REVIEW PAPER

Associative Nitrogen Fixation in Lowland Rice

Raj K Shrestha and Surya L Maskey

Soil Science Division-NARC, Khumaltar-Lalitpur-Nepal

ABSTRACT

Nitrogen (N), a most limiting nutrient, is the input required in the largest quantity for lowland rice production. The concerns on N economy and efficiency and its impact on environment have renewed interest in exploring alternative or supplementary N source for sustainable agriculture. Several studies have indicated the existence of significant rice genotypic differences in N_2 fixation stimulating traits (NFS). Rice genotypes with high NFS are desirable because they add N to the soil-water-plant system without additional farm inputs and reduce dependence on fertilizer. Large genotypic differences in percent N derived from air (% Ndfa) like 1.5% in Abang Basur, medium maturing genotype, to 21% in Oking Seroni, late maturing genotype, indicates potential of isolating genotypes with high NFS for sustainable agriculture. The exogenous supply of nitrogenous fertilizer to lowland rice significantly inhibited N fixation but improved plant growth. Where as phosphorous fertilizer did not affect atom % ^{15}N excess and % Ndfa significantly but slight decrease in atom % ^{15}N excess and increase in N_2 fixation was observed. Inhibitory effect of exogenous supply of N fertilizer indicates limited potential of associative N_2 fixation to significantly benefit agriculture. Farmers would have to withhold N fertilizer from their rice crop in order to increase biological N_2-fixation associated with rice. If they do such practice the plants will be N deficient and might have a lower yield. However, the development of N fixation in response to a deficiency of available N may well be an integral part of the N cycle of natural ecosystem and low input farming system there by maintaining a N balance in the environment.

Key words: Fertilizer, fixation, nitrogen, rice

INTRODUCTION

Rice is the most important cereal crop. In the next three decades, the world will need to produce about 60% more rice than today's global production to feed the extra billion people. Nitrogen is the major nutrient limiting the high yield potential of modern rice cultivars. Development of fertilizer-responsive varieties, coupled with the realization by farmers of the importance of nitrogen, has led to high rates of N fertilizer use on rice. But unfortunately a substantial amount of the N fertilizer is lost through different mechanisms causing environmental pollution problems. Utilization of biological N fixation (BNF) technology can decrease the use of N fertilizer, reducing the environmental problems to a considerable extent. BNF technologies must be economically viable, ecologically sound, and socially acceptable to be successful (Ladha and Reddy 2003).

Current environmental protection requirements make it necessary to develop ecologically clean technique of crop production that make maximum use of natural sources of bound N. Thus, biological fixation of atmospheric N and especially non-symbiotic N_2-fixation in the soil has been subject of continuing interest in recent decades. In addition, scare and the increasing cost of nonrenewable chemical fertilizers necessitates the greater use of renewable indigenous biological N_2-fixation system as source of N for the rice.

In flooded soil, N is available to rice even in fields that have been planted for many years without fertilizer application. Sen (1928) reported the presence of heterotrophic N_2-fixing bacteria in the rice root. However, the significance of his suggestion was neglected till Yoshida and Ancajas (1971) found that some N_2-fixing activity was associated with wetland rice root. Later, evidence of N_2 fixation by wetland rice roots was confirmed by ^{15}N studies (Eskew et al 1981, Yoshida and Yoneyama 1989). Subsequently, several studies have reported the evidence and significance of associative N_2-fixation (ANF) using different methods ie N balance studies (App et al 198l), acetylene reduction assay (ARA) (Sano et al 1981, Ladha et al 1986, 1987, 1988, Tirol-Padre et al 1988), and ^{15}N studies (Buenaventura et al 1984, Watanabe et al 1987, Chalk 1991). Moderate but sustainable yield of wetland rice can be obtained without N fertilizer (Koyama and App 1979). Thus long maintenance of soil fertility has been attributed to associative and free living biological N_2-fixation (BNF) (Yoshida and Ancajas 1973, Hirota et al 1978, Watanabe 1986). It has been recognized for a long time that associative N_2-fixing biological systems in wetlands enrich the soil organic N pool and supply up to 113 kg N/ha to rice crop depending upon the ecosystem, cultural practices and rice variety grown (Watanabe et al 1977, Rao et al 1998, Ariosa et al 2004). Researchers have agreed since long time that the high fertility of lowland rice field is because of biological N fixation (Grist 1965). Yoshida and Ancajas (1971, 1973) have given convincing evidence on efficient N fixation in the rhizosphere of rice by bacteria contributing N economy of the rice soil. Flooded soil planted to rice had a significant positive N balance. The positive N balance was found to be the result of phototrophic and heterotrophic N_2-fixing agents (App et al 1980).

