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Effects of *Azospirillum* isolates from paddy fields on the growth of rice plants

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Inoculation study in pot culture showed that *Azospirillum* inoculation could significantly increase all the plant growth parameters including germination. To observe the plant growth promoting effects of *Azospirillum* isolates, ten isolates isolated from paddy fields of North Bengal were used to inoculate "BRRI dhan-28" seeds. Inoculated rice seeds were germinated on petri dish and compared with control. Both inoculated and controlled germinated rice seeds were sown in the soil earthen pots. Some plant growth parameters were considered and compared with control. All of the isolate inoculations increased rice seed germination (%) compared to control. Inoculation with most of the isolates showed that *Azospirillum* inoculation could significantly increase the growth in terms of height; no. of leaf/plant; length and breadth of leaf; and fresh and dry weight/plant of rice plant.

Keywords: *Azospirillum*, isolates, inoculation, rice plant, North Bengal, growth parameters, biofertilizer.

Bangladesh, one of the most densely populated countries, mostly depends on agriculture for living. Population explosion has created a tremendous pressure on agriculture. Rice is the main cereal food for the people of Bangladesh, the production of which is insufficient hampering Bangladesh to ensure food security. Although the country is now trying to achieve self-sufficiency in cereal food production, it will not be able to ensure food security for an ever-increasing population, as the requirement will double in the next 25 years while the natural resource base will shrink. To keep pace with population growth and the shrinking land resource base, total production of food crops will have to be increased by 60–70% within that period (Sattar *et al.*, 2008). Increasing total yield of rice through horizontal expansion (i.e. using more land for production) is not possible, so vertical increase (yield/unit area) is the only option. In this situation the Bangladesh Rice Research Institute (BRRI) and Bangladesh Institute of Nuclear Agriculture (BINA) are developing high yielding modern rice varieties requiring high amounts of N for biomass production. Rice requires 20-40 kg soil N, during 3-5 month, for every ton of grain produced (Rasul, 1999). N-deficiency of most of the rice soils of Bangladesh, the high cost of chemical nitrogenous fertilizers, the low purchasing power of most of the farmers and increased environmental concern restrict its use in proper amounts, hampering crop production. So, worldwide attention has been developed to use biological approaches for increasing crop production. Biological approaches are usually less expensive, harmless and in the reach of all the countries. The utilization of biological nitrogen fixation (BNF) technology can also decrease the use of urea-N, prevent the depletion of soil organic matter and reduce environmental pollution to a considerable extent. *Azospirillum* a plant growth prtomoting bacteria is being used as biofertilizer in several countries of the world. It is a soil bacterium capable of producing associative symbiosis in the roots of various plants including grain crops including rice. *Azospirillum* promotes plant growth by fixing atmospheric nitrogen and by some other ways like production of growth promoting substances and influencing root development, causing increased uptake of nutrients from the land, and inhibiting pathogenic fungi and bacteria in the rhizosphere. *Azospirillum* inoculation increases percentage of rice seed germination (treated 50% : untreated 20%) (Kannan and Ponmurugan, 2010; Ravikumar *et al.*, 2002). Inoculation of plants with *Azospirillum* has been found to cause significant increases in growth and yield which is equivalent to that is attainable by application of 15-20 kg N/ha (Rodrigues *et al.*, 2008). A yield increase in rice due to inoculation of *Azospirillum* is reported to be in the 5–60% range (Kumar and Balasubramanian, 1986). The aims and objectives of the present study were to determine rice seed germinating efficiency and the contributing capacity of the isolated *Azospirillum* isolates to increase rice growth.

Materials and Methods

Collection of seeds - Fresh and healthy seeds of "BRRI dhan-28 (BR-28)" variety of rice were collected from Bangladesh Rice Research Institute (BRRI), Joydevpur, Gazipur.

Collection of soil - The soil for pot experiment was collected from Dhamrai, Dhaka. Such soil was selected because the soil has almost neutral pH value.

Filling the experimental earthen pots - 33 pots with height of 12" were taken for 11 treatments and filled with selected soils that are homogenous. Each pot contained 6.5 kg of soil. Each treatment has three replications.

