PHYSIOLOGICAL CHARACTERISTICS AND BIOLOGICAL NITROGEN FIXATION OF *AZOSPIRILLUM* SPP., ISOLATED FROM THREE LOCATIONS IN BANGLADESH

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Abstract

Some important physiological characteristics and nitrogen fixing potential of 6 *Azospirillum* spp., isolated previously at Jahangirnagar University (JU) from the rhizosphere of the plants growing on sandy lands, were investigated. Thirty different colonies of *Azospirillum* spp. were isolated from the particular locations of the banks of 3 different rivers of Bangladesh, Jamuna, Padma and Atrai from which 6 strains (F-1, F-2, F-3, F-4, F-5 and F-6) were finally selected on the basis of better growth in semi solid nitrogen free bromothymol blue (Nfb) medium. And also among these 6 selected *Azospirillum* strains, 2 (F-2, F-4) were identified as *A. lipoferum* and 4 (F-1, F-3, F-5, F-6) as *A. brasilense* by comparing with the reference strains on the basis of colony, cell morphology and biochemical tests. In the present study, all of the strains preferred neutral pH and temperature 40° C for showing maximum activity. Growth of all of these strains gradually decreased with the increase of NaCl concentrations in the medium. Nitrogen fixing potential and microbial activity of these strains varied significantly. The nitrogen fixing potential ranged from 10.80 to 13.16 mg nitrogen/g substrate in the semi solid nitrogen free malate medium and the activity of the strains measured in terms of the amount of CO_2 evolved by them after incubation for 5 days and varied from 7.70 to 37.40 mg. Finally, F-2 was found to be superior to other 5 selected strains considering nitrogen fixing potential, microbial activity and physiological characteristics.

Keywords: *Azospirillum*, Nitrogen Fixation, Microbial Activity, Physiological Characteristics.

Introduction

To increase the food production through intensive agriculture, among other things, heavy input of inorganic fertilizers is required. However, the energy crisis, which is looming large in all spheres of human endeavor, has hampered chemical fertilizer production and availability with the result that attention is being increasingly paid to alternate ways of increasing crop yields. Scientists have recently identified two major research thrusts to augment crop yields. One of them aims at improving the photosynthetic efficiency of plants and the other envisages strengthening research on biological nitrogen fixation.

In fact, photosynthesis and biological nitrogen fixation are closely linked because nitrogen fixation consumes a good deal of energy captured from the sun in the process of photosynthesis. Accurate estimates of annual turn over of nitrogen in the biosphere vary from 100 million (Delwiche, 1970) to 200 million metric tons (Burns and Hardy, 1975) of which 2/3 come from biological sources. It is interesting to note that the ratio between chemically fixed nitrogen and biologically fixed nitrogen ranges approximately from 1:4 to 1:2.5 (Subba Rao, 1979). The global demand for chemically fixed nitrogen in the years to come for sustaining the ever increasing need for agricultural products has already alerted specialists in the field of environmental pollution, since accumulation of undesirable nitrate in the environment in future years would contribute to severe eutrophication, infant and aminal methemoglobenemia and the formation of nitrosamines.

In a developing country like Bangladesh, the existing gap between supply and demand of nitrogen fertilizer is likely to increase with added constraints on technological development of fertilizer industries, inadequate power supply, non-availability of raw-materials, poor storage, improper distribution and extension services (Rahman, 2002).

For the cultivation of leguminous and other crops, nitrogen fixing bacteria *Rhizobium, Azospirillum* and *Azotobacter* are being used as biofertilizer in several countries of the world. In Bangladesh, works on *Rhizobium* have received much dimension and this organism is now being used as biofertilizer for the cultivation of a number of leguminous crops. The importance of other nitrogen fixing bacteria should also be considered for other crops especially for grain crops. *Azospirillum* is a soil bacterium, capable of producing associative symbiosis in the roots of various plants including grain crops. The plants that grow in sandy lands obtain their mineral nutrient form the sands. In such places nitrogen content is almost nil and biological nitrogen fixation seems to be the main mechanism of the production and supply of combined

nitrogen to the plants. Nitrogen fixation is also thought to be a major input to nitrogen budget of deserts (Zaddy *et al.*, 1998).

