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Contribution of non-leguminous biofertilizers to rice biomass, nitrogen fixation and fertilizer-N use efficiency under flooded soil conditions

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Abstract

A field experiment was conducted to study the effect of root-associated diazotrophic rhizobacteria and *Azolla*, alone and in combinations, on rice grown in a flooded saline soil. ^{15}N labelled ammonium sulphate at the rate of 30 kg N/ha was used to monitor its recovery and computation of biological nitrogen fixation (BNF) using an isotopic dilution method. The bacterial population of nitrogen-fixing bacteria generally increased more for inoculated treatments compared with uninoculated ones. The rice biomass (straw + grain) was maximum for *Azolla* cover followed by the bacterial treatment. The fertilizer-N recovery in rice was maximum for *Azolla* cover + bacteria followed by *Azolla* cover treatment, which may be due to lowering of floodwater pH by *Azolla*. The maximum amount of BNF (46 kg N/ha) was obtained for bacterial treatment, followed by bacteria + *Azolla* incorporated treatment. The study indicated that use of biofertilizers, along with a low input of chemical-N fertilizer, is useful for increasing rice yield, fertilizer-N use efficiency and BNF in rice grown in flooded soil conditions.

Introduction

Among fertilizer inputs, nitrogen is the major limiting nutrient for crop production. It can be supplied through chemical or biological means, but the production of chemical nitrogen fertilizer is energy intensive as 2 tonnes of fuel oil are consumed for production of 1 tonne of fertilizer nitrogen (Regan, 1988). Both the legume and non-legume nitrogen biofertilizers can convert atmospheric nitrogen to plant-usable form through biological nitrogen fixation. Despite the high N_2-fixing potential and positive effect on soil properties, the use of green

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manure legumes for lowland rice has declined dramatically world-wide over the past 30 years, and at present they cover less than 5 million ha in tropical Asia (Becker *et al.*, 1995). Most of the rice in the world (128 million ha rainfed lowland and irrigated vs. 19 million ha upland) is grown in flooded conditions (IRRI, 1994). The legume green manures have certain weaknesses for the rice ecosystem, as most of them do not grow or fix atmospheric nitrogen in flooded conditions. Unlike legumes, non-leguminous biofertilizers such as *Azolla* and rhizobacteria are adapted to flooded conditions of lowland rice and do not need separate time, water and land for their multiplication, as *Azolla* can be grown on the water surface as an intercrop with rice, while rhizobacteria can rapidly multiply in the rhizosphere.

Based on data from various workers, Peoples *et al.* (1995) estimated that *Azolla* can fix 22–40 kg N/ha per month, while driving 52–99% of its nitrogen from the atmosphere. Its growth rate is very high (3–5 days under optimal conditions), and its long-term use not only increased rice yield but also improved soil fertility (Ventura and Watanabe, 1993). *Azolla* has been used traditionally as green manure for rice production in Southeast Asia and is still considered an important biofertilizer for rice crops (Lumpkin and Plucknett, 1982; Roger *et al.*, 1993; Peoples *et al.*, 1995). The application of *Azolla* has been reported to increase rice yield by 0.4–1.5 t/ha over the control, in most of the experimental sites in China, Vietnam, India, Thailand, Philippines and USA (Kikuchi *et al.*, 1984).

According to Frankenberger and Arshad (1995), many of the root-associated bacteria not only fix nitrogen but also produce some metabolites which influence the growth of the plant. Okon (1981) inoculated corn with nitrogen-fixing bacteria and found 77 kg more N/ha than in uninoculated plants. Considering all the data of inoculation with nitrogen-fixing and plant growth-promoting rhizobacteria, an average increase in rice grain yield was estimated to be 28% for pot and 14% for field conditions (Roger *et al.*, 1993).

About 14 million acres of land are salt-affected in Pakistan. Since rice can grow under varying degree of flooding, and has shown some salt tolerance, most of the salt-affected soils, whether reclaimed through chemical or biological means, are invariably sown to rice as the first crop. These soils are usually saline sodic, and due to a high floodwater pH there is a significant loss of applied fertilizer-N through ammonia volatilization (Hussain and Malik, 1983). The loss of applied mineral N in lowland rice, calculated on ^{15}N balance by various researchers, was up to 62% (Becker *et al.*, 1995) and a recovery of only 30–40% of applied N by rice has been reported in paddy soils (Cooke, 1982; Crawswell and Vlek, 1979).

