Inoculation effects of *Azotobacter* and *Azospirillum* on N_2-fixation and its transformations in wetland rice soil

A. C. Das* and D. Saha

Department of Agricultural Chemistry and Soil Science, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur –741 252, West Bengal, India

ABSTRACT

The effect of inoculation of Azotobacter (strain BS_9) and Azospirillum (strain BM_9), either singly or in combination, on N-accretion and its transformation in the rhizosphere of wetland rice was investigated. Inoculation of Azotobacter and Azospirillum either alone or in a combination along with N-fertilizer (50 kg ha^{-1}) stimulated the growth and activities of non-symbiotic N_2-fixing bacteria in the rhizosphere soil of wetland rice. Inoculation with the diazotrophs increased the nitrogenase activity (C_2H_2 reduction) of rice roots, availability of inorganic N (exchangeable NH_4^+-N and soluble NO_3^--N), accumulation and mineralization of hydrolysable organic N (hydrolysable NH_4^+-N, amino acid-N and hexosamine-N) in the rhizosphere soil, resulting in a greater yield of the crop. In general, single inoculation of the diazotrophs exhibited better performance than their combined inoculation. Between the two non-symbiotic N_2-fixing bacteria, Azotobacter was more effective than Azospirillum in the rice rhizosphere.

Key words: Azotobacter, Azospirillum, *nitrogenase activity, N-transformation, rice, yield*

Wetland rice soils are more dependent on soil-N than the dryland crops. But the N-use efficiency of wetland rice soils generally does not exceed 40% of applied inorganic N due to losses occurring through leaching, denitrification and ammonia volatilization (Watanabe *et al.*, 1988; De Datta, 1995). Moreover, the increased demand for soil-N by high yielding rice varieties makes the situation more vulnerable. To compensate the N demand in wetland rice soils, a significant amount of N is to be replenished for sustaining higher crop productivity. The incremental application of mineral N-fertilizer and limited incorporation of organic matter not only deteriorate the soil health as a whole but also aggravate the environmental pollution to a great extent (Alexander, 1978). To overcome this, it becomes necessary to maintain N-balance in wetland rice soils through biological means for a greater sustainability.

Biological fixation of atmospheric-N by heterotrophic microorganisms plays a vital role in the N economy of wetland rice soils (Watanabe, 1986). Besides blue green algae (Ghosh and Saha, 1997), the high potential N_2-fixation by free living Azotobactor and root associative microaerophilic Azospirillum in the rhizosphere soil of rice has been well documented (Gopalaswamy *et al.*, 1988; Rao *et al.*, 1998; Das and Saha, 2000). Ammonia is an inhibitor of nitrogenease enzyme (Alexander, 1978). But incorporation of small amount of inorganic N does not inhibit N_2-fixation by non-symbiotic N_2-fixing bacteria (Kanungo et al., 1998) rather enhance their population (Koch and Oya, 1974), resulting in a greater fixation of atmospheric-N in soil (Vendan and Sundaram, 1997). In addition, the association of N_2-fixing bacteria also improves N transformation and contributes a significant amount of growth promoting substances to the standing crop (Rao *et al.*, 1998) resulting in a greater yield (Sarwar *et al.*, 1998).

To explore the characteristics of heterotrophic non-symbiotic N_2-fixing bacteria for augmenting N nutrition and crop yield, an experiment was conducted to investigate the effect of inoculation of two non-symbiotic nitrogen fixing bacteria namely, Azotobacter and Azospirillum, either singly or in combination, along with partial application of inorganic-N on N-accretion and N-transformation in the rhizosphere of wetland rice.

MATERIALS AND METHODS

Two efficient strains of non-symbiotic N_2-fixing bacteria viz. *Azotobacter* (strain BS_9) and *Azospirillum* (strain BM_9) were isolated from the rhizosphere of rice (*Oryza sativa* L) in N-free sucrose-calcium carbonate agar (Das and Mukherjee, 1994) and N-free semisolid malate agar (Baldani and Dobereiner, 1980) media, respectively following the methods as outlined by Dey and Bhattacharyya (1975), and Saha *et al.* (1985). The organisms were identified to their generic levels following Bowie *et al.* (1969). Both the bacteria were grown on potato infusion agar (Lucia *et al.*, 1980) plates at 30 ± 1^0C for 72 hours. The cultures were subsequently scraped out and suspended in their respective N-free liquid media for use as inoculants to rice seedlings for further studies.

