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Original Article

Effect of Some Environmental Factors on the Growth of *Azospirillum* Species Isolated from Saline Soils of Satkhira District, Bangladesh

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Twelve isolates of *Azospirillum*, recovered and identified from non-rhizosphere soil, rhizosphere soil of *Cynodon dactylon* and the roots of the same plant, were collected from five different locations of saline area in the district Satkhira, Bangladesh. The isolates were belonged to *Azospirillum lipoferum, A. brasilense, A. halopraeferans* and *A. amazonense*. All isolates, except MR-5, preferred neutral to alkaline condition for their optimal growth. Three isolates (MR-3, MR-4 and MR-8) showed maximum growth at 41°C, while the others grew best at temperature between 35° and 37°C. The optimal NaCl concentration for the growth was found to be 3% for 5 isolates (MR-1, MR-3, MR-4, MR-8 and MR-11) and 5% for the rest 7 isolates (MR-5, MR-6, MR-7, MR-13, MR-14, MR-15 and MR-16). All isolates were true halophilic since they did not show grow in absence of salinity, and all of them were also fungicide (Perfeckthion and Indofil M-45) tolerants to some extent.

Keywords: *Azospirillum*, Growth characteristics, Halophilic, Tolerance to fungicide

Introduction

Microbial colonization on plant roots is affected by biotic and abiotic factors such as root exudates, competition, inorganic nutrients, soil pH, temperature, etc. Among the agriculturally important abiotic stresses, soil salinity, alkalinity, drought and heat rank as the most detrimental and cause loss of crop productivity all over the world[1]. The investigation of their effects on the rhizosphere microflora may help to get a better insight into the dynamics of microbial populations in the rhizosphere.

Azospirillum species are free-living N_2-fixing bacteria that live in close association with roots of cereals and other grasses and are able to promote plant growth and yields in many crops of agronomic importance[2]. It is assumed that the bacteria affect plant growth mainly by the production of plant growth promoting substances, which leads to an improvement in root development and an increase in the rate of water and mineral uptake[3]. *Azospirillum* species support plant growth indirectly by improving growth-restricting conditions either via production of antagonistic substances or by inducing resistance against plant pathogens[4]. Since associative interactions of plants and microorganisms must have come into existence as a result of co-evolution, the use of this group as bio-inoculants must be pre-adapted, so that it fits into a long-term sustainable agricultural system. *Azospirillum* have been suggested as an alternative or supplement to chemical fertilization[5].

The agricultural land use in the coastal areas is very poor. In the virtual absence of any tested technology suited to the coastal conditions, the farmers in most of the coastal areas rely on the age-old traditional methods of agriculture. There, however, exists scope of improvement of the situation. The nitrogen status of these soils is poor and the most appropriate solution to this situation is to exploitation of biological nitrogen fixation to supply nitrogen from the renewable source to the crops. To develop inoculants of *Azospirillum* for the crops of saline ecosystem in Bangladesh, isolation and screening of efficient isolates of this organism from saline soils of this country are most necessary. A few works on the distribution and N_2-fixing potential of *Azospirillum* of normal agricultural soils have been done[6-7]. However, no systematic and comprehensive study has so far been conducted in Bangladesh in order to screen the efficient indigenous *Azospirillum* for the use as biofertilizer for crop production in saline soil. In this paper the *in vitro* growth of *Azospirillum* isolated from the saline area of Bangladesh has been reported.

Materials and Methods

Isolated and selected isolates of *Azospirillum* from saline soils of Bangladesh were characterized and identified according to Krieg and Döbereiner[8]. The growth of each isolate was determined in terms of optical density at 600 nm of the 72-h-old culture in 10 ml bromothymol blue-free nitrogen containing broth[8] under various temperatures, pH values and concentrations of NaCl and fungicides. To study the effect of temperatures, NaCl, and fungicides (Perfeckthion and Indofil M-45) on growth of

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Azospirillum, malic acid was used as the carbon source in the bromothymol blue-free nitrogen containing broth of neutral pH. However, for examining the effect of various pH values on the growth of *Azospirillum*, malate was replaced by galactose (0.5%) in the bromothymol blue-free nitrogen containing broth to avoid changes in pH by the utilization of malate[9]. Statistical analysis was done for the results of each experiment.