Although sufficient work is reported on the nitrogen fixation by *Azolla* and root-associated nitrogen fixing bacteria only meagre information is available on the conjoint use of these biofertilizers and particularly in saline soils. We therefore tried to use these bacteria and *Azolla* alone and in different combinations to study the effect on rice yield, nitrogen fixation and fertilizer-N use efficiency in a flooded saline soil using ^{15}N labelled fertilizer.

Materials and methods

In this field study a marginally saline soil was used in the cemented microplots, and the chemical analysis of this soil is presented in Table 1. The total N was estimated with the regular Kjeldahl method, and KCl extractable NH_4 and NO_3-N by using MgO-Devarda alloy steam distillation methods (Keeney and Nelson, 1982).

The soil was put into 1.5 × 1.5 × 0.6 m (LxWxD) cemented plots and was flooded for a few days before rice transplanting. The field experiment was carried out in a randomized complete block design, with four replications of 11 treatments (Table 2), having ^{15}N-labelled ammonium sulphate in T6–T11.

For bacterial inoculation a mixture of previously isolated root-associated bacteria (Malik *et al.*, 1994); comprising *Azoarcus* (K-1), *Flavobacterium* (96–57), *Pseudomonas* (96–51) and *Azospirillum* (N-4) was used. These bacteria were grown in semisolid nitrogen-free culture medium, and suspension of each strain was prepared at 10^8 cells/ml. Then an equal volume of these suspensions was mixed for inoculation of rice. The inoculum was applied to rice by dipping the roots of the seedling for ½ h before transplanting.

One-month-old seedlings of rice (NIAB-6) were transplanted at two seedlings per hill with 20 × 20 cm spacing. Seven days after rice transplanting (DAT), mixed culture of *Azolla* spp.; comprising *A. pinnata var. pinnata, Rong Ping* (hybrid *Azolla*), *A. microphylla, A. caroliniana, A. filiculoides,* and *A. pinnata* (local); was inoculated at 177 g (f.w.)/m^2.

To enhance *Azolla* growth superphosphate was applied at 5 kg P_2O_5/ha in *Azolla*-inoculated plots and also in all the other plots. ^{15}N-labelled ammonium sulphate (5% abundance) was applied in a single dose, in solution form at 3–4 cm below the soil surface 7 DAT at 30 kg N/ha for treatments 6 to 10, and at 60 kg n/ha for treatment 11, which was kept as the non-fixing reference treatment.

To study the colonization of inoculated diazotrophic bacteria on rice roots the most probable number (MPN) and direct plate count methods, using combined carbon medium (CCM), in vials and plates were used (Rennie, 1981). For enumeration of these bacteria in soil and associated with rice roots, serial dilutions in saline solution (0.9%) were prepared and 100 µl from every dilution was inoculated into vials and plates (Bilal *et al.*, 1990).

Table 1. Chemical properties of the experimental soil.

EC (saturation extract)	4.87 ds/m
pH (soil paste)	7.8
K (saturation extract)	0.15 mEq/L
Na (saturation extract)	66.0 mEq/L
Ca (saturation extract)	4.1 mEq/L
Total N	0.04%
Available NH_4-N	11.5 mg/kg
Available NO_3-N	13.4 mg/kg

