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Effect of diazotrophic inoculations on nitrogen fixation and its mineralization in rice (*Oryza sativa*) soil

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ABSTRACT

A field experiment was conducted during 2002 to investigate the effect of inoculation of 2 non-symbiotic N_2-fixing bacteria, *Azotobacter* (strain AS_8) and *Azospirillum* (strain AM_1), in presence of urea-N (50 kg/ha) and FYM (5 t/ha) on the performances of the diazotrophs with respect to availability and transformation of inorganic nitrogen in the rhizosphere soils of wetland rice (*Oryza sativa* L, cv. PNR-381). In most cases, a successful inoculation of the diazotrophs was recorded, with the proliferation of *Azotobacter* and *Azospirillum*, either alone or in combination, in the rhizosphere soils, and nitrogenase activity (C_2H_2 reduction) of the microbes was present in the rice roots. Inoculation of the diazotrophs substantially increased the content and availability of different fractions of inorganic (exchangeable NH_4^+ and soluble NO_3^-) nitrogen in the rhizosphere soils. In general, combined inoculation of the diazotrophs was most stimulative in augmenting the nitrogen status of the rhizosphere soils, which was comparable to the effects under the uninoculated soil series receiving 100 kg N/ha as urea-N. Between the two organisms, *Azotobacter* was more effective than *Azospirillum* in stimulating growth and activities of the diazotrophs, in relation to accumulation and transformation of different fractions of inorganic and total nitrogen content in the rhizosphere soils. Total and exchangeable NH_4^+ nitrogen content increased at maximum tillering to flowering stages of the crop, followed by a decline at maturity.

Key words : *Azospirillum* population, *Azotobacter* population, Diazotrophic inoculation, Inorganic nitrogen, Nitrogenase activity (C_2H_2 reduction), Total nitrogen.

Wetland rice soils are more dependent on soil nitrogen than dry land crops. But the nitrogen use efficiency of wetland rice soils does not exceed 40% of applied inorganic nitrogen due to considerable losses that occur through leaching, denitrification and ammonia volatilization (Watanabe et al., 1988). Moreover, the increasing demand for soil nitrogen by high yielding rice varieties makes the situation more critical. To meet this nitrogen demand in wetland rice soils, a significant amount of nitrogen has to be replenished for sustained crop production. The increasing demand for inorganic nitrogenous fertilizer application and limited incorporation of organic matter not only cause soil health as a whole to deteriorate, but also aggravate environmental pollution to a great extent. To overcome this adverse situation, it becomes necessary to maintain the nitrogen balance in wetland rice soils through biological means with maximum potentiality and adequate techniques.

Biological fixation of atmospheric nitrogen by heterotrophic microorganisms plays a vital role in the nitrogen economy of wetland rice soils. Besides blue green algae (Ghosh and Saha, 1993; 1997), the high potential nitrogen fixation by free living *Azotobacter* and root associative microaerophilic *Azospirillum* in the rhizosphere soil of rice has been well documented (Das and Saha, 2000). Ammonia is an inhibitor of nitrogenase enzyme, but the incorporation of small amounts of inorganic nitrogen does not inhibit nitrogen fixation by non-symbiotic nitrogen fixing bacteria (Kanungo et al., 1998). Rather it enhances their populations resulting in a greater fixation of atmospheric nitrogen in soil (Vendan and Sundaram, 1997). In addition, the association of nitrogen fixing bacteria also improves nitrogen transformation and contributes a significant amount of growth promoting substances to the standing crop (Rao et al., 1998) resulting in a greater yield. However, limited information is available regarding the enhancement of growth of cereals under field condition.

To explore the potential heterotrophic non-symbiotic N_2-fixing bacteria for the improvement of nitrogen nutrition, an experiment was conducted to investigate the effect of inoculation of the non-symbiotic nitrogen fixing bacteria, *Azotobacter* and *Azospirillum*, either alone or in combination, and in the presence of a low application of inorganic nitrogen, on nitrogen accumulation and transformation in the rhizosphere of wetland rice.