Nitrogen fixing bacteria make up a large percentage of the total micro-flora in the rhizosphere of lowland rice. Using acetylene reduction method, Ishizawa et al (1987) and Yoshida and Ancajas (1971) found high nitrogenase activity in roots of lowland rice. Submergence seems to provide suitable conditions for N_2-fixation on rice roots grown under lowland conditions. The estimated amounts of N fixed in the dry season were 63 kg ha^{-1} N in planted flooded soil, 28 kg ha^{-1} in unplanted flooded soil, and only negligible in upland soil (Trolldeneir 1975). Form of available N and the status of potassium nutrition influence the number of bacteria and their activity around rice root grown in solution culture (Trolldeneir 1973).

Biological N_2 fixation is gaining importance in rice ecosystem because of current concern on the environmental and soil health that are caused by the continuous use of nitrogenous fertilizers and the need for improved sustainable rice productivity. Thus, biological fixation of atmospheric N, especially non-symbiotic N_2-fixation in the soil, has been subject of continuing interest in recent decades especially for low input agriculture. Therefore, the objectives of this paper are to review prospect and contribution of ANF, free living and associative system in flooded rice soil, genotypic differences, contribution and effect of chemical fertilizer on N fixation.

PROSPECT AND CONTRIBUTION OF ASSOCIATIVE NITROGEN FIXATION

Chalk (1991) reported that ANF can potentially contribute agronomically significant amount of N (>30-40 kg N ha^{-1} yr^{-1}) to the N nutrition of plants of importance in tropical agriculture when grown in uninoculated, N-deficit soils. Nitrogen fixation by some diazotrophic bacteria like *Azotobacter*, *Clostridium*, *Azospirillum*, *Herbaspirillum* and *Burkholderia* can substitute for N fertilizer, while *Rhizobium* can promote the growth physiology or improve the root morphology of the rice plant (Choudhury and Kennedy 2004).

A number of studies have summarized the advantages of rice plant-associative N_2 fixation:

1. Part of fixed N is available to the plant immediately (Ito et al 1980, Yoshida and Yoneyama, 1980, Eskew et al 1981, Watanabe and Roger 1984).

2. Plant associated N_2 fixation is less sensitive to N fertilizer application (Watanabe et al 1981).
3. Most of the plant associated fixed N is probably not readily amenable to loss process as it is microbially bound in the rhizosphere (Ladha et al 1987).

Studies conducted in different parts of the world have shown that percent N derived from air (%Ndfa) by N_2 fixation is 0 to 35 percent in rice (Table 1). Acetylene reduction assay, ^{15}N studies and N balance studies showed that the contribution of N_2 fixation associated with rice root is about 20% of the N of rice (Watanabe et al 1979). Similarly, Boddy and Dobereiner (1984) reported that root associated BNF is one of the major sources of N for wetland rice and it is estimated at 30 kg N ha^{-1} $crop^{-1}$, on around 20% of the total plant N.

