60	5.12	5.82	0.70	
Maize	0	3.43	3.90	0.47	Konde and
	33	3.95	5.81	1.86	Shinde, (1986)
	66	3.98	7.20	3.22	
	100	4.15	7.27	3.12	

The response to inoculation was reported at all levels of fertiliser addition. However, the degree of response varied at different fertiliser levels. Higher yields were obtained even with higher dose of fertilisers and *Azospirillum* inoculation in certain cases. Tien *et al.* (1979) attributed the yield increases in such situations to the production of plant hormones by *Azospirillum* sp. Savings in nitrogen use to the extent of 42 and 39 kg ha^{-1} have been reported in field experiments with millets and guinea grass, respectively, in Florida, USA when *A. lipoferum* was used as inoculant (Smith *et al.* 1976). Some plantation crops have also responded to *Azospirillum* inoculation. Govindan and Chandy (1985) reported the superiority of *Azospirillum* inoculation over IBA treatment in inducing rooting in black pepper cuttings. Increase in the number of leaves, root biomass, shoot biomass, root length and shoot weight has been reported in cocoa due to *Azospirillum* inoculation (Govindan and Nair, 1987).

5.2. Blue Green Algae

Positive response of rice to inoculation with BGA has been reported in India, China, Japan and Philippines. Inoculations have been effective in increasing grain yields both in the presence and absence of fertiliser nitrogen in different agroclimatic conditions and soil types. Nitrogen transfer from BGA to higher plants has been demonstrated using ^{15}N tracer technique. The production of growth promoting substances by algae is also reported to be an important factor responsible for increasing crop yields. The physical properties of soil particularly those of salt affected areas are greatly improved by algalisation.

Kuksa and Orleanskii (1965) reported 24 percent increase in grain yield in rice due to inoculation of *Anabaena azotica* in China. Venkataraman and Goyal (1968) reported 15 percent increase in grain yield in rice variety ASD^5 due to algalisation in the absence of fertiliser nitrogen. Srinivasan and Ponnaya (1978) found that the algal application increased yields to levels equivalent to that obtainable by addition of 25 kg N ha^{-1}. Field experiments conducted in different states in India demonstrated the effectiveness of BGA application under different agroclimatic conditions and soil types (Table 5).

Table 5. Effect of blue green algal inoculation on grain yield of rice in different states in India

State	N treatment kg ha^{-1}	Grain yield (t ha^{-1}) Control	Inoculated
Orissa	-	2.98	3.71
Bihar	-	2.30	3.06
U.P.	-	3.52	4.36
	60	-	5.76
	120	5.83	-
Maharashtra	-	2.07	2.55
	50	-	3.44
	75	3.44	3.73
	100	-	3.66
Tamil Nadu	50	-	5.11
	75	5.24	5.21
	100	4.70	5.48
Kerala	60	-	3.84
	90	3.56	-

Source : Kaushik, 1985

Algalisation with reduced levels of nitrogen fertiliser application gave the same effect as that with full dose of fertiliser application. The fertiliser responsive high yielding varieties of rice gave 10-15 percent increase in grain yield when inoculation was done with higher levels of fertiliser application (Kaushik, 1985). The results obtained by algalisation of rice fields in several tropical countries revealed the possibility of using blue green algae as a biofertiliser in rice cultivation.

5.3. *Azolla-Anabaena* Association

Azolla is used as a biofertiliser for rice production in several rice growing countries such as India, Philippines, China, Vietnam, North America, West Africa, Sri Lanka and Thailand. Pande (1979) reported that incorporation of 10 t ha^{-1} of fresh *Azolla* was as efficient as basal application of 25- 30 kg N ha^{-1}. Kannaiyan (1984) reported rice yield increase equivalent to that of 30 kg fertiliser nitrogen application due to the application of *Azolla* as a biofertiliser. Experiments conducted in several countries revealed the feasibility of using *Azolla* in combination with fertiliser nitrogen to increase rice yields. Azolla cannot be used in areas where water is not available in plenty. It has been reported that upto 50 percent nitrogen requirement of rice is met by *Azolla* in China (FAO, 1977). *Azolla* is also known to suppress the weed populations in wet land rice and, hence, have an additional economic advantage in rice production technology. Thus, there is tremendous potential to use this fern as nitrogen rich green manure in rice cultivation.

5.4. Combined Inoculation with other Micro-organisms

Inoculation trials with nitrogen fixing bacteria and other beneficial micro-organisms such as phosphate solubilisers and mycorrhiza revealed the synergistic nature of interaction between these organisms. Oblisami (1980) obtained maximum yield increase in rice in simultaneous inoculation with *Azotobacter chroococcum* and the phosphate solubilising bacteria, *Bacillus polymyxa* and *B. Megaterium* when compared to their individual inoculations. Manjunath *et al.* (1981) observed synergistic beneficial effects from inoculation with nitrogen fixing *Beijerinckia mobilis*, phosphate solubilising fungus, *Aspergillus niger* and the VAM fungus, *Glomus fasciculatum* in onion. Seed inoculation of *A. brasilense* and soil inoculation with VAM fungi like *Glomus fasciculatum*, *Glomus mosseae*, *Acaulospora* sp., *Gigaspora margarita* and *Gigaspora calospora* significantly increased the yield and phosphorus content of barley (Subba Rao *et al.*, 1985) when compared to their individual inoculations. Simultaneous inoculation of *A. brasilense* and *G. fasciculatum* was better than their individual inoculations in sorghum. Combined inoculation of nitrogen fixing micro-organisms and VAM fungi enables the plants to derive maximum benefits in their nitrogen and phosphorus nutrition. But VAM fungi have not so far been cultured on synthetic media. This remains as a major constraint in large scale production of mycorrhizal inoculum.