MATERIALS AND METHODS

A field experiment was conducted during 2002 in microplots (7 m by 7 m) following a randomized block design (RBD) in the experimental farm of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India. The soil was classified as a *Typic Fluvaquent*, and the general characteristics are presented in Table 1. Farmyard manure (FYM) at 5 t/ha and fertilizers consisting 25 kg/ha urea-N, 22 kg/ha single superphosphate-P and 42 kg/ha potassium chloride-K were mixed thoroughly with the soil during land preparation. Thirty-day old rice seedlings (*Oryza sativa* L, variety PNR-381) previously raised in a seedbed receiving 25 t/ha FYM, 60 kg/ha urea-N, 13 kg/ha single superphosphate-P and 25 kg/ha potassium chloride-K, were uprooted and the soil adhering to the roots was washed off carefully in clean water. The seedlings were inoculated with 2 efficient strains of *Azotobacter* (strain AS_8) and *Azospirillum* (strain AM_1), either alone or in combination, previously isolated from roots of same rice variety (Das and Saha 2003), by dipping rice roots in bacterial suspensions containing viable cell numbers 10.4×10^9/ml for 1 hr, followed by air drying in shade for 30 min. Seedlings inoculated with bacterial cultures were then transplanted separately at 4 seedlings/hill, with a spacing of 15 cm × 20 cm between hill and row. There was an uninoculated control. Thirty days after transplanting, another 25 kg/ha urea-N was applied as a top dressing to all the plots. Thus nitrogen was applied at 50 kg/ha, which was 50% of recommended field rates for the crop. There was also an uninoculated treatment receiving 100 kg/ha urea-N but no FYM. All the treatments were replicated 3 times. The crop was cultivated following usual cultural practices.

Rhizosphere soil samples were collected from each plot at maximum tillering [35 days after transplanting (DAT)], flowering (70 DAT), and maturity (105 DAT) stages of the crop growth, by uprooting plants carefully as outlined by Das and Mukherjee (2000). After the pieces of plant roots had been removed, rhizosphere soils from replicated plots of each treatment were analyzed immediately to enumerate colony-forming units (cfu) of *Azotobacter* in sucrose-calcium carbonate agar medium (Das and Mukherjee 1994) through plate count method (Zuberer 1994) and most probable numbers (mpn) of *Azospirillum* in semi-solid malate agar medium (Baldani and Döbereiner 1980) through mpn method (Woomer 1994), following incubation at 30 ± 1°C, using a serial dilution technique. Nitrogenase activity associated with roots of rice plants was determined at different growth stages of crop through an acetylene reduction assay (ASA) (Ghosh and Saha, 1993), with the help of a gas chromatograph (HP model 5730A) fitted with a glass column packed with porapak-R (80-100 mesh) and equipped with a flame ionization detector. The operating temperature of the oven and the flow rate of carrier gas (N_2) were adjusted to 80°C and 60 ml/min, respectively.

Soil samples were analyzed to determine available inorganic nitrogen (exchangeable NH_4^+ and soluble NO_3^-) in potassium chloride extract through distillation (Mulvaney, 1996). The total nitrogen content of the rhizosphere soils was estimated following the method outlined by Bremner (1996). Results were evaluated by 2-way analysis of variance and the statistical significance of effects within the factors (diazotrophs and sampling days) and their interactions were evaluated.

RESULTS AND DISCUSSION

Effect on N_2-fixing bacteria and their activities

Inoculation with *Azotobacter* and *Azospirillum*, either alone or in combination, stimulated the growth and multiplication of *Azotobacter* in the rhizosphere soils of rice (Fig. 1A); the effect was most pronounced with a single inoculation of *Azotobacter* followed by the combined inoculation of *Azotobacter* and *Azospirillum*. The uninoculated treatment receiving 100 kg N/ha also accelerated the population of *Azotobacter* in soil. A similar trend was recorded for *Azospirillum* populations in soil (Fig. 1B). Among the treatments, the soils inoculated with *Azospirillum* in the presence of FYM and partial application of fertilizer-N exhibited the greatest stimulation towards the proliferation of these microaerophilic N_2-fixing bacteria in the rhizosphere soil. Inoculation with *Azotobacter* and *Azospirillum* resulted in greater nitrogenase activity (C_2H_2 reduction) of the microbes present in the rice roots (Fig. 1C). This enzymatic activity of the diazotrophs was most pronounced with the single inoculation of *Azospirillum*, followed by the combined inoculation. This indicated that association of microaerophilic *Azospirillum* with rice roots influenced nitrogenase activity of roots to a greater extent (Kanungo et al., 1997). Incidentally, there was a significant positive correlation (r = 0.977) between the population of *Azospirillum* and root nitrogenase activity. The uninoculated soil series receiving 100 kg N/ha also exerted a significant effect on nitrogenase activity of the rice roots as compared to the uninoculated control. The population densities of the non-symbiotic N_2-fixing bacteria as well as their nitrogenase activity in rice roots were highest at the flowering stage of the crop. At this stage, plant roots released the highest amount of exudates rich in growth promoting substances and these organic substances could be utilized by the rhizosphere microorganisms for their growth and multiplication, resulting in an increase in their population and activities in rhizosphere soil (Dey and Bhattacharyya, 1975). This was in agreement with the earlier reports of Vendan and Sundaram (1997) and Das and Saha (2003).