Isolates collection - Ten *Azospirillum* isolates, isolated and identified at species level, from rhizosphere soils and roots of rice (*Oryza sativa*) growing on rice fields of particular locations of three different districts of North Bengal- Bogra, Naowgoan and Dinajpur were selected for inoculation. The locations were three villages (Bezura, Chalkvalli and Pattakur) of Shahjahanpur Upzilla and one village (Adorsha para) of Adamdighi Upzilla of Bogra; three villages (Sahapur, Dhamkuri and Vobanipur) of Naowgaon sadar Upzilla of Naowgaon and three villages (Sheikhpur, Hindupara and Gunjapara) of Dinajpur sadar Upzilla of Dinajpur (Hossain *et al.*, 2015)(Table 1). Nitrogen fixing efficiency and physiological characteristics of these isolates were studied (Hossain *et al.*, 2014).

Table 1 ***Azospirillum*** **isolates used for inoculation**

Selected isolates	Identified species
M-1	*Azospirillum brasilense*
M-2	*Azospirillum brasilense*
M-3	*Azospirillum lipoferum*
M-4	*Azospirillum lipoferum*
M-5	*Azospirillum brasilense*
M-6	*Azospirillum lipoferum*
M-7	*Azospirillum lipoferum*
M-8	*Azospirillum lipoferum*
M-9	*Azospirillum halopraeferens*
M-10	*Azospirillum lipoferum*

Preparation of inoculums - The selected isolates were grown in 250 ml Erlenmeyer flasks containing 100 ml malate liquid medium (Nfb medium without agar) supplemented with 0.25% NH_4Cl and 0.1% yeast extract. The culture was incubated for

48 hours at 32°C±2°C with agitation (100 rpm) in a water bath.

Sterilization of seeds - Surface sterilization of rice seeds was accomplished by treatment with 0.1% $HgCl_2$ for 2/3 minutes. The seeds were washed four times with sterilized distilled water.

Inoculation of seeds - Inoculation was performed by immersing of the surface sterilized seeds (75 rice seeds per selected isolate) for 3 hours into the 48 hours culture containing an inoculum concentration of 10^7 cfu/ml. Seeds (75 rice seeds) immersed in sterile medium for 3 hours served as control.

Observation of seed germination - After inoculation, the seeds were aseptically placed on a sterilized petri dish (25 rice seeds per petri dish) containing a sterilized filter paper with the help of sterilized forceps. Then sterilized distilled water was added regularly to prevent drying of filter paper. This would help the seeds to germinate. Then the petri dishes containing seeds were placed on dark place, sterilized with ethanol. Then the data of seed germination were taken regularly.

Sowing of seeds - The germinated seeds were sown (25 seeds per pot) in the soil earthen pots. The pots were watered regularly in a way that the pots never overflowed or to prohibit the water logging conditions.

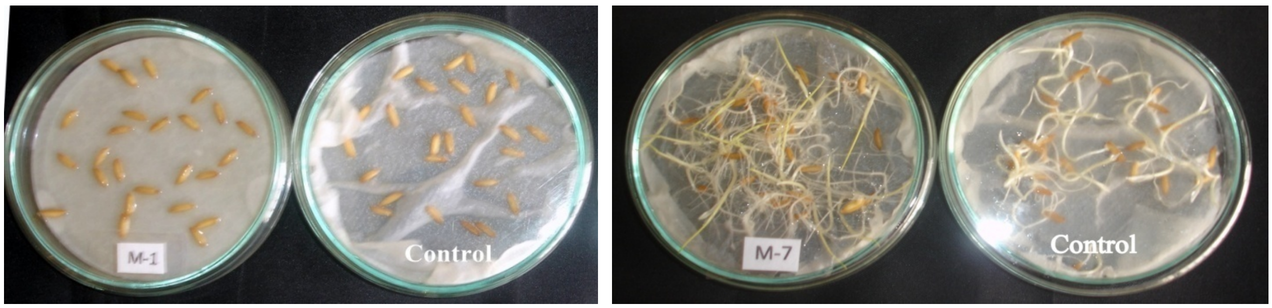
Cultural practice - Thinning was done 15 days after emergence of seedlings. 15 seedlings were kept. It was made in such a way that healthy seedlings of uniform size and vigor were allowed to grow. Regular watering and weeding were performed to ensure equal environmental condition throughout the pot. No chemical fertilizer was applied.

Observation and data collection - During present investigation some growth parameters were noted in the pots. To study the morphological and physiological changes, the following characteristics were studied at different stages of growth and development of plants. Five randomly selected plants were used for each pot for collecting data. Most of the results were statically analyzed by Duncan's multiple range tests.