Table 2. Effect of biofertilizers on dry matter and nitrogen yield of rice

Treatment	Dry matter yield (kg/ha)			Nitrogen yield (kg/ha)		
	Straw	Grain	Straw+grain	Straw	Grain	Straw+grain
T1. Control	7556^{b}	7583	15139^{ab}	40^{ab}	112	152^{a}
T2. *Azolla* cover	7265^{b}	7691	14956^{ab}	37^{bc}	108	145^{ab}
T3. *Azolla* incorporated	7786^{ab}	8181	15967^{ab}	41^{ab}	109	150^{a}
T4. Bacteria	7392^{b}	7656	15048^{ab}	37^{b}	107	144^{ab}
T5. *Azolla* cover + bacteria	7470^{b}	7752	15223^{ab}	40^{ab}	108	148^{ab}
T6. 30 kg N/ha	7657^{ab}	7884	15541^{ab}	41^{ab}	110	151^{a}
T7. 30 kg N + *Azolla* cover	8433^{a}	7781	16214^{a}	43^{a}	110	152^{a}
T8. 30 kg N + bac. + *Azolla* incorporated	7814^{ab}	7878	15691^{ab}	43^{a}	110	153^{a}
T9. 30 kg N + bac. + *Azolla* cover	7619^{ab}	7710	15329^{ab}	42^{a}	111	153^{a}
T10 30 kg N + bacteria	8449^{a}	7753	16202^{a}	44^{a}	113	157^{a}
T11. 60 kg N/ha	7044^{b}	7732	14776^{b}	33^{c}	103	136^{b}
		N.S.			N.C.	

^{15}N-labelled ammonium sulphate was applied to soil for treatments T6–T11.
Means followed by the same letter are not statistically different a 5% *P*.

For identification of inoculated bacteria, morphological methods (shape, size, colour of colony) and biochemical tests (using QTS strips), were used (Bilal *et al.*, 1990). In addition to these tests, strain-specific fluorescent antibody (FA) stains for the four bacterial strains were prepared in rabbits according to Somasegaran and Hoben (1985). The quality and specifity of FA was tested according to Schmidt (1974), and used for identification of inoculated strains. To minimize non-specific binding, the root was covered with gelatine/rhodamine/isothiocynate conjugate as described by Bohlool and Schmidt (1968).

In case of *Azolla* cover treatments *Azolla* was allowed to grow without incorporation, while in *Azolla*-incorporated treatments approximately half of the *Azolla* was incorporated into soil when it grew to full cover, and in total two incorporations were made, being at 55 and 91 DAT. When plots were completely covered with *Azolla*, the fresh weight of *Azolla* was recorded by using the quadrate method. At 1 week after first incorporation, pest attack was observed on *Azolla* and Furadan (1 kg carbofuran/ha) as granules was broadcast onto all *Azolla* as well as other plots to kill the insects. After 4 days of Furadan application, superphosphate (5 kg P_2O_5/ha) was again applied to all the plots, to enhance *Azolla* growth.

At maturity, 36 central rice hills of each plot were harvested, and one outermost line on each side was left as border. Total fresh weight of straw and grain was recorded and then dried at 70°C for dry weight. The ^{15}N analysis was performed by converting ammonium to dinitrogen with alkaline sodium hypobromite (Hauck, 1982) using a mass spectrometer with an inlet system (Mat GD150).

Results and discussion

Bacterial population of rhizosphere and rice roots

The population of nitrogen-fixing bacteria in rhizosphere-soil of rice, estimated by the ARA-based MPN method, increased more in inoculated treatments (T8 and T10) than in uninoculated (30 kg N/ha) treatment (T6) (Figure 1). The bacterial number increased with rice growth period for nitrogen (T6), and nitrogen+*Azolla* incorporated+bacteria (T8), nitrogen+bacteria (T10) treatments; but decreased for nitrogen+*Azolla* cover+bacteria (T9) treatment (Figure 1), which may be due to differences in bacterial ecology such as lesser diffusion of air/nitrogen into soil, through the *Azolla* mat.

The bacterial population on rice roots, also enumerated by the ARA-based MPN method, showed higher numbers for inoculated treatments than only nitrogen, at the initial stage, but it decreased with time during the rice growth period (Figure 1). The decline in population after inoculation has also been reported by other workers (Albrecht *et al.*, 1983; Chan *et al.*, 1963).

The population of inoculated bacteria, as shown by FA staining, showed that in rhizosphere soil it was generally higher for inoculated treatments than uninoculated control at panicle as well as at maturity stage of rice (Table 3).

The FA staining of roots of rice showed higher numbers for inoculated than uninoculated treatments for K-1, N-4 and 95–57 strains, whereas the population of 96–51 was not found at panicle and maturity stage of rice (Table 3). The absence of *Pseudomonas* strain 96–51 on rice roots may be due to its poor survival, or competition, or the antagonistic effect of other microorganisms in the soil (Chan *et al.*, 1963).