Table 1. ^{15}N dilution estimates of N_2 fixation associated with flooded rice in pot experiment (modified form Chalk 1991)

Soil Treat	Test plant		Reference plant		% Ndfa	Reference
	Cultivar	Inoculum	Cultivar	Inoculum		
U, C	R26	Pseudomona sp. Azospirillium sp.	IR 26	Nil	0	Watanabe and Lin 1984
U	-	-	-	-	20-23	Zhu et al 1986
U, C	IR 42	-	R 42	-	32-35	Buenaventura et al 1984
H, C	C5444	Klebsiella oxytoca	C5444	-	11-19	Yoo et al 1986
U, C	C5444	Klebsiella oxytoca Enterobactor cloacae	C5444 T65	K. oxytoca	0-18	Fujii et al 1987
-	IR 42	Rice covered soil	-	-	20-30	Ventura and Watanabe 1983
-	Japonica and Indica rice	Alcaligenes faecalis	-	-	20-30	You et al 1991
-	-		-	-	19-25	Yoshida and Yoneyama 1980
-	IR 42		Palawan	-	35	Wu 1993

U, Unsterilized. H, Heat sterilized. C, Covered with black code, aluminium foil or lid.

FREE LEAVING AND ASSOCIATIVE SYSTEM

Diverse N_2-fixing microorganisms (aerobic, facultative anaerobes, heterotrophs, phototrophs) are found in wetland rice ecosystem and contribute to soil N pools. The major BNF systems in the flooded rice soils include cynobacteria, photosynthetic bacteria and heterotrophic bacteria.

The contributions of cynobacteria BNF are estimated to be 10-80 kg N ha^{-1} crop $^{-1}$, averaging about 30 kg N ha^{-1} $crop^{-1}$ (Roger and Watanabe 1986). Since the discovery of the cynobacteria in N gain under flooded conditions, many inoculation experiments have been conducted using cultured cynabacteria to improve soil fertility and grain yields of rice. Roger and Watanabe (1986) calculated that cyanobacterial inoculation increase rice yields only by an average of 337 kg grain ha^{-1} $crop^{-1}$.

Heterotrophic bacterial BNF is 7 kg N ha^{-1} (App et al 1986), ranging from 11-16 kg N ha^{-1} which contributes 16-21% of total rice N requirement (Zhu et al 1984, Shrestha and Ladha 1996a).

GENOTYPIC DIFFERENCES IN NITROGEN FIXATION

Identification of rice genotypes capable of stimulating associative N_2 fixation is an important goal for rice agriculture. It is important to document the differences between different genotypes, and select genotypes that have greater ability to stimulate N_2 fixation. A genotype possessing high N_2-fixing stimulating traits (NFS) would diminish the need for fertilizer N but would have no further impact on other cultural practices.

Several studies indicate that significant genotypic differences exist in NFS for rice (Yoshida and Ancajas 1971, Lee et al 1977, Hirota et al 1978, App et al 1986, Ladha et al 1987, 1988) (Table 2). The following reasons have been suggested to explain genotypic differences in NFS: specificity of plant-bacterial associations, differences in root exudations and gaseous diffusion efficiency (Ladha et al 1986). Through the exudates of rice roots different genotypes play an important role in the effectiveness of ANF (Lin and You 1989).

Table 2. Rice genotypic variation in associative nitrogen fixation and related characteristics

Variety	N_2-fixation estimation method	Enhanced N gain level	
IR42	ARA	High (Barraquio et al 1986, Watanabe et al 1987)	Medium (App et al 1986)
	^{15}N dilution	High (Buenaventura et al 1984)	-
Hua-cho-chi-mo-mor	^{15}N	Low (Watanabe 1986)	High (App et al 1986)
IS4	ARA	Low (Ladha et al 1986) Buenaventura et al 1984	Low (App et al 1986)
	^{15}N dilution	Low (Buenaventura et al 1984)	-
BG 367-4	ARA	Low (Ladha et al 1988)	Average (Ladha et al 1988)
Dular	-	-	High (App et al 1986)
Palawan	ARA	-	Low (App et al 1986)
	^{15}N dilution	Low (Wu 1993)	-
Pokkali	^{15}N dilution	Low (Buenaventura et al 1984)	-
Ma-Wei-chan	-	-	High (App et al 1980)
Cigalon	-	-	Low (App et al 1980)
C5444	-	Low (App et al 1986)	-
Oking Seroni	^{15}N dilution	High (Buenaventura et al 1984)	High (Buenaventura et al 1984, App et al 1986)