Physiological parameters or plant growth status - The following parameters were considered as plant growth status- time taken for germination; height of the plant; No., length and breadth of leaf; fresh weight and dry weight of plant. Germination records were taken up to 5 days of inoculation. For each measurement data were recorded at 15 days interval of sowing of germinated seeds and data were taken with the help of centimeter scale and electric balance. For obtaining fresh weight, 5 plants were uprooted at 15 days of sowing of germinated seeds and measured. For dry weight plants were collected at 25 days of sowing of germinated seeds and kept in the oven at 75°C for 48 hours until a constant was attained.

Results and Discussion

The pot experiment was undertaken to observe the effect of *Azospirillum* inoculation on the growth of rice plant. Response to inoculation was observed at initial developmental stages. Effect of inoculation was significant in almost all parameters. Inoculation treatment of rice seed with different *Azospirillum* isolates showed variation in germinating efficiency of seeds in the petri plates. Germination started from the 2nd day after inoculation and completed on 5th day after inoculation. Kannan and Ponmurugan (2010) studied rice seed germination and found that the percentage germination of seeds were higher in *Azospirillum* treated seeds than in control. Ravikumar *et al*. (2002) showed that pretreatment of rice seeds with *Azospirillum brasilense* increased percentage of seed germination (treated 50% : untreated 20%). The reason for this may be due to the tremendous pressure developed inside the seeds, which is responsible for breaking of the seed coat quickly (Sifton, 1959). This pressure may be induced by phytohormones especially auxin, indole acetic acid, cytokinin and gibberelic acid like substances secreted by *Azospirillum* (Okon, 1985). In this study, inoculation with *Azospirillum* caused statistically significant effect on the germination of rice seeds. Most of the selected isolates was found highly effective and M-4, M-8 and M-10 were to be most efficient (Figure 1). They caused 96% germination on 5th day after inoculation. In the control on the same day the germination was only 88% (Table 2).



a) Seeds of 1 day after inoculation b) Germinated seeds of 6 days after inoculation

Figure 1 Observation of seed germination

Table 2 Effect of *Azospirillum* inoculation on the germination (%) of rice seeds

Treatment	% of germinated seeds at different days after inoculation*					
	1st	2nd	3rd	4th	5th	Difference with control at 5th day
Control	0	66.67±6.11	86.67±8.33	88.00±8.00	88.00±8.00	--
M-1	0	84.00±4.00	89.33±8.31	90.67±6.11	90.67±6.11	2.67
M-2	0	92.00±4.00	93.33±4.62	93.33±4.62	93.33±4.62	5.33
M-3	0	86.67±2.31	89.33±2.31	90.67±2.31	90.67±2.31	2.67
M-4	0	86.67±2.31	96.00±4.00	96.00±4.00	96.00±4.00	8.00
M-5	0	84.00±4.00	88.00±4.00	89.33±2.31	90.67±2.31	2.67
M-6	0	77.33±6.11	88.33±4.62	88.00±4.00	89.33±6.11	1.33^{ns}
M-7	0	67.33±11.0	89.33±2.31	90.67±2.31	90.67±2.31	2.67
M-8	0	93.33±6.11	94.67±4.62	96.00±4.00	96.00±4.00	8.00
M-9	0	86.67±6.11	89.33±8.33	90.67±6.11	90.67±6.11	2.67
M-10	0	88.00±8.00	94.67±6.11	94.67±6.11	96.00±4.00	8.00
						Sig.
CD at 5% level						2.46

*Average of three replications, Mean ± SD; Sig.= significant, ns = non significant

Rice physiologically responded to *Azospirillum* inoculation increasing its aerial biomass (Salamone *et al.*, 2010). El-Khawas and Adachi (1999) found that inoculation of rice root with *Azospirillum* resulted in significant increase in root elongation, root surface area, root dry matter and development of lateral roots. Isawa *et al.* (2010) showed that inoculation of rice seeds with *Azospirillum* sp. isolate B510 significantly enhanced the growth of newly generated leaves and shoot biomass under greenhouse conditions. Rice seedlings inoculated with *Azospirillum* sp. B510 also significantly increased growth in terms of tiller numbers and shoot length in paddy field (Boa *et al.*, 2013). The seed dressing by *Azospirillum* induced the production of plant growth promoting substances and led to the increase of shoot and root length. *Azospirillum* treated seedling appeared taller than control (Chi *et al.,* 1998). In pot culture, inoculation treatment of rice with different *Azospirillum* isolates significantly affected plant height and number of leaf/plant at initial stage of growth. At initial stage, plants inoculated with selected isolate M-10 showed the maximum height (24.38 cm) and was followed by the plants inoculated with the isolate M-1 (23.67 cm) and the isolate M-7 (22.74 cm). Plant inoculated with the selected isolate M-1 showed highest number of leaf per plant (4.2) and this was followed by the plants inoculated with M-4, M-5, M-6, M-7, M-8, M-9 and M-10 (4.0) (Table 3; Figure 2, 3).