The above-mentioned bacterial population and ARA studies indicated that inoculation of nitrogen-fixing bacteria into rice and rice–*Azolla* culture helped in increasing the desirable bacterial population in rice ecosystem.

Azolla *biomass and N content*

Periodic visual observations indicated that the growth of inoculated *Azolla* increased after application of phosphorus. The fresh *Azolla* biomass, recorded after 49 days of its inoculation, was 0.4–0.8 kg/m^2 (4–8 t/ha), whereas the average *Azolla* biomass was reported to be 1.1 kg/m^2 in INSFER trials of various countries (Watanabe, 1987). In this study nitrogen content in the *Azolla* mat was estimated to be 6–15 kg/ha, and slightly higher N contents were observed for treatments having *Azolla* alone, or for *Azolla*+ nitrogen than for *Azolla*+bacteria. Kikuchi *et al.* (1984) have reported that in open fields one layer of *Azolla* containing 10 tonnes of green matter may have about 25–30 kg N/ha. The lower values for fresh biomass and nitrogen recorded in the present study may be due to the adverse effect of high summer temperature and high sodium or salts in this soil, as high temperature (Cary and Weerts, 1992) and salinity reduces the growth and nitrogen fixation in *Azolla* (Ali *et al.*, 1990).

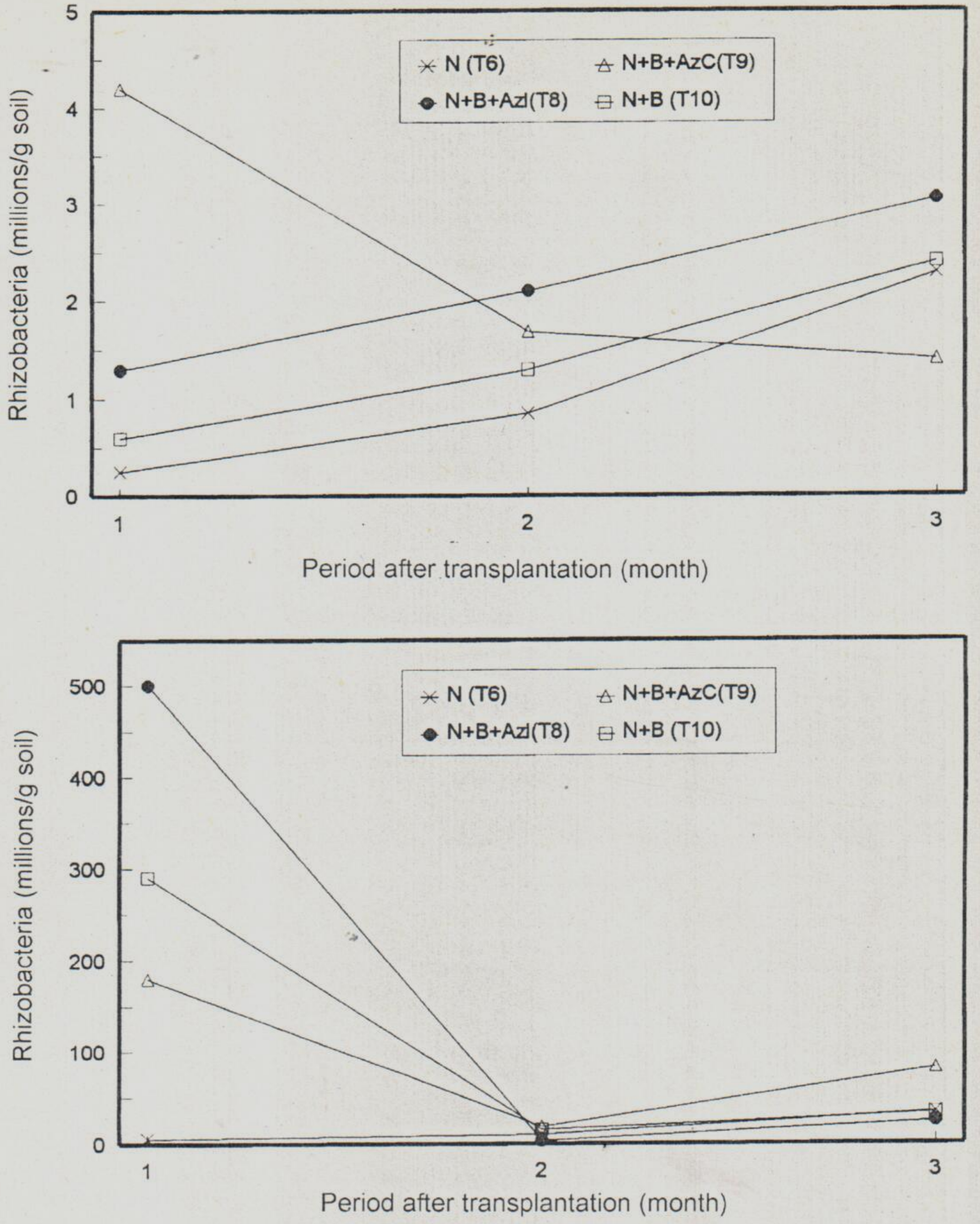

Figure 1. Diazotrophic bacterial population in the rhizosphere soil (top) and on the rice roots (bottom). (N=30 kg N/ha, B=rhizobacteria, Azl=*Azolla* incorporated, AzC=*Azolla* cover)

Rice yield

The rice yield (straw + grain) was maximum for nitrogen+*Azolla* cover (T7) and nitrogen+bacteria (T10) and minimum for 60 kg N/ha (T11), while a higher grain yield was observed for T3 (Table 2). The higher rice yield for the above-mentioned biofertilizer + 30 kg N/ha treatments indicates the usefulness of *Azolla*