Shrestha and Ladha (1996a) observed significantly high %Ndfa in Hsiang-Ai-Tsao 7 (20%), Yeolsulbeyo (17%), Pokkali (18%) and Biron (18%) among 22 early maturing genotypes studied in a green house experiment with 70 rice genotypes of different growth duration. These genotypes also showed the highest specific N_2 fixation of 2.08, 1.7, 1.59 and 1.56 mg g^{-1} biomass, respectively, among 70 genotypes. Oking Seroni (21%), IR2937-36-3 (16.8%), and OR-142-99 (15.3%) had the highest % Ndfa among 25 late maturing genotypes. The genotypes with low NFS were PTB 18 (2.7%), Brontok (2.7%) and Abang Busur (1.5%) among early, medium and late maturing genotypes, respectively. Shrestha and Ladha (1996a) also reported that some of the rice genotypes with high NFS also had significantly higher grain yield and N uptake: for example, Pankaj and MTU15 (medium duration) and Oking Seroni and IR29337-36-3 (late duration). But some of the genotypes superior in NFS were not superior for grain yield like Hsiang Ai Tsao 7 (early). It is therefore important to consider grain yield in addition to Ndfa for selecting rice genotypes (Vincent 1984). In another experiment, Shrestha and Ladha (1996b) again observed highest % Ndfa of about 8% in Oking Seroni followed by Murungakayan 30, Pankaj, Gogo Putih, BG380-2 and OR1420-99 as in earlier study (Shrestha and Ladha 1996a). Oking Seroni showed highest % Ndfa at all level of N applications.

EFFECT OF FERTILIZER ON NITROGEN FIXATION

Nitrogen

Since combined form of N control nitrogenase activity in the living organisms, it would be interesting to know whether exogenous supply of fertilizer N counteracts N_2-fixation in the rhizosphere. Several studies have illustrated almost complete and long lasting inhibitory effect of N fertilizers on the N_2-fixing activity of free-living cynobacteria (Roger and Kulasooriya 1980). On the other hand, a systematic study on the effect of exogenous supply of N on associative N_2 fixation is still lacking.

We know that N_2-fixation take place in the soil when there is readily available organic carbon and the concentration of mineral N is low. While in vitro experiments, long ago showed that N_2-fixation is retarded when mineral N is present, there has been little study whether it would affect N_2-fixation in the soil when the plants are present.

The rhizosphere of rice was found to be an ideal location for beneficial reduction process, the microbial reduction of molecular N to ammonia (Ishizawa et al 1970, Yoshida and Ancajas 1973). Root associated heterotrophic bacteria with N_2-fixing potential develop nitrogenase activity in response to low concentration of combined N in their environment (van Berkum and Sloger 1981, 1983).

Reduction of BNF with increasing fertilizer N has been reported long time ago (McAuliffe 1958, Boller and Heichel 1983, Henson and Heichel 1984). Evidence exists on the inhibition of N_2-fixation due to higher level of combined N in pure culture and water logged soils (Knowles and Denike 1974, Charyulu and Rao 1980). Van Berkum and Sloger (1983) reported inhibitory effect of combined N in the fixation process of bacteria associated with the root of grasses as well as N_2-fixation in root nodules on legumes. The inhibitory effect of combined N especially nitrate was observed on root hair infection, nodule initiation, nodule development (Munnus 1977), nitrogenase activity in legumes (Mengel 1994, Cherney and Duxbury 1994) and in rice (Trolldeneir 1987). The root split technique with half root dipped in a nutrient solution with 40 ppm N and other half in the solution deficient in N. Nitrogen fixation on some roots of the same plant is inhibited by high concentration of combined N and remaining other roots in an N-free medium. The N_2-fixation on these roots was lower than that of plants where entire root system was growing in a free solution. Trolldeneir (1977) in a laboratory experiment has clearly demonstrated the repression effect of N at 10 ppm combined N in the form of urea on rhizosphere N_2-fixation. On the other hand, he also reported no inhibitory effect of fertilizer N in a fertility trial with lowland rice, presumably because of rapidly decrease in N concentration of the soil solution. Ladha et al (1989) did not observe any correlation between increase or decrease in ARA per plant and the amount of N applied.