Due to inoculation with *Azospirillum* isolates, significant variations in leaf length and breadth of the plants were observed in the pots of different treatments. Plants inoculated with isolate M-1 showed maximum leaf length (16.48 cm) and were followed by the plants inoculated with the isolate M-7 (15.74 cm) and the isolate M-10 (15.20 cm). In case of breadth of leaf, plant inoculated with the isolate M-10 showed the highest breadth (0.44 cm) and were followed by plant inoculated with the isolate M-1 (0.43 cm) and isolate M-7 (0.40 cm) (Table 4; Figure 2, 3).

Table 3 Effect of inoculation with various isolates of *Azospirillum* spp. on the height and number of leaf/plant of rice plant at initial stage of growth

Treatment	Height of the plant (cm)*		No. of leaf/plant*	
	Initial stage	Difference with control	Initial stage	Difference with control
Control	19.68±1.42	--	3.8 ± 0.45	--
M-1	23.67±2.31	3.99	4.2 ± 0.45	0.40
M-2	19.94±1.52	0.26^{ns}	3.8 ± 0.45	0.00^{ns}
M-3	19.83±1.72	0.15^{ns}	3.8 ± 0.45	0.00^{ns}
M-4	20.26±2.36	0.58^{ns}	4.0 ± 0.00	0.20
M-5	20.19±1.69	0.51^{ns}	4.0 ± 0.00	0.20
M-6	21.59±2.77	1.91	4.0 ± 0.00	0.20
M-7	22.74±2.63	3.06	4.0 ± 0.00	0.20
M-8	19.89±1.99	0.21^{ns}	4.0 ± 0.00	0.20
M-9	19.85±2.63	0.17^{ns}	4.0 ± 0.00	0.20
M-10	24.38±2.83	4.7	4.0 ± 0.00	0.20
		Sig.		Sig.
CD at 5% level		1.49	CD at 5% level	0.10

*Average of three replications, Mean ± SD; Sig.= significant, ns = non significant

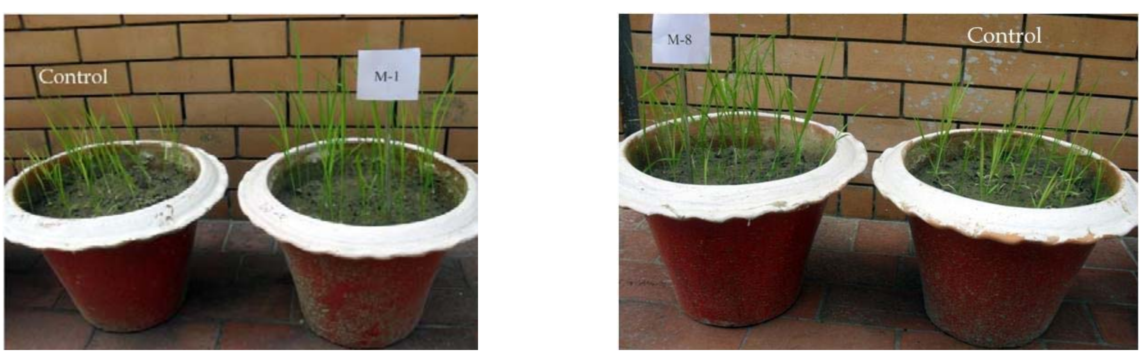


Figure 2 Initial stage of rice plants inoculated with *Azospirillum* isolates M-1 and M-8

Figure 3 Initial stage of rice plants inoculated with *Azospirillum* isolates M-7 and M-10

Table 4 Effect of inoculation with various isolates of *Azospirillum* spp. on the length and breadth of leaf of rice plant at initial stage of growth