Table 3. FA staining of rice roots from control and PGPR-inoculated plots at different stages of rice growth

Rice growth stage	Bacterial FA stain used			
-	K-1	N-4	96-51	96-57
Rhizosphere soil				
Tillering				
Control	ND	ND	ND	ND
Inoculated	5–10	5–10	5–10	5–10
Panicle				
Control	ND	ND	5–10	5–10
Inoculated	5–10	11–50	5–10	11–50
Maturity				
Control	ND	ND	5–10	ND
Inoculated	11–50	11–50	11–50	11–50
Rice roots				
Tillering				
Control	ND	ND	ND	ND
Inoculated	5–10	5–10	5–10	5–10
Panicle				
Control	5–10	5–10	ND	5–10
PGPR	11–50	11–50	ND	5–10
Maturity				
Control	5–10	ND	ND	ND
PGPR	100–200	50–100	ND	5–10

Number of bacteria/microscopic field is given; at least 10–12 fields were observed (ND = not detected)

and bacteria for enhancing rice biomass and grain yield. The positive effect of *Azolla* on rice yield has been observed in various countries (Kikuchi *et al.*, 1984, Kumarasinghe and Eskew, 1993) and its use with chemical-N fertilizer is also considered feasible (FAO, 1988). The total nitrogen accumulated in rice plants was slightly higher for nitrogen+bacteria (T10), indicating a useful effect of bacteria on nitrogen uptake by rice.

The benefit of *Azolla* for increasing rice yield was reported to be positively correlated with the number of its incorporations and the amount of incorporation, and a greater increase in rice yield was observed for *Azolla* incorporated before rice transplanting than after transplanting (Kikuchi *et al.*, 1984; Watanabe, 1987). The very low positive effect of *Azolla* on rice yield in our experiment may be due to suboptimum *Azolla* growth, and only two incorporations made during the later stage of rice growth.