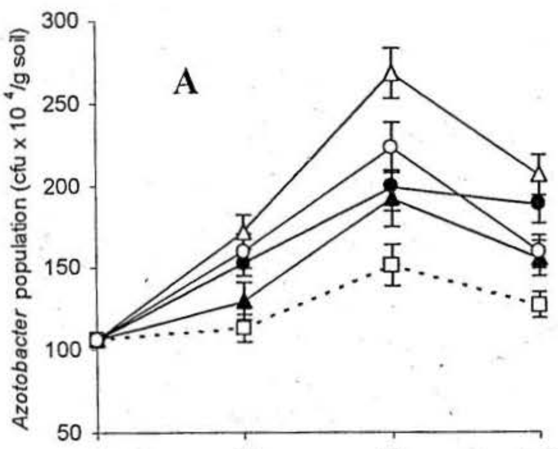
Effect on mineral and total nitrogen status

The stimulation of growth and activities of *Azotobacter* and *Azospirillum* due to inoculation resulted in greater availability of inorganic nitrogen (exchangeable NH_4^+ and soluble NO_3^-) in the rhizosphere soil (Figs. 2A and 2B). The uninoculated soil series receiving 100 kg N/ha also significantly augmented the availability of mineral nitrogen in the rhizosphere soils but the stimulation tended to be more pronounced when the crop was inoculated with *Azotobacter* and *Azospirillum* in combination. This indicated that inoculation of non-symbiotic N_2-fixing bacteria in presence of FYM (5 t/ha) promoted the activities of ammonifying and nitrifying bacteria, which were responsible for the mineralization of organic nitrogen to NH_4^+ and oxidation of NH_4^+ to NO_3^-, respectively (Ghosh et al., 1990; Das and Mukherjee, 1998), resulting in a greater accumulation of mineral nitrogen in soil. This was also in agreement with earlier reports (Rao et al., 1998). The availability of NH_4^+-N was most highly increased at the flowering stage (70 DAT) of the crop, followed by a gradual decrease up to maturity stage (Fig. 2A). These data suggest that greater microbial activities at the flowering stage of the crop greatly augmented the formation of NH_4^+ (ammonification) which was subsequently utilized by the standing crop as well as by microbes for their growth and metabolism and nitrified to form NO_3^- (fig.2B), resulting in a gradual decrease in exchangeable NH_4^+-N. Soluble NO_3^--N, on the other hand, was decreased during maximum tillering stage (35 DAT) of the crop followed by a gradual increase up to maturity (105 DAT) of the crop, except the uninoculated soils treated with full dose (100 kg/ha) of fertilizer-N (Fig. 2B). This indicated that at maximum tillering stage, the rate of oxidation of NH_4^+ by the nitrifying bacteria was less than the absorption of soluble NO_3^- by the crop as well as by the microbes (Das and Saha, 2004). However, the demand was compensated with the age of the crop due to higher microbial activities in the rhizosphere soil. Data also revealed that rhizosphere soils, in general, retained higher amounts of exchangeable NH_4^+-N than soluble NO_3^--N, indicating that inoculation with *Azotobacter* and *Azospirillum* induced the process of ammonification more than nitrification in rice soil (Das and Saha, 2000). The increased microbial activities due to inoculation of *Azotobacter* and *Azospirillum*, stimulated by the greater availability of nutrients including growth promoting substances significantly increased the amount of total nitrogen in the rhizosphere soils up to maximum tillering followed by a gradual decline until maturity of the crop (Fig. 2C). A similar trend was recorded with uninoculated soil series treated with 100 kg N/ha. The uninoculated control recorded a steady decline in the content of total nitrogen until the end of the experiment. In general, the greatest accumulation of total nitrogen in the rhizosphere soils of rice was recorded with combined inoculation of the diazotrophs, followed by uninoculated soils receiving 100 kg N/ha. Between the two diazotrophs, single inoculation of *Azotobacter* retained higher amount of total nitrogen in the rhizosphere soils of rice as compared to that of *Azospirillum* stain. Das and Saha (2003) also reported higher accumulation of total nitrogen in the rice rhizosphere under *Azotobacter* and *Azospirillum* inoculations.