A field experiment was conducted in microplots (7m x 7m) following randomized block design (RBD) in the experimental farm of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India. The soil was a *typic Fluvaquent* having the general characteristics as: textural class, clay loam; density, 1.08 g cm^{-1}; water holding capacity, 64.2%; pH (1:2.5 w/v) in water, 7.4; cation exchange capacity, 15.0 cmol (p^+) kg^{-1}; electrical conductivity, 0.35 dS m^{-1}; organic C, 7.18 g kg^{-1}; total N, 0.76 g kg^{-1}; C: N ratio, 9.4. Fertilizers consisting of 25, 22 and 42 kg ha^{-1} of N, P and K as urea, single superphosphate and muriate of potash respectively, were mixed thoroughly with the soil during land preparation. Thirty-day old rice seedling (*Oryza sativa* L, variety IET 4094) were uprooted from the seedbed and the soils adhering to the roots were washed carefully in clean tap water. The seedlings were then inoculated with *Azotobacter* (BS_9) and *Azospirillum* (BM_9), singly as well as in combination by dipping the roots in heavy suspensions (cells number ~10^9 ml^{-1}) of 72-hours old cultures of inocula for one hour followed by drying in shades for thirty minutes. The seedlings inoculated with the bacterial cultures were then transplanted separately at four seedlings per hill with a spacing 15 cm × 20 cm. There was also an uninoculated control. All the treatments were replicated three times. Thirty days after transplanting, 25 kg ha^{-1} of N as urea was applied as top dressing to all the plots and thus N was applied at 50 kg ha^{-1} which was 50% of recommended field rates for the crop. The crop was grown following recommended agronomic practices and harvested at maturity.

Rhizosphere soil samples were collected from each plot at the maximum tillering [45 days after transplanting (DAT)], flowering (85 DAT) and maturity (110 DAT) stages of the crop growth by uprooting plants carefully. After the pieces of plant roots had been removed, rhizosphere soils from the replicated plots of each treatment were analyzed immediately.

Soil samples were analyzed to enumerate the colony forming units (cfu) of *Azotobacter* in sucrose-calcium carbonate agar medium (Das and Mukherjee, 1994) following serial dilution technique and plate count method (Salle, 1973). The agar plates were incubated at 30 ± 1°C for 7 days in a BOD incubator. The cfu of *Azospirillum* was counted in the semisolid malate agar medium (Baldani and Dobereiner, 1980) following serial dilution technique and most probable numbers (mpn) method (Woomer, 1994) by growing organisms at 30 ± 1°C for 72 hours in a BOD incubator. All the cultures were examined microscopically to establish their morphological identity.

The nitrogenase activity associated with roots of rice plants were determined at different growth stages of the crop through acetylene reduction assay (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 ionizing detector. The operating temperature of oven and flow rate of carrier gas (N_2) was adjusted to 80 °C and 60 ml min^{-1}, respectively.

Soil samples were also analyzed to determine available inorganic nitrogen (exchangeable NH_4^+ and soluble NO_3^-) in 0.1 M potassium chloride extract through distillation (Bremner and Keeney, 1966). The soils were further hydrolyzed with hydrochloric acid to estimate different fractions of hydrolysable organic nitrogen (Stevenson 1996).

The grain and straw yield were measured from replicated field plots.

RESULTS AND DISCUSSION

Inoculation of *Azotobacter* and *Azospirillum* either alone or in a combination stimulated the growth and activities of both the non-symbiotic N_2-fixing bacteria in the rhizosphere soil of rice (Tables 1 and 2). The stimulation was more pronounced with *Azotobacter* as compared to *Azospirillum*. It was also observed

Table 1. Effect of inoculation of diazotrophs on the population of *Azotobacter* and *Azospirillum* in the rhizosphere soil of rice

Treatments	Stages of crop growth			
	Tillering (45 DAT)	Flowering (85 DAT)	Maturity (110 DAT)	Mean
	Number of *Azotobacter* (cfu x $10^4 g^{-1}$ soil)			
Control	97.1 ± 8.2	178.5 ± 10.8	152.2 ± 5.8	142.6
Azotobacter (BS_9)	123.6 ± 9.2	254.1 ± 13.2	205.1 ± 7.8	194.2
Azospirillum (BM_9)	121.8 ± 7.1	203.2 ± 11.4	187.2 ± 9.2	170.7
$BS_9 + BM_9$	138.6 ± 6.8	325.1 ± 6.9	167.1 ± 11.2	210.2
Mean	120.2	240.2	177.9	
CD (P = 0.05)	Treatment 16.4; growth stage 14.2; interaction 28.4			
	Number of *Azospirillum* (cfu x $10^4 g^{-1}$ soil)			
Control	76.5 ± 5.6	184.5 ± 8.9	103.1 ± 7.2	121.3
Azotobacter (BS_9)	106.7 ± 4.2	153.9 ± 9.2	129.4 ± 6.2	130.0
Azospirillum (BM_9)	116.3 ± 3.1	249.9 ± 10.0	195.1 ± 5.3	187.1
$BS_9 + BM_9$	66.2 ± 4.5	127.2 ± 9.1	228.5 ± 4.5	140.6
Mean	91.4	178.8	164.0	
CD (P = 0.05)	Treatment 12.1; growth stage 10.5; interaction 21.1			