There was not much increase in rice yield even for fertilizer-N application at both 30 and 60 kg N/ha as normally observed for fertilizer application. The overall low response to biofertilizers and chemical-N fertilizer may be due to the high levels of available NH_4 and NO_3-N in this soil (Table 1). Recently, Hussain *et al.* (1994) have estimated the N availability index of 50 different local soils, and they have concluded that 18 and 21 mg N/kg soil, of KCl-extractable NO_3-N and (NH_4+NO_3)-N respectively; were the critical levels. In our soil NO_3-N was 13 and (NH_4+NO_3)-N was 25 mg/kg, thus it is very likely that this soil was not much deficient in nitrogen and therefore a very significant fertilizer response was not observed. The high total N content of rice (152 kg N/ha) and a very high rice grain yield (7.6 t/ha), even in control (Table 2), also indicates that sufficient nitrogen was available in this soil; as removal of about 123 kg N/ha has been reported by a similar crop (7.9 t/ha) in a tropical country (De Datta, 1981). The high level of available-N in this soil may be due to the fact that soil used in this experiment was left uncultivated for some period.

Fertilizer-N recovery and losses

As traced with ^{15}N-labelled fertilizer applied in T6–T11, the fertilizer-N recovery in rice straw, grain and straw + grain was maximum for T9 followed by T7 (Table 4). The higher fertilizer-N recovery for these two treatments indicates the usefulness of *Azolla* for increasing fertilizer-N use efficiency. Similarly, a 10–92% increase in ^{15}N recovery in rice from urea has been reported from various countries due to *Azolla* inoculation (Kumarasinghe and Eskew, 1993). The presence of *Azolla* also helped in increasing fertilizer-N retention in soil, as it was up to 14% higher for T9 as compared to T6. Thus, due to *Azolla*, the total fertilizer-N recovery in rice plant and soil was maximum (51%) for T9, and this recovery was significantly lower for non-*Azolla* treatments.

The fertilizer-N losses were 49% for T9, and 66% and 72% for 30 and 60 kg N/ha treatments, respectively (Table 4). Thus the use of biofertilizers reduced N losses by 17% and 23% as compared to low and high amounts of fertilizer application, respectively, Reduction in urea-N losses (11–19%) due to *Azolla* were also noted in a pot study by Ali and Malik (1993).

Table 4. Fertilizer N recovery of straw, grain and soil in rice field

Treatment	Fertilizer N recovery				
	Straw (%)	Grain (%)	Soil (%)	Total (%)	N lost (%)
T6. 30 kg N/ha	3.35^{b}	10.32^{bc}	20^{c}	34^{c}	66^{b}
T7. 30 kg N/ha + *Azolla* cover	4.17^{a}	11.53^{ab}	25^{b}	41^{b}	59^{c}
T8. 30 kg N/ha + bacteria. + *Azolla* incorporated	3.36^{b}	9.33^{c}	23^{b}	36^{c}	64^{b}
T9. 30 kg N/ha + bacteria + *Azolla* cover	4.33^{a}	12.43^{a}	34^{a}	51^{a}	49^{d}
T10 30 kg N/ha + bacteria	2.79^{c}	7.56^{d}	18^{cd}	28^{d}	72^{a}
T11. 60 kg N/ha	3.18^{bc}	9.30^{c}	16^{d}	28^{d}	72^{a}

^{15}N-labelled ammonium sulphate was applied to soil for treatments T6–T11.
Means followed by the same letter are not statistically different at 5% *p*.
Total = (straw + grain + soil).

Floodwater pH

The floodwater pH showed a large variation during daytime. It was low during the morning (7 am) reached maximum value in the afternoon (2 pm) and again decreased in the evening (6 pm) in all treatments (Figure 2). The increase in pH at noon was attributed to algal photosynthesis, during which the concentration of carbon dioxide and bicarbonates decreased in flood water (Mikkelsen *et al.*, 1978); Tuan and Thuyet, 1979). In general the pH remained lower in *Azolla* cover treatments than in the fertilizer treatments. The lowering of midday floodwater pH (Tuan and Thuyet, 1979; Mabbayad, 1987) as well as of floodwater temperature (Ali and Malik, 1988) has also been reported due to *Azolla*.

In this study the improvement in fertilizer-N use efficiency in rice plant and retention of higher fertilizer-N for *Azolla* treatments may be due to lowering of floodwater pH by *Azolla* cover (Figure 2), and reduction in N losses due to *Azolla* cover has also been observed in some *Azolla*–rice studies (Kumarasinghe and Eskew, 1993). The ammonia volatilization losses have been widely recognized as an important mechanism for loss of mineral N, due to high temperature and elevated floodwater pH (Mikkelsen and De Datta, 1979; Becker *et al.*, 1995), and maximum ammonia concentration and losses of applied fertilizers were reported during the daytime (10–16 hs) in a flooded soil (Ferdrazzini and Tarsitano, 1987).