The use of beneficial microorganisms associated with roots may accelerate the restoration of disturbed areas (Carrillo-Garcia *et al.*, 2000). Sandy soils with more than 90 percent sand require high fertilizer input. The use of biofertilizer such as nitrogen fixing bacteria can reduce chemical fertilizer requirement and consequently lower production cost (Saad *et al.* 1999). Under such stress condition *Azospirillum* might be naturally adapted to attain remarkable efficiency in fixing atmospheric nitrogen and in enhancing plant growth by this and by some other ways like production of growth promoting substances and influencing root development and causing increased uptake of nutrients from the land. So, the present work was undertaken to study the physiological characteristics and nitrogen fixing potential of *Azospirillum* spp. isolated from the different locations of Bangladesh.

Materials and Methods

Azospirillum strains

In the present study, 6 *Azospirillum* spp., collected from the Department of Botany, Jahangirnagar University, Savar, Dhaka, which were previously isolated and identified by Khan and Rahman (2003) from three locations, Bhuapur of Tangail at the bank of Jamuna; Bhagyakul of Munshiganj at the bank of Padma and Srikanthapur of Dinajpur at the bank of Atrai on the basis of colony, cell morphology and biochemical tests. The best 6 *Azospirillum* spp. were selected for further study by Rahman (2002) from initially isolated 30 *Azospirillum* colonies on the basis of their ability to grow better and faster in Nfb semi solid medium. The reference (control) strain, *Azospirillum brasiliense* (ATCC No. 29145) was collected form the American type culture collection to compare the characteristics of these 6 strains.

Determination of microbial activity

Microbial activity of the reference strain and selected strains was determinated by measuring the amount of CO_2 evolved by the culture. CO_2 evolution was estimated according to the method described by Pramer and Schmidt (1964). Conical flasks of 1000 ml capacity were filled with 250 ml semi solid Nfb medium. A sterile test tube containing sterile 10 ml N/2 NaOH solution was hung in each flask with the help of a sterile thread. The flasks were closed with rubber stoppers (Sterilized with alcohol and ether) sealed with petroleum jelly. The flasks were incubated at 35° C. CO_2 evolve were absorbed by the alkali in the test tube and measured by the titrimetrically after 5 days. During each estimation the contents of the test tube were quantitively transferred to a flask followed by the addition of 5 ml of saturated solution of the $BaCl_2$ to precipited as $BaCO_3$. The reaction was as follows:

$2NaOH + CO_2 = Na_2CO_3 + H_2O$; $Na_2CO_3 + BaCl_2 = BaCO_3 + 2NaCl$

The residual amount of NaOH in the flask was measured by titrating against N/2 HCl using 2-3 drops of phenopthelene as indicator. The calculation was done according to following formula:

1 ml of 1(N) HCl ≡ 1 ml of 1 (N) NaOH ≡ 22 mg of CO_2

Effect of pH on the activity of the Azospirillum strains

Semi solid Nfb medium was prepared and pH of the medium was adjusted to 5, 6, 7, 8 and 9 just prior to adding agar. Effect of different pH on the activity of reference strain and selected strains was determined by CO_2 evolution method (Pramer and Schmidt, 1964).

Effect of temperature on the activity of the Azospirillum strains

To find the maximum, minimum and optimum temperature, each strain was allowed to grow in Nfb semi solid medium at temperature 30°, 35°, 40° and 45° C for 5 days. Then the activity of each strain was estimated by CO_2 evolution method (Pramer and Schmidt, 1964).

Effect of salinity on the activity of the Azospirillum strains

Tubes of liquid malate medium without bromothymol blue but contains 1.0% NH_4Cl were used. Tubes of the medium containing various concentration of NaCl (1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%) were inoculated with the referecne strain and selected strains and total viable count of the organism was determined after 48 hours.

Determination of nitrogen fixing potential of the Azospirillum strains

Nitrogen fixation was determined in terms of the quality of nitrogen gained in the 72 hours old cultures of each strains developed in 25 ml semi solid nitrogen free malate medium (without bromothymol blue). The total nitrogen content in culture was estimated by microkjeldahl method. The total nitrogen content in culture of the organism was determined by digesting the sample with concentrated H_2SO4 together with digestion mixture ($CuSO_4.5H_2O$+K_2SO4 with ratio of 10:1) in kjeldahl flask and distilling the content in a microjeldahl distillation apparatus with 30% NaOH solution. The ammonia evolved was absorbed in 4% boric acid with mixed indicator and treated with a standard 0.01 % (N) HCl solution. The total amount of nitrogen was calculated using the following formula:

1 ml of 1 (N) HCl = 0.014 g nitrogen.