DAT, days after transplanting

Table 2. Effect of inoculation of diazotrophs on nitrogenase activity (C_2H_2 reduction) of rice roots

Treatments	Stages of crop growth			
	Tillering (45 DAT)	Flowering (85 DAT)	Maturity (110 DAT)	Mean
	Amount of C_2H_2 reduced (nMh^{-1} g^{-1} root)			
Control	10.9 ± 1.2	23.2 ± 1.2	23.1 ± 1.3	19.0
Azotobacter (BS_9)	13.9 ± 0.6	61.2 ± 1.7	32.3 ± 1.1	35.8
Azospirillum (BM_9)	14.3 ± 1.0	117.2 ± 1.6	51.8 ± 1.4	61.1
$BS_9 + BM_9$	23.9 ± 0.7	74.5 ± 2.4	30.0 ± 0.9	42.8
Mean	15.7	69.0	34.3	
CD (P = 0.05)	Treatment 2.4; growth stage 2.1; interaction 4.2			

DAT, days after transplanting

that the population of *Azotobacter* was highly increased due to the combined inoculation of the diazotrophs while the single inoculation of *Azospirillum* significantly accentuated the proliferation of these root associative microaerophilic N_2 fixing bacteria in soil. The synergistic effect of both the diazotrophs on growth and multiplication of *Azotobacter* was due to their protocooperative association in soil (Alexander, 1978). Similar observation was also reported earlier by Das and Saha (2000). The greater association of *Azotobacter* and *Azospirillum* in rice rhizosphere resulted in greater fixation of atmospheric-N in soil and this was evidenced by greater nitrogenase activity (C_2H_2 reduction) of rice roots (Table 2). The enzymatic activity for nitrogen fixation was more pronounced with the inoculation of *Azospirillum* indicating that association of *Azospirillum* to rice roots influenced the nitrogenase activity of roots to a greater extent (Saha *et al.*, 1985; Kanungo *et al.*, 1997). Incidentally, there was a significant positive correlation (r = 0.949) between the population of *Azospirillum* and nitrogenase activity of roots. The results also supported the earlier findings (Kanungo *et al.*, 1997; Das and Mukherjee, 2000). The microbial activities as well as their populations were highest at flowering stage of the crop. At the flowering stage of the crop, plant roots released highest amount of root exudates (Rovira, 1969) rich in growth promoting substances (Alexander, 1978) which were preferentially

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).

The stimulation of growth and activities of *Azotobacter* and *Azospirillum* due to inoculation of the diazotrophs resulted into a greater availability of inorganic N (exchangeable NH_4^+ and soluble NO_3^-) in the rhizosphere soil (Table 3). The stimulation was more pronounced when the crop was inoculated with either *Azotobacter* or *Azospirillum* resulting in greater accumulation of mineral N in soil. This was in agreement with the earlier reports (Saha *et al.*, 1985; Rao *et al.*, 1998; Sarwar *et al.*, 1998). From the results (Table 3), it was also revealed that the availability of NH_4^+-N increased at flowering stage of the crop followed by a gradual decrease to maturity stage. This clearly pointed out that greater microbial activities at the flowering stage of the crop highly augmented the mineralization of organic N (Saha *et al.*, 1982), which were subsequently utilized by the standing crop as well as by the enhanced microbes for their growth and metabolism resulting in a gradual decrease of exchangeable NH_4^+-N up to the maturity stage of the crop. Soluble NO_3^--N, on the other hand, was gradually decreased with the age of the crop. Earlier workers (Pal et al., 1987; De Datta, 1995) also recorded the decrease in inorganic N due to cropping. It was also revealed that rhizosphere soil, in general, retained higher amount of exchangeable NH_4^--N than soluble NO_3^--N. This indicated that inoculation of *Azotobacter* and *Azospirillum* induced the process of ammonification than that of nitrification in rice soil (Das and Saha, 2000).