Nitrogen fixation

The amount of fixed nitrogen in rice plants, computed using the ^{15}N isotope dilution technique and the high N treatment (T11) as non-fixing control, indicated

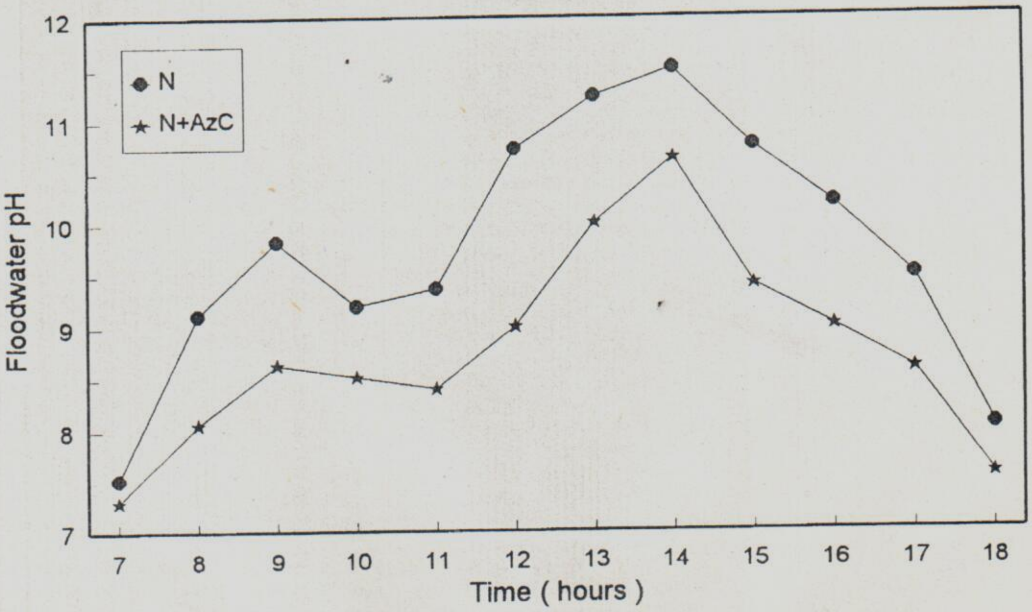

Figure 2. Kinetics of floodwater pH during daytime in rice–*Azolla* culture (N=Nitrogen treatments, average of T6 and T11; N+AzC=nitrogen+*Azolla* cover treatments, average of T7 and T9).

significantly higher values for bacterial treatments than control. Maximum BNF in rice (46 kg N/ha) was obtained for T10 where bacteria were inoculated along with 30 kg N/ha of ammonium sulphate, followed by T8 having 30 kg N/ha+bacteria+*Azolla* incorporation (Figure 3). A stimulation in the associative nitrogen fixation due to starter nitrogen fertilizer has also been reported in maize (Vlassak and Reynders, 1979). The figure shows that a greater amount of nitrogen fixed by bacteria was assimilated by rice than from *Azolla*. The higher availability of fixed N from bacteria may be due to active nitrogen fixation and an early release of N from microbial biomass than *Azolla*. Since *Azolla* has some woody tissue, and may have 18–30% lignin (Watanabe *et al*., 1991), therefore after incorporation into soil its decomposition and mineralization may take 5–8 weeks to release 70% of its total N (Watanabe *et al*., 1977). As *Azolla* was incorporated at later stages of rice growth (19 and 54 days before rice harvest) and it also needs a longer time for decomposition and release of its fixed N than microbes, almost no fixed N by *Azolla* was available to rice. Variable estimates of BNF have been given for nitrogen-fixing bacteria by various workers. In India seed inoculation, with *Azospirillum*, of various grasses and oats, gave a response equivalent to 20 and 40 kg N/ha respectively, and in Nepal inoculation of rice