Treatment	Length of leaf (cm)*		Breadth of leaf (cm)*	
	Initial stage	Difference with control	Initial stage	Difference with control
Control	11.60 ± 3.68	--	0.310 ± 0.079	--
M-1	16.48 ± 2.16	4.88	0.430 ± 0.081	0.120
M-2	11.65 ± 3.00	0.05^{ns}	0.313 ± 0.076	0.003^{ns}
M-3	12.37 ± 3.52	0.77^{ns}	0.324 ± 0.083	0.014^{ns}
M-4	11.62 ± 2.55	0.02^{ns}	0.325 ± 0.084	0.015^{ns}
M-5	11.63 ± 2.36	0.03^{ns}	0.347 ± 0.086	0.037^{ns}
M-6	11.63 ± 3.21	0.03^{ns}	0.360 ± 0.080	0.050
M-7	15.74 ± 2.31	4.14	0.400 ± 0.084	0.090
M-8	11.62 ± 3.39	0.02^{ns}	0.347 ± 0.078	0.037^{ns}
M-9	11.62 ± 2.86	0.02^{ns}	0.340 ± 0.076	0.030^{ns}
M-10	15.20 ± 2.16	3.60	0.440 ± 0.086	0.130
		Sig.		Sig
CD at 5% level		1.68	CD at 5% level	0.040

*Average of three replications, Mean ± SD; Sig.= significant, ns = non significant

Kannan and Ponmurugan (2010) found that the fresh and dry weights per plant of rice were also increased to a considerable extent in *Azospirillum* treated seedlings. Pereira *et al.* (1988), Murty and Ladha (1988) and Gunarto *et al.* (1999) found similar result. This may be due to the formation and development of numerous root branching; root hairs; and primary and secondary lateral roots which increases the nutrient uptake capacity of roots (Gopalswamy and Vidhy asekaran, 1988; Hartmann *et al*., 1983). This effect on the root system as well as more root colonization and root proliferation are probably due to the growth hormones secreted by the bacteria and also nitrogen fixation by it. The increased nitrogen uptake from the soil might have correspondingly increased the biomass to some extent. In the present study, fresh and dry weights per plant were presented in the following table. The selected isolate M-7 recorded better results than the other isolates. The fresh weight per plant (0.23 g) was recorded from M-7 treated plant and lowest fresh weight was (0.10 g) recorded from uninoculated control plant. Similarly, the dry weight per plant (0.171 g) was recorded from M-7 and M-5 treated plant and lowest fresh weight was (0.099 g) recorded from uninoculated control plant (Table 5).

Table 5 Effect of inoculation with various isolates of *Azospirillum* spp. on the fresh weight and dry weight of rice plant at initial stage of growth

Treatment	Fresh weight/plant (g)*		Dry weight/plant (g)*	
	Initial stage	Difference with control	At 25 days of sowing	Difference with control
Control	0.10 ± 0.006	--	0.099 ± 0.017	--
M-1	0.21 ± 0.040	0.11	0.133 ± 0.028	0.034
M-2	0.11 ± 0.020	0.01^{ns}	0.127 ± 0.035	0.028
M-3	0.11 ± 0.050	0.01^{ns}	0.146 ± 0.026	0.047
M-4	0.15 ± 0.040	0.05	0.142 ± 0.033	0.043
M-5	0.12 ± 0.010	0.02^{ns}	0.171 ± 0.021	0.072
M-6	0.13 ± 0.020	0.03^{ns}	0.103 ± 0.008	0.004^{ns}
M-7	0.23 ± 0.010	0.13	0.171 ± 0.021	0.072
M-8	0.14 ± 0.030	0.04	0.161 ± 0.033	0.062
M-9	0.15 ± 0.020	0.05	0.115 ± 0.001	0.016^{ns}
M-10	0.22 ± 0.010	0.12	0.157 ± 0.009	0.058
		Sig.		Sig.
CD at 5% level		0.04	CD at 5% level	0.023

*Average of three replications, Mean ± SD; Sig.= significant, ns = non significant

Conclusions

Among growth promoting rhizobacteria, *Azospirillum* is known to be a very active nitrogen fixer under laboratory as well as soil conditions providing fast growth, better health of the plant and higher yield. From this work, it became clear that *Azospirillum* has significant growth promoting effect on rice plant. Of the selected isolates M-10, M-1 and M-7 were found to be better in enhancing growth of rice. Due to shortage of time, effect of these isolates on yield of rice could not be investigated. This research might be helpful for developing biofertilizer for rice using these *Azospirillum* isolates. Different biological nitrogen fixation (BNF) systems including *Azospirillum* are used on a limited scale in Bangladesh agriculture. Before large-scale extension of biological nitrogen fixation (BNF) systems at the farm level, further research is needed to determine their N supplement potentials.

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References

Bao, Z, Sasaki K, Okubo T, Ikeda S, Anda M, Hanzawa E, Kakizaki K, Sato T, Mitsui H, Minamisawa K. (2013). Impact of *Azospirillum* sp. B510 inoculation on rice-associated bacterial communities in a paddy field, *Microbes Environ* 4, 487–490.