The accumulation of hydrolysable organic N in the rhizosphere soil of rice has also been increased due to the inoculation of Azotobacter and Azospirillum to rice seedlings (Table 4). This suggested that increased number of N_2-fixing bacteria not only fixed high amount of atmospheric N in their cells but also released greater amount of growth promoting substances (Arshad and Frankenberger, 1998) which were organic in nature and contained high amount of hydrolysable organic N (Watanabe *et al.*, 1988). The other microorganisms present in the rhizosphere soil utilized available N from the dead cells of the microorganisms including the diazotrophs as well as from native organic matter present in soil (Alexander, 1978; Mukhopadhyay *et al.*, 1985) resulting in higher immobilization of hydrolysable organic N in their cells. Moreover, the higher availability of plant nutrients as influenced by enhanced microbial activities stimulated the growth of rice plants which also released a significant amount of root exudates containing greater amount of hydrolysable organic N (Rovira 1969) in rhizosphere soil. The proportions of

Table 3. Effect of inoculation of diazotrophs on the availability of inorganic nitrogen in the rhizosphere soil of rice

Treatments	Stages of crop growth			
	Tillering (45 DAT)	Flowering (85 DAT)	Maturity (110 DAT)	Mean
	Amount of exchangeable NH_4^+-N (mg kg^{-1})			
Control	20.5 ± 3.2	81.2 ± 2.3	65.3 ± 6.1	55.6
Azotobacter (BS_9)	25.6 ± 2.1	96.3 ± 3.4	88.2 ± 3.1	70.0
Azospirillum (BM_9)	22.6 ± 1.4	90.4 ± 4.9	80.7 ± 3.2	64.5
BS_9 + BM_9	19.2 ± 1.1	80.5 ± 5.2	72.8 ± 5.2	57.5
Mean	21.9	87.1	76.7	
CD (P = 0.05)	Treatment 6.6; growth stage 5.7; interaction NS			
	Amount of soluble NO_3^--N (mg kg^{-1})			
Control	11.6 ± 1.2	20.2 ± 2.2	25.6 ± 2.3	19.1
Azotobacter (BS_9)	18.7 ± 1.5	26.3 ± 2.3	36.6 ± 1.2	27.2
Azospirillum (BM_9)	22.7 ± 2.1	24.4 ± 2.1	32.2 ± 1.6	26.4
BS_9 + BM_9	19.8 ± 1.2	22.5 ± 1.2	26.1 ± 1.6	22.8
Mean	18.2	23.3	30.1	
CD (P = 0.05)	Treatment 3.1; growth stage 2.7; interaction NS			

DAT, days after transplanting; NS, not significant

Table 4. Effect of inoculation of diazotrophs on hydrolysable organic nitrogen in the rhizosphere soil of rice

Treatments	Stages of crop growth			
	Tillering (45 DAT)	Flowering (85 DAT)	Maturity (110 DAT)	Mean
	Amount of hydrolysable NH_4^+ - N (mg kg^{-1})			
Control	131.2 ± 4.1	69.3 ± 2.8	59.7 ± 2.8	86.7
Azotobacter (BS_9)	142.4 ± 3.9	89.6 ± 4.9	42.9 ± 2.7	91.6
Azospirillum (BM_9)	164.1 ± 3.1	65.2 ± 3.2	65.0 ± 3.1	98.1
BS_9 + BM_9	164.5 ± 4.9	82.5 ± 3.2	46.4 ± 3.4	97.8
Mean	150.5	76.6	53.5	
CD (P = 0.05)	Treatment 6.3; growth stage 5.5; interaction 11.0			
	Amount of total amino acid-N (mg $kg^{-1)}$			
Control	107.2 ± 2.1	92.2 ± 3.1	85.2 ± 2.2	94.8
Azotobacter (BS_9)	160.3 ± 3.2	117.1 ± 1.7	53.5 ± 2.1	110.3
Azospirillum (BM_9)	120.4 ± 2.1	115.4 ± 3.2	52.3 ± 1.8	96.0
BS_9 + BM_9	120.2 ± 3.2	118.7 ± 2.5	52.8 ± 2.6	97.2
Mean	127.0	110.8	60.9	
CD (P = 0.05)	Treatment 4.5; growth stage 3.9; interaction 7.8			
	Amount of hexosamine-N (mg kg^{-1})			
Control	26.5 ± 1.6	17.8 ± 2.1	14.5 ± 1.1	19.6
Azotobacter (BS_9)	47.8 ± 1.9	24.5 ± 2.3	14.6 ± 1.2	28.9
Azospirillum (BM_9)	44.9 ± 2.1	30.7 ± 1.6	13.8 ± 1.3	29.8
BS_9 + BM_9	36.6 ± 2.2	24.8 ± 0.9	16.7 ± 0.8	26.0
Mean	38.9	24.4	14.9	
CD (P = 0.05)	Treatment 2.9; growth stage 2.5; interaction 5.1			