The exogenous supply of all levels of nitrogenous fertilizer to lowland rice significantly inhibited N_2-fixation but improved plant growth (Shrestha and Ladha 1996b). The inhibitory effect of combined N in Ndfa was reported in different crops like alfalfa (Lamb et al 1995), pigeonpea (Tobita et al 1978, Tsai 1993). Nelson and Knowles (1978) observed delay in the appearance of N_2-fixation when N fertilizers were applied to the soil. They found a slight lag in N_2-fixation by growing culture of *Azospirillium brasillence* when nitrate was added to medium. They found a negative correlation between the level of N application and N_2-fixation activity (r = -0.7*), generally resulting a negative N balance in biological N balance (difference in N_2-fixation).

Increasing concentration of N, from 20 to 100, 200 and 400 kg N ha^{-1} reduced 85 to 75, 60 and 43 % Ndfa respectively. Thus, complete suppression effect of higher rates of N than normally applied in farming practices was not observed in N_2-fixation of fababean (Hardarson et al 1991). Kotera et al (1992) also reported significant inhibitory effect of N on N_2-fixation of gray forest soil. But in the presence of corn, which consume mineral N, the inhibitory effect of N was less pronounced. Merbach

(1995) also observed inhibitory effect of mineral N application on symbiotic N_2 fixation. Instead of fixed N, the plants took up mineral N. On the other hand he did not observed greater effect of mineral N in the species with an efficient atmospheric N_2-fixation which last till the end of the growth stage (such as *Vicia faba* and *Lupinus luteus*).

Most of the study has reported synergistic effect of low N application and suppressive effect of high N application in N_2-fixation. Balasendaram and Sen (1971) obtained increase in grain yield with Beijerinckia when inoculums and urea at the rate of 40 kg ha^{-1} was applied. The yield response was comparable to that with 80 kg N h^{-1} alone. McAuliffe (1959) observed 65% of the N fixed from atmosphere in the clover at the first cutting on the clay when 25 pounds of N per acre had been added to the soil and only 10% was fixed when the 200-pound treatment had been used. Similar decrease was also observed in Norfolk sandy loam. With time (second and third cutting) the clover fixed more N as the level in the soil declined. Increase in N_2-fixation from the first to third cuttings is apparently due to the reduction of soil N to a low level. The effect of the combined N on microbial nitrogenase varied with the concentration of the applied fertilizer N to the soil (Yoshida et al 1973, Knowles and Denire 1974, Rao 1976). High-Jensen and Schjoerring (1994) showed that application of 400 kg N ha^{-1} significantly reduced dinitrogen fixation by both enriched ^{15}N dilution and the natural ^{15}N abundance method.

Phosphorous

Some of the author (Robson 1983) have reported that phosphorus (P) increase the symbiotic N_2-fixation by stimulating host plant growth rather than exerting a direct effect of N_2-fixation per se, but some have reported P availability strongly affect traits related to N_2-fixation.

Application of P stimulates the soil N_2-ase in an alluvial soil and in a P-deficient soil under both flooded and Non-flooded conditions. The estimation of N_2-ase by P was more pronounced under non-flooded conditions. A corresponding increase in N_2-ase occurred with an increase in the P level at least up to 80 ppm level. A depression effect of P on N_2-ase occurred after 16 d under unflooded condition when the level of P was increased to 100 ppm. But under flooded conditions, the stimulation was almost continuous. Addition of P had little effect on the population of N_2-fixing microorganisms in alluvial soil. On the contrary addition of P stimulated the population of *Azospirillium* and *Azotobacter* in a P deficient soil. Data suggested that the alteration in the N_2-fixing microbial populations and the levels of available P might be responsible for changes in the N_2-ase activity in the soils. Result indicated that the level of applied P exhibited differential influence on N_2-ase and N_2-fixers in tropical paddy soil. Shrestha and Ladha (1996b) reported that phosphorous fertilizer did not affect atom % ^{15}N excess and % Ndfa significantly but slight decrease in atom % ^{15}N and increase in N_2 fixation was observed. Phosphorus fertilizer is found to increase N uptake significantly.