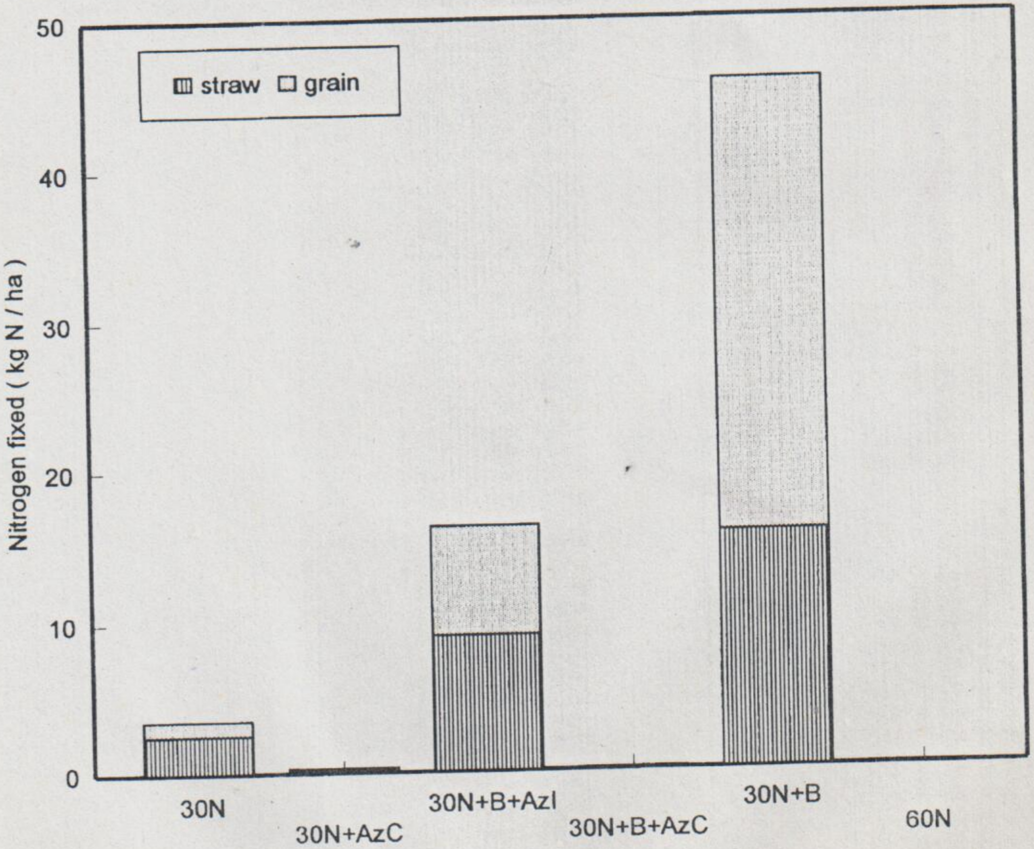

Figure 3. Amount of nitrogen fixed during rice crop, estimated by ^{15}N dilution technique. (N=nitrogen, Az=*Azolla*, B=bacteria, C=cover, I=incorporated)

seedlings increased rice yield comparable to 20 kg N/ha (FAO, 1988). Okon (1981) inoculated corn with nitrogen-fixing bacteria and found 77 kg more N/ha than in uninoculated plants. The 46 kg nitrogen fixation/ha for T10 (Figure 3) indicated that the inoculated bacteria were able to fix sufficient amounts of nitrogen even in the presence of 30 kg N/ha of fertilizer-N. Similar findings for lower sensitivity of associative nitrogen fixation to N-fertilizer application have been reported by Roger and Watanabe (1986).

Practical significance

This study indicated that the use of biofertilizers such as *Azolla* and nitrogen-fixing rhizobacteria can not only help in increasing rice yield and nitrogen fixation, but also enhance fertilizer-N use efficiency and reduce N losses of applied fertilizer, under flooded soil conditions. As they continued fixing nitrogen even in the presence of low amounts of fertilizer-N, as well as a high level of available-N in the soil, these biofertilizers can be used along with low doses of chemical-N fertilizer, to minimize the depletion of soil-N reserves.

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