The results of the present investigation clearly indicated that inoculation of *Azotobacter* and *Azospirillum*, either alone or in combination, and in the presence of a partial application of fertilizer-N and FYM, stimulated the growth and activities of free living *Azotobacter* and root associative *Azospirillum* in the rhizosphere soil of rice. The enhanced microbial activity augmented the availability of inorganic and total nitrogen in the rhizosphere soil, which was comparable to the recommended full dose (100 kg N/kg) of fertilizer-N. The results also indicated that combined inoculation of the diazotrophs was more stimulative than their single inoculation in improving the nitrogen status of the rhizosphere soils. Of the two organisms, *Azotobacter* was more effective than *Azospirillum*.

Table 1 General characteristics of the soil of the experimental plots

Type and classification	Alluvial (Typic Fluvaquent)
Textural class	Clay loam
Density (g/cm^3)	1.08
Water holding capacity (%)	64.2
pH (1 : 2.5 w/v) in water	7.4
Cation exchange capacity [cmol (p^+)/kg]	15.0
Electrical conductivity (dS/m)	0.35
Organic C (g/kg)	7.18
Total N (g/kg)	0.75
C: N ratio9.6	
Inorganic N	
Exchangeable NH_4^+ N (mg/kg)	58.2
Soluble NO_3^- N (mg/kg)	26.4
Available P (mg/kg)	12.3