6. INFLUENCE OF ORGANIC AMENDMENTS ON NITROGEN FIXATION

One of the major factors restricting the development and activity of nitrogen fixing bacteria in the tropics is the low organic matter content of the soils. The organic materials applied through the organic amendments serve as carbon and energy source for the nitrogen fixers. Addition of easily decomposable organic compounds leads to an increase in the number of asymbiotic nitrogen fixers and fixation of dinitrogen at significant rates. Incomplete oxidation of organic matter by soil micro-organisms in wet soil might cause the accumulation of organic intermediate products including organic acids which are considered as favourable carbon sources for *Azospirillum*, *Clostridium* and nitrogen fixing rods. Rao (1976) reported enhanced nitrogenase activity in several paddy soils due to the application of paddy straw. Wani (1985) observed 9 and 12 percent increase in yield of pearl millet when *A. lipoferum* and *A. chroococcum* inoculations were done along with the application of FYM at the rate of 5 t ha^{-1} when compared to the response to the treatment of FYM alone. Inoculation of rice with *Azotobacter* along with the application of green manures such as Sesbania, Glyricidia, Sunnhemp and paddy straw increased grain yield by 7 -17 percent when compared to organic amendments alone (Table 6).

Table 6. Increase in yield of rice in response to organic amendment (6 t ha^{-1}) and *A. chroococcum* inoculation

Organic	Grain yield (t ha^{-1})		Increase due to
amendment	Uninoculated	Inoculated	inoculation (%)
Unamended	3.14	3.53	12.4
Sesbania	3.84	4.12	7.3
Glyricidia	3.53	4.11	16.4
Sunnhemp	3.47	3.86	11.2
Paddy straw	3.09	3.61	16.8

Source : Prasad, 1986

The treatment of *A. chroococcum* + 60 kg N + 7.5 t glyricidia gave significantly higher grain yield in rice when compared to the treatment of 90 kg N ha^{-1} (Prasad, 1986). The grain yield increase obtained in rice due to the application of 60 kg N ha^{-1}, FYM (10 t ha^{-1}) and *Azotobacter* was equal to that obtained with 120 kg N ha^{-1} (Jagtap and Shingte, 1982).

7. PLANT BREEDING AND SELECTION FOR ENHANCED NITROGEN FIXATION

It has been suggested that associative nitrogen fixation is under genetic control of the host plants. Varietal difference in nitrogen fixation has been observed in lines of pearl millet (Wani, 1988), wheat (Boddey and Dobereiner, 1984), sugarcane (Ruschel and Ruschel, 1981), sorghum (Wani, 1988) and rice (Watanabe, 1981). By using ^{15}N dilution technique, Wani (1988) brought out the variation in nitrogen fixing potential of six lines each of sorghum and millet (Table 7). Watanabe (1986) suggested that screening for rice varieties having a great stimulatory effect on nitrogen fixation would be the most efficient way of harnessing the biological nitrogen fixation. Urquiaga *et al.* (1989) suggested that it might be possible to breed for high nitrogen fixing sugarcane varieties using *Saccharum spontaneum* cv. Krakatau as donor parent. The idea of breeding for enhanced associative nitrogen fixation is an attractive one for high value crops in tropical countries. Large scale planting of cultivars or ecotypes which are reported to have high nitrogen fixing potential in tropical farming systems can provide long term economic advantage, particularly in soils of low nitrogen status.

Table 7. Variation in nitrogen fixing potential of different genotypes of sorghum and millet when determined by ^{15}N dilution technique

Genotype	% N fixed in relation to IS 3003	mg N fixed in relation to IS 3003	Genotype	% N fixed in relation to ICH 107	mg N fixed in relation to ICH 3003
Sorghum					
IS 801	27	49	D 180	17	22
IS 84	22	40	PHB 12	16	19
CSV 5	21	37	Melzongo	14	15
IS 2980	17	26	IP 2787	11	14
IS 5218	13	22	DS 395	6	6
IS 3003	0	0	ICH 107	0	0

Source : Wani, 1988.

8. CONCLUSIONS

In future, it will be difficult to rely on industrial production of nitrogenous fertilisers as nitrogen source in crop production since the process is energy intensive and depends on the fossil fuels which are getting depleted at a fast rate. Biological nitrogen fixation holds promise as an alternative source of nitrogen. Convincing evidences have been obtained on the contribution of significant proportion of nitrogen to the nitrogen nutrition of many agricultural plants by biological fixation. Field experiments conducted in several countries demonstrated the potential of nitrogen fixing bacteria and blue green algae as biofertilisers in crop production. The *Rhizobium*-leguminous plant association is the most efficient and reliable nitrogen fixing system capable of contributing significant quantities of nitrogen in tropical and temperate conditions. Eventhough several non-symbiotic bacteria are reported to be capable of fixing atmospheric nitrogen, the potential of only *Azotobacter* and *Azospirillum* has been recognised in crop production on the basis of large number of field trials in different countries. Blue green algae and *Azolla-Anabaena* association can form an important nitrogen source in tropical rice fields. The possibility of reducing fertiliser application by using biofertilisers has also been shown in several crops. Incorporation of organic manures and agricultural wastes can go a long way in improving non-symbiotic nitrogen fixation and the soil fertility status. It is now necessary to develop strains of bacteria suitable for different crops in different agroclimatic conditions and soil types to fully harness the benefit from the nitrogen fixers. These biofertilisers are highly attractive in developing countries where low external input agriculture is promoted.

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