Results and Discussion

A total of 12 *Azospirillum* isolates recovered from saline soils were selected for this study, which belonged to 4 different species of *Azospirillum*. Isolates MR-1, MR-11, MR-14, MR-15 and MR-16 belonged to the species *Azospirillum lipoferum*, isolates MR-6, MR-7 and MR-13 belonged to *Azospirillum brasilense*, isolates MR-3, MR-4 and MR-8 belonged to *Azospirillum halopraeferans*, and only isolate MR-5 belonged to *Azospirillum amazonense*. The isolates MR-1, MR-3, MR-4, MR-7, MR-8 and MR-13 showed optimum growth at pH 8.0 and the isolates MR-6, MR-11, MR-14, MR-15 and MR-16 showed optimum growth at pH 7.0 (Table 1). *A. amazonense* (MR-5) showed optimal growth at pH 6.5. Döbereiner[10] mentioned that the pH of soil and of root surface might affects the microbial equilibrium decisively. Earlier observations indicated that *Azospirillum*, like *Azotobacter*, requires near-neutral pH for abundant occurrence[9]. The optimal growth of different species of *Azospirillum*, *viz*., *A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. halopraeferans* occur at pH range 6.0-7.8, 5.7-6.8, 5.7-6.5 and 6.8-8.0, respectively[11].

Temperature is one of the most important factors that governs the physiology and growth of the living organisms. The high temperature requirements of azospirilla with growth optima between 32° and 36°C might explain the much more generalized occurrence of these organisms in subtropical and tropical regions[9]. In the present study, the isolates MR-3, MR-4 and MR-8 showed maximum growth at 41°C and rest of the isolates showed maximum growth between 35° and 37°C (Table 2).

Table 1. *Effect of pH on the growth of selected isolates of Azospirillum*

Isolate No.	Growth (OD_{600}) at various pH												Mean	CD at 5% level
	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5		
MR-1	0.03	0.05	0.41	0.41	0.42	0.42	0.42	0.45	0.48	0.42	0.30	0.01	0.31	0.12
MR-3	0.0	0.03	0.38	0.51	0.60	0.65	0.75	0.80	1.0	0.80	0.70	0.02	0.52	
MR-4	0.02	0.04	0.29	0.35	0.41	0.52	0.65	0.70	0.74	0.40	0.25	0.01	0.36	
MR-5	0.03	0.05	0.32	0.80	1.0	1.2	0.80	0.75	0.74	0.45	0.26	0.02	0.53	
MR-6	0.02	0.04	0.26	0.26	0.26	0.30	0.30	0.28	0.22	0.15	0.01	0.0	0.17	
MR-7	0.01	0.03	0.3	0.65	0.70	0.75	0.79	0.80	0.80	0.60	0.45	0.01	0.49	
MR-8	0.01	0.04	0.29	0.30	0.36	0.65	0.70	0.75	0.80	0.58	0.50	0.03	0.41	
MR-11	0.01	0.04	0.23	0.50	0.60	0.70	0.78	0.45	0.38	0.30	0.20	0.0	0.34	
MR-13	0.02	0.05	0.27	0.27	0.28	0.70	0.75	0.78	0.80	0.50	0.25	0.01	0.39	
MR-14	0.02	0.05	0.28	0.70	0.80	0.85	0.95	0.93	0.92	0.74	0.64	0.02	0.57	
MR-15	0.0	0.03	0.22	0.70	0.90	1.0	1.1	0.65	0.38	0.30	0.21	0.0	0.45	
MR-16	0.01	0.06	0.19	0.70	0.81	0.95	1.0	0.95	0.90	0.85	0.70	0.05	0.59	
Mean	0.01	0.04	0.28	0.52	0.62	0.72	0.75	0.68	0.65	0.50	0.37	0.02		
CD at 5% level	0.12													

Table 2. *Effect of temperature on the growth of the selected isolates of Azospirillum*