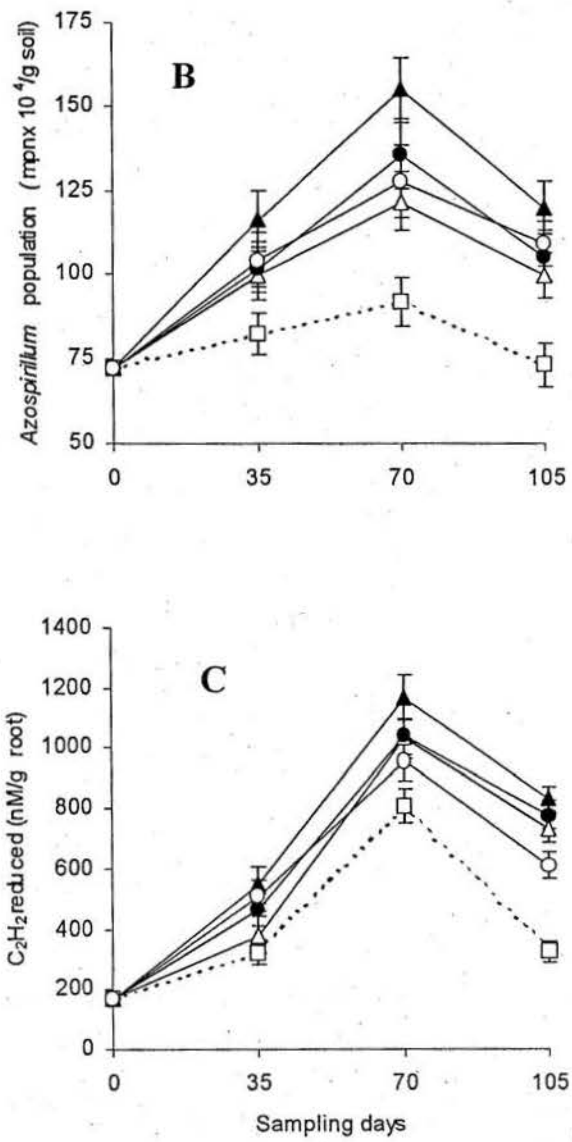
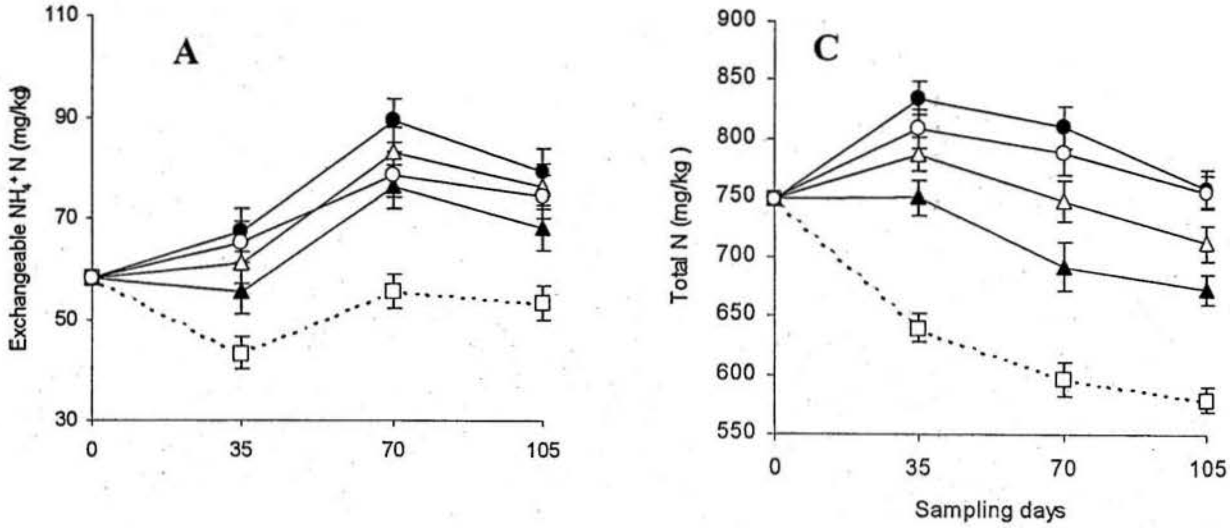


Fig 1 Effect of inoculation of diazotrophs on their population in the rhizosphere soils and nitrogenase activity of rice roots. Treatments: □, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) (uninoculated control); ○, Fertilizer-N (100 kg/ha) (uninoculated); Δ, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) + *Azotobacter* (AS_8); ▲, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) + *Azospirillum* (AM_1); ?, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) + (AS_8) + (AM_1). Values are means ± s.e



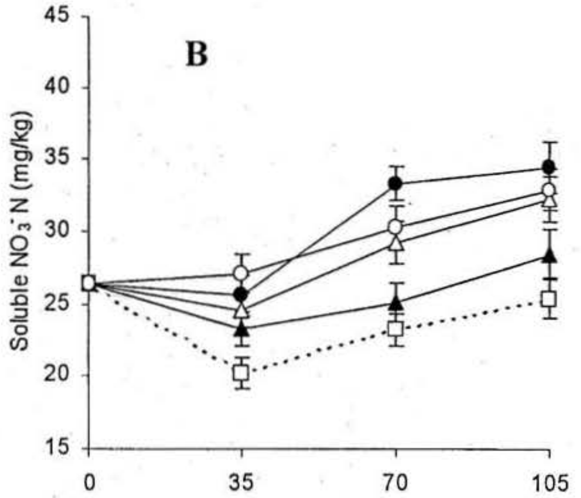


Fig 2 Effect of inoculation of diazotrophs on inorganic and total nitrogen status in the rhizosphere soil of rice. Treatments: □, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) (uninoculated control); ○, Fertilizer-N (100 kg/ha) (uninoculated); △, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) + *Azotobacter* (AS_8); ▲, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) + *Azospirillum* (AM_1); ●, Fertilizer-N (50 kg/ha) + FYM (5 t/ha) + (AS_8) + (AM_1). Values are means ± s.e.

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