DAT, days after transplanting

hydrolysable NH_4^+-N and hydrolysable amino acid N were higher than that of hydrolysable hexosamine N (Mukhopadhyay et al., 1985). It was also recorded that mineralization of hydrolysable organic N, in general, increased with the age of the crop (Pal et al., 1987; De Datta, 1995) depicting 69.9%, 66.6% *and 69.5%* mineralization of hydrolysable NH_4^+-N, amino acid N and hexosamine N, respectively during the period of tillering to maturity stages of the crop with Azotobacter inoculation. The corresponding values for Azospirillum and their combined inoculation were 60.4%, 56.6%, 69.3% and 71.8%, 56.1% and 54.4%, respectively. This pointed out that single inoculation of Azotobacter and Azospirillum mineralized higher amounts of hydrolysable organic N as compared to their combined inoculation in the rhizosphere soil. This was also true for their accumulation of hydrolysable organic N in soil.

Higher microbial activities as influenced by inoculation of *Azotobacter* and *Azospirillum* in presence of partial application of N-fertilizer augmented greater availability of N in the rhizosphere soil. As a result, the yield of the crop was increased (Table 5). The similar observations of increase in yield of the crop due to inoculation of *Azotobacter* and/or *Azospirillum* in presence of partial application of inorganic N were also evidenced by Vendan and Sundaram (1997) and Sarwar *et al.* (1998). Single inoculation of the diazotrophs manifested better performance than their

Table 5. Effect of inoculation of diazotrophs on yield of rice

Treatments	Grain yield (kg $plot^{-1}$)	Straw yield (kg $plot^{-1}$)
Control	4.2 ± 0.3	31.8 1.4
Azotobacter (BS_9)	5.1 ± 0.3	36.6 ± 2.0
Azospirillum (BM_9)	4.6 ± 0.2	35.2 ± 2.5
BS_9 + BM_9	4.6 ± 0.4	33.6 ± 1.6
Mean	4.6	34.2
CD (P = 0.05)	0.3	2.1

combined inoculation and more specifically, *Azotobacter* was more stimulative as compared to *Azospirillum*. This was also in agreement with the reports of earlier workers (Venden and Sundaram, 1997; Das and Saha, 2000).

The results of the present investigation thus clearly indicated that inoculation of *Azotobacter* and *Azospirillum* either alone or in a combination stimulated the growth and activities of free living *Azotobacter* and root associative *Azospirillum* in the rhizosphere soil of rice. The enhanced microbial activities augmented the availability of inorganic and hydrolysable organic N in the rhizosphere soils resulting in greater yield of the crop. The results also indicated that single inoculation of the diazotrophs was more stimulative than their combined inoculation and between the two organisms, *Azotobacter* was more effective than *Azospirillum*.

REFERENCES

Alexander M 1978. Introduction to soil microbiology. Wiley Eastern Ltd, New Delhi, India.

Arshad M and Frankenberger WT 1998. Plant growth-regulating substances in the rhizosphere: microbial production and functions. Adv Agron 62: 45 -151

Baldani VLD and Dobereiner J 1980. Host-plant specificity in the infection of cereals with *Azospirillum* spp. Soil Biol Biochem 12: 433 - 439

Bowie IS, Loutit MW and Loutit JS 1969. Identification of aerobic heterotrophic soil bacteria to the generic level by using multipoint inoculation techniques. Can J Microbiol 15: 297-302

Bremner JM and Keeney DR 1966. Determination of exchangeable ammonium, nitrite and nitrite by extraction distillation methods. Soil Sci Soc Amer Proc 50: 577-587

Das AC and Mukherjee D 1994. Effect of insecticides on the availability of nutrients, nitrogen fixation and phosphate solubility in the rhizosphere soil of rice. Biol Fertil Soils 18: 37- 41

Das AC and Saha D 2000. Inoculation of diazotrophs on nitrogenase activity (C_2H_2) of roots and N-transformations in the rhizosphere soils of rice. pp. 644 – 646, *In*: Proceedings of the International Conference on Managing Natural Resources for Sustainable Agricultural Production in the 21st Century, New Delhi, India.