Isolate No.	Growth (OD_{600}) at various temperatures (°C)											Mean	CD at 5% level
	25	27	29	31	33	35	37	39	41	43	45		
MR-1	0.20	0.24	0.29	0.32	0.38	0.42	0.41	0.20	0.07	0.01	0.0	0.23	0.11
MR-3	0.25	0.30	0.41	0.45	0.50	0.55	0.60	0.68	0.85	0.41	0.02	0.45	
MR-4	0.19	0.25	0.34	0.40	0.50	0.54	0.60	0.64	0.74	0.21	0.01	0.40	
MR-5	0.19	0.20	0.24	0.26	0.35	0.40	0.34	0.10	0.05	0.02	0.01	0.19	
MR-6	0.20	0.21	0.23	0.29	0.38	0.40	0.34	0.20	0.05	0.02	0.01	0.21	
MR-7	0.36	0.38	0.42	0.54	0.65	0.80	0.80	0.35	0.07	0.04	0.02	0.40	
MR-8	0.21	0.30	0.35	0.38	0.48	0.54	0.60	0.65	0.70	0.22	0.01	0.40	
MR-11	0.26	0.29	0.35	0.38	0.58	0.60	0.54	0.45	0.20	0.01	0.0	0.33	
MR-13	0.35	0.40	0.46	0.68	0.70	0.79	0.80	0.65	0.07	0.02	0.01	0.44	
MR-14	0.30	0.38	0.45	0.71	0.80	0.95	0.91	0.32	0.02	0.01	0.0	0.44	
MR-15	0.29	0.35	0.58	0.79	0.85	1.10	1.0	0.56	0.35	0.20	0.02	0.55	
MR-16	0.25	0.35	0.54	0.70	0.85	1.0	0.95	0.56	0.20	0.05	0.02	0.49	
Mean	0.25	0.30	0.38	0.48	0.58	0.67	0.62	0.44	0.28	0.10	0.01		
CD at 5% level	0.11												

All isolates of *Azospirillum* preferred saline condition for proper growth (Table 3). The optimal NaCl concentration was found to be 3% for the isolates MR-1, MR-3, MR-4, MR-8 and MR-11 and 5% for the isolates MR-5, MR-6, MR-7, MR-13, MR-14, MR-15 and MR-16. All isolates were unable to show recognizable growth on normal media. The results clearly confirmed that the isolates of *Azospirillum* were halophilic. Ravikumar *et al*.[12] reported the isolation of *A. brasilense* from mangrove environment that showed better growth and indole acetic acid (IAA) production at 3% NaCl. *A. halopraeferans* was reported to occur in the rhizoplane of plants growing in saline soils in Brazil[10]. In this study, three isolates, MR-3, MR-4 and MR-8, were identified as *A. halopraeferans* occurring in the non-rhizosphere saline soil and root of *Cynodon dactylon* growing in saline soil of the district Satkhira, Bangladesh. *A. halopraeferans* as deduced from its name seems adapted to saline soils[10]. Low concentrations of NaCl exerted an accelerating effect on the growth of bacteria[13]. Obligate halophiles possess specialized enzymes that become in their active configuration only at high salt concentrations[1].

In this study, the significant effect of the fungicides, Perfeckthion and Indofil M-45, on the growth of the *Azospirillum* isolates was observed (Table 4 and 5). The bacteria grew better in Perfeckthion than in Indofil M-45. Two isolates MR-1 and MR-5 could tolerate up to 250 ppm of the fungicides showing growth that was comparable to the growth in the control medium without fungicide. The MR-1 and MR-5 were belonged to *A. lipoferum* and *A. amazonense* respectively. Application of pesticides has become an integral part of present agriculture. Very little is known about their effect on non-target microorganisms. The effect of various pesticides on the growth and N_2-fixation was studied using pure culture of bacteria by Wood and MacRae[14] and Kulkarni *et al*.[15].

Table 3. *Effect of salinity (NaCl) on the growth of the selected isolates of Azospirillum*