Sulaiman (1971) claimed that inoculation with *Azotobacter chroococcum* resulted in increased paddy yield in the presence of phosphorous fertilizer or lime. Cadisch et al (1993) reported that P limits growth and N_2-fixation to a greater extent than did potassium. Phosphorous supply increased % Ndfa by 15% at 5 kg P ha^{-1} to 259% at 75 kg P ha^{-1} at 14 weeks after sowing. App et al (1980) found significant increase in positive N balance with addition of P and iron to flooded soil planted with rice.

In leguminous crop, among the essential nutrients required by N_2-fixing symbiosis, P is a key element. It is involved in energy transfer and the supplying ATP for nitrogenase activity in nodules. Therefore, leguminous plants dependent on symbiotically fixed N for growth require more P than non N_2-fixing plants which are essentially depend on combined N. High ARA and high P content in the nodules of *Acacia mangium*, suggests that P was used preferably to enhance N_2-fixing activity even when the nodulation capacity was low. This finding supports the general hypothesis that the highly effective nodules are strong sink for P (Robson et al 1981). Israel (1987) indicated that severe P deficiency markedly impaired both host plant growths. Symbiotic dinitrogen fixation has a higher P requirement for optimal functioning. Beck and Vadez (1994) also reported the ability of some lines of common bean to fix increased amounts of N_2 at low levels of P indicate that plant improvement to enhance N_2 fixation under P limiting conditions is possible.

Phosphorous deficiency impairs N_2 fixation in young pea plants indirectly by impairing metabolisms of the shoots, not by direct action on nodule formation (Jakobsen 1985) or functions correcting P deficiency. In soybean by supplying increasing amount of P has been found to increase nodulation, nodule mass, activity of nitrogenase (Cassman et al 1980, Ganry et al 1985, Israel 1887, Raut and Kohire 1991). Addition of 90 mg of P kg^{-1} soil significantly increased the amount of N_2 fixed by 31% at the late pod filling stage (Pongsakul and Jensen 1991).

Rice genotypes with high NFS are desirable because they add N to the soil-water-plant system without additional farm inputs and reduce dependence on fertilizer N. Some of the rice genotypes with high NFS also had significantly higher grain yield and N uptake: for example, Pankaj and MTU15 (medium duration) and Oking Seroni and IR29337-36-3 (late duration). But some of the genotypes superior in NFS were not superior for grain yield like Hsiang Ai Tsao 7 (early). It is therefore important to consider grain yield in addition to Ndfa for selecting rice genotypes.

The exogenous supply of nitrogenous fertilizer to lowland rice significantly inhibited N fixation but improved plant growth. Where as phosphorous fertilizer did not affect atom % ^{15}N excess and % Ndfa significantly but slight decrease in atom % ^{15}N excess and increase in N_2 fixation was observed. Inhibitory effect of exogenous supply of N fertilizer indicates limited potential of associative N_2 fixation to significantly benefit high input agriculture. Farmers would have to reduce N fertilizer from their rice crop in order to increase biological N fixation associated with rice. If they do, then the plants might have N deficiency and might have a lower yield.

However, the development of N fixation in response to a deficiency of available N may well be an integral part of the N cycle of natural ecosystem and low input farming system there by maintaining a N balance in the environment. Development of genetically altered bacteria for root-associated nitrogen fixation, which can fix dinitrogen in the presence of repressive levels of combined nitrogen, is essential.

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