De Datta SK 1995. Nitrogen transformation in wetland rice ecosystems. Fert Res 42: 193 - 203

Dey BK and Bhattacharyya P 1975. Effect of *Azotobacter* inoculation on free amino acid and reducing sugar composition of plant tissues of wheat (*Triticum aestivum* L). Indian Agric 19: 351-354

Gapalaswamy P, Vidhyasekharan P and Chelliah S 1988. Effect of *Azospirillum lipaferum* inoculation and inorganic nitrogen on wetland rice. Oryza 26: 378-380

Ghosh TK and Saha KC 1993. Effect of inoculation with N_2-fixing cyanobacteria on the nitrogenase activity in soil and rhizosphere of wetland rice (*Oryza sativa* L). Biol Fertil Soils 16: 16-20

Kanungo PK, Ramakrishnan B and Rao VR 1997. Placement effects of organic sources on nitrogenase activity and nitrogen fixing bacteria in flooded rice soils. Biol Fertil Soils 25: 103-108

Kanungo PK, Ramakrishnan B and Rao VR 1998. Nitrogenase activity of *Azospirillum* sp. isolated from rice as influenced by combination of NH_4^+-N and insecticide, carbofuran. Chemosphere 36: 339-349

Koch BL and Oya J 1974. Non-symbiotic nitrogen fixation in some Hawaiian pasture soils. Soil Biol Biochem 6: 363-367

Lucia V, Baldani D and Dobereiner J 1980. Host plant specificity in the infection of cereals with *Azospirillium* spp. Soil Biol Biochem 12: 433-439

Mishra RK and Sen SP 1996. Associative symbiosis and nitrogen fixation of rice. Curr Agric Res 9: 71-76

Mukhopadhyay AK, Saha D and Ghosh R 1985. Effect of NH_4^+- producing nitrogenous fertilizers and N-serve on transformation of organic forms of N in soil under different incubating moisture levels. Trop Agric 62: 157-160

Pal SK, Saha D and Mukhopadhyay AK 1987. Influence of cropping and N-fertilization on changes in different forms of nitrogen in an alluvial soil. Plant Soil 101: 137-139

Rao VR, Ramakrishnan B, Adhya TK and Nayak DN 1998. Review: Current status and future prospects of associative nitrogen fixation in rice. World J Microbiol Biotech 14: 621-633

Rovira AD 1969. Plant root exudates. Bot Rev 35: 35-37

Saha D, Mukherjee R and Mukhopadhyay AK 1982. Availability of fixed NH_4^+ to crops. I. Native fixed form. Plant Soil 65: 345-349

Saha KC, Sannigrahi S and Mandal LN 1985. Effect of inoculation of *Azospirillum lipoferum* on nitrogen fixation in rhizosphere soil, their association with root, yield and nitrogen uptake by mustard (*Brassica juncia*). Plant Soil 87: 273-280

Salle AJ 1973. Laboratory manual on fundamental principles of bacteriology. McGraw-Hill Book Company, New York, USA.

Sarwar KS, Rahman M and Khan S 1998. Effect of *Azospirillum lipoferum* on growth, yield and nutrient content of rice (*Oryza sativa* cv. BR-11). Bull Inst Trop Agric Kyuslu Univ. 21: 9-17

Stevenson FJ 1996. Nitrogen-organic forms. *In*: Methods of soil analysis, part 3. Sparks *et al.* Eds, Soil Science Society America Inc., American Society of Agronomy Inc., Madison, Wisconsin, USA. p. 1185-1200

Vendan RT and Sundaram MD 1997. Effect of biological nitrogen fixation in relations to growth and yield of certain rice varieties. J Ecobiol 9: 281-287

Watanabe I 1986. Nitrogen fixation by non-legumes in tropical agriculture with special reference to wetland rice. Plant Soil 90: 343-357

Watanabe I, De Datta SK and Roger PA 1988. Nitrogen cycling in wetland rice soils. pp 239-256, *In*: Advances in nitrogen cycling in agricultural ecosystems. Wilson JR, Ed. CAB International, Willingford, Oxon, UK.

Woomer PL 1994. Most probable number counts. *In*: Methods of soil analysis, Part 2. Weaver *et al.* Ed, Soil Science Society of America Inc, Madison, Wisconsin, USA. p. 59-79