Isolate No.	Growth (OD_{600}) at various concentrations of NaCl (%)																Mean	CD at 5% level
	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5		
MR-1	0.01	0.17	0.24	0.29	0.32	0.35	0.38	0.31	0.21	0.2	0.1	0.02	0.0	0.0	0.0	0.0	0.15	0.12
MR-3	0.02	0.14	0.37	0.4	0.48	0.49	0.68	0.48	0.09	0.03	0.01	0.0	0.0	0.0	0.0	0.0	0.19	
MR-4	0.0	0.11	0.3	0.41	0.45	0.5	0.58	0.68	0.45	0.3	0.04	0.01	0.01	0.0	0.0	0.0	0.23	
MR-5	0.0	0.03	0.2	0.24	0.27	0.3	0.32	0.35	0.4	0.42	0.5	0.54	0.25	0.12	0.08	0.01	0.25	
MR-6	0.0	0.1	0.14	0.26	0.29	0.3	0.36	0.41	0.46	0.51	0.64	0.59	0.45	0.3	0.2	0.05	0.31	
MR-7	0.0	0.2	0.31	0.39	0.39	0.42	0.45	0.49	0.51	0.58	0.74	0.64	0.4	0.34	0.21	0.09	0.38	
MR-8	0.0	0.08	0.3	0.35	0.38	0.4	0.56	0.55	0.44	0.43	0.23	0.21	0.09	0.01	0.0	0.0	0.24	
MR-11	0.01	0.09	0.3	0.35	0.38	0.4	0.78	0.71	0.36	0.25	0.08	0.0	0.0	0.0	0.0	0.0	0.23	
MR-13	0.0	0.1	0.28	0.3	0.38	0.42	0.45	0.48	0.5	0.8	1.0	1.2	0.9	0.21	0.08	0.01	0.43	
MR-14	0.0	0.01	0.3	0.39	0.4	0.45	0.49	0.5	0.55	0.59	0.57	0.57	0.45	0.21	0.11	0.05	0.35	
MR-15	0.0	0.2	0.29	0.32	0.42	0.45	0.51	0.7	0.75	0.8	0.85	0.72	0.65	0.25	0.05	0.01	0.41	
MR-16	0.0	0.2	0.3	0.35	0.42	0.49	0.5	0.55	0.71	0.8	0.86	0.73	0.54	0.32	0.1	0.02	0.42	
Mean	0.0	0.11	0.27	0.33	0.38	0.41	0.5	0.51	0.45	0.47	0.46	0.43	0.31	0.14	0.06	0.02		
CD at 5% level	0.14																	

Table 4. *Effect of fungicide (Perfeckthion) on the growth of the selected isolates of Azospirillum*

Isolate No.	Growth (OD_{600}) at various concentrations of Perfeckthion (ppm)				Mean	CD at 5% level
	Control	250	375	500		
MR-1	0.38	0.40	0.25	0.12	0.28	0.14
MR-3	0.68	0.45	0.23	0.10	0.36	
MR-4	0.58	0.48	0.20	0.09	0.33	
MR-5	0.50	0.55	0.48	0.45	0.49	
MR-6	0.64	0.58	0.38	0.22	0.45	
MR-7	0.74	0.52	0.35	0.20	0.45	
MR-8	0.56	0.46	0.26	0.11	0.34	
MR-11	0.78	0.42	0.25	0.12	0.39	
MR-13	1.0	0.62	0.46	0.45	0.63	
MR-14	0.57	0.63	0.40	0.30	0.47	
MR-15	0.85	0.64	0.26	0.05	0.45	
MR-16	0.86	0.60	0.30	0.21	0.49	
Mean	0.67	0.52	0.31	0.20		
CD at 5% level	0.08					

Table 5. *Effect of fungicide (Indofil M-45) on the growth of the selected isolates of Azospirillum*

Isolate No.	Growth (OD_{600}) at various concentrations of Indofil M-45 (ppm)				Mean	CD at
	Control	250 ppm	375 ppm	500 ppm		5% level
MR-1	0.38	0.38	0.18	0.01	0.23	0.15
MR-3	0.68	0.39	0.22	0.09	0.34	
MR-4	0.58	0.27	0.17	0.11	0.28	
MR-5	0.50	0.35	0.23	0.14	0.30	
MR-6	0.64	0.34	0.25	0.13	0.34	
MR-7	0.74	0.29	0.19	0.08	0.32	
MR-8	0.56	0.30	0.21	0.11	0.29	
MR-11	0.78	0.39	0.23	0.12	0.38	
MR-13	1.0	0.41	0.29	0.14	0.46	
MR-14	0.57	0.44	0.27	0.13	0.35	
MR-15	0.85	0.38	0.24	0.08	0.38	
MR-16	0.86	0.42	0.32	0.15	0.43	
Mean	0.67	0.36	0.23	0.11		
CD at 5% level	0.09					

In the present investigation, variations in characteristics of the isolates of *Azospirillum* were observed. Statistical analysis showed that pH, temperature, NaCl and pesticide influenced significantly the growth of the *Azospirillum* isolates. It was evident from this work that saline soil harbours halophilic azospirilla and they could colonize in the roots of the plants growing in saline habitats. Further works are required to ascertain the potentiality of these organisms for use them as biofertilizer for improving crop yield in saline agricultural fields.

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