Statistical analysis

All the data were arranged in Completely Randomized Design (CBD) and analyzed statistically using ANOVA.

Results and discussion

Microbial activity of Azospirillum strains

The activity of the selected strains under nitrogen fixing condition measured in terms of CO_2 evolution is shown in Table 1. Among the selected strains, highest amount of CO_2 was evolved by the strain F-2 followed by the strain F-5. Strain F-1, F-3 and F-4 evolved lower amount of CO_2. The extent of the amount of evolved CO_2 in all the six selected strains after 5 days, ranged from 7.7 to 37.40 mg. As per their activity the selected strains could be arranged as F-2>F-5>F-1=F-3=F-4=F-6. The reference strain *Azospirillum brasilense* (ATCC NO. 29145) evolved 24.20 mg CO_2 and F-2 could evolve more CO_2 than this reference strain. Carbondioxide is one of the principle metabolic products of microorganisms and CO_2 evolution during microbial growth has frequently been used as a measure of microbial activity (Waksman and Starkey, 1957).

Table 1. Activity of the reference and selected *Azospirillum* strains in nitrogen fixing condition at 35° C and pH 7 after 5 days

Strain	Evolved CO_2 (mg)
F-1	8.80
F-2	37.40
F-3	8.80
F-4	8.80
F-5	9.90
F-6	7.70
Ab	24.40
LSD at 5% level	0.26

Ab: *Azospirillum brasilense* (ATCC NO. 29145)

Effect of pH on the activity of the Azospirillum strains

At different pH, variation in the activity of the selected strains of *Azospirillum* under nitrogen fixing condition was observed (Table 2). All of the strains showed maximum activity at pH 7 and the activity decreased at both acidic and alkaline pH. At pH 7 the strain F-2 showed the highest activity evolving 37.40 mg CO_2 after incubation for 5 days. The least activity was showed by F- 6 at pH 7 and at this pH this strain evolved 7.70 mg CO_2 after 5 days. The strain F-1, F-3 and F-4 evolved same amount of CO_2, which was 8.80 mg after 5 days. Day and Dobereiner (1976) reported that nitrogenase activity of *Azospirillum* is very pH sensitive and optimum nitrogen fixation & nitrogen dependent growth occur only between pH 6.8 and 7.8 and there happen little growth & nitrogen fixation below pH 5.5 or above 9.0. Khan and Akond (1996) reported that 5 strains *A. brasilense* fixed maximum amount of nitrogen at pH 7.0 and these strains exhibited almost no nitrogen fixation at pH 5.0 and 9.0. Similarly in the present study, optimum activity of all these selected strains were observed at pH 7.0 and activities were least at pH 5.0 and 9.0 (Table 2). Tilak *et al.* (1988) found that optimum pH values for 3 strains of *Azospirillum* for nitrogen fixation ranged from pH 6.5 to 8.5.

Table 2. Effect of pH on the activity of the reference and selected *Azospirillum* strains after 5 days

Strain	Evolved CO_2 (mg) at different pH				
	5.0	6.0	7.0	8.0	9.0
F-1	0.00	4.31	8.80	6.60	4.79
F-2	5.39	11.86	37.40	13.20	7.60
F-3	6.47	9.58	8.80	6.60	0.21
F-4	7.55	10.32	8.80	7.60	2.24
F-5	4.31	7.55	9.90	4.49	1.79
F-6	4.31	7.55	7.70	4.40	4.49
Ab	11.86	15.30	24.20	16.50	13.20
LSD at 5% level	0.84	0.99	0.26	0.45	0.02

Ab: *Azospirillum brasilense* (ATCC NO. 29145)

Effect of temperature on the activity of the Azospirillum strains

The organisms showed variation in activity at different tempetature values (Table 3). All of the selected strains showed lowest activity at 30° C and highest at 40° C. At 35° C and 45° C the activity of the strains was more or less similar. At 40° C the values of activity ranged from 22 mg CO_2 by F-6 to 42.90 mg CO_2 by F-2 after incubation for 5 days. Temperature is one of the most important factors that governs the physiology and growth of the living organisms. Day and Dobereiner (1976) reported that the optimum temperature for nitrogen dependent growth of *Azospirillum lipoferum* was between 32° C and 40° C and nitrogen fixation was very little at temperatures below 24° C. At 42° C the nitrogenase of this organism was inactivated. Tilak *et al.* (1988) reported that