Chi HNT, Thanh HH, Dzung NN. (1998). Response of rice plants to inoculation with *Azospirillum* sp. under field conditions. K.A. Malik *et al*. (eds). Kluwer Academic Publishers. Nitrogen fixation in non-legumes. 237-240.

El-Khawas H, Adachi K. (1999). Identification and quantification of auxins in culture media of *Azospirillum* and *Klebsiella* and their effect on rice roots, *Biology and Fertiity of Soils* 28, 377–381.

Gopalawamy G, Vidhyasekaran P. (1988). Effect of *Azospirillum lipoferum* inoculation and inorganic nitrogen on wetland rice, *Oryza* 26, 378-380.

Gunarto L, Adachi K, Senboku T. (1999). Isolation and selection of indigenous *Azospirillum* spp. from a subtropical island, and effect of inoculation on growth of lowland rice under several levels of N application, *Biology and Fertility of Soil* 28, 129–135.

Hartmann A, Mahavir S, Kligmaller W. (1983). Isolation and characterization of *Azospirillum* mutants excreting high amounts of Indole acetic acid, *Canadian Journal of Microbiology* 29, 916-922.

Hossain MM, Akter S, Hasan MM, Hasan A, Uddin KR, Parvin A, Jahan I, Rahman MN, Rahman SMB. (2014). Nitrogen fixing efficiency and physiological characteristics of *Azospirillum* isolates from the paddy fields of North Bengal, *Jahangirnagar University J Biol Sci* 3(1), 47-53.

Hossain MM, Jahan I, Akter S, Rahman MN, Rahman SMB. (2015). Isolation and identification of *Azospirillum* isolates from different paddy fields of North Bengal, *Indian Journal of Research in Pharmacy and Biotechnology* 3(1), 74-80.

Isawa T, Yasuda M, Awazaki H, Minamisawa K, Shinozaki S, Nakashita H. (2010). *Azospirillum* sp. isolate B510 enhances rice growth and yield, *Microbe. Environ* 25(1), 58-61.

Kannan T, Ponmurugan P. (2010). Response of paddy (*Oryza sativa* L.) varieties to *Azospirillum brasilense* inoculation, *Journal of Phytoogy* 2(6), 08–13.

Kumar K, Balasubramanian B. (1986). Field response of rice to *Azospirillum* biofertilizer, *Current Research* 15, 74–76.

Okon Y. (1985). *Azospirillum* as a potential inoculant for agriculture, *Trends in Biotechnology* 3, 223-228.

Murty MG, Ladha JK. (1988). Influence of *Azospirillum* inoculation on the mineral uptake and growth of rice under hydroponic conditions, *Plant Soil* 108, 281-288.

Pereira JAR, Cavalcante VA, Baldani JI, Dobereiner J. (1988). Field inoculation of sorghum and rice with *Azospirillum* spp. and *Herbaspirillum seropedicae, Plant Soil* 110, 269-274.

Rasul G. 1999. Production of growth hormones and nitrogenase by diazotrophic bacteria and their effects on plant growth. Unpublished PhD Thesis. Institute of Biochemistry and Biotechnology, University of Punjab, Lahore, Pakistan.

Ravikumer S, Ramanthan G, Suba N, Jeyaseeli L. (2002). Quantification of hlophilic *Azospirillum* from mangroves, *Indian Journal of Marine Science* 31(2), 157-160.

Rodrigues PE, Rodregues LS, Oliveira ALM de, Baldani VLD, Teixeira KR dos S, Urquiaga S, Reis VM. (2008). *Azospirillum amazonense* inoculation: effects on growth, yield and N_2-fixation of rice (*Oryza sativa* L.), *Plant Soil* 302, 249-361.

Salamone IEG de, Salvo LPD, Ortega JSE, Sorte PMFB, Urquiaga S, Teixeira RS. (2010). Field response of rice paddy crop to *Azospirillum* inoculation: physiology of rhizosphere bacterial communities and the genetic diversity of endophytic bacteria in different phases of plants, *Plant Soil* 336, 351-362.

Sattar MA, Rahman MF, Das DK, Choudhury TMA. (2008). Prospects of using *Azotobacter*, *Azospirillum* and cyano-bacteria as supplements of urea nitrogen for rice production in Bangladesh. 59-60. *In: Efficient nutrient use in rice production in Vietnam achieved using inoculant biofertilisers.* Part-3. Australian center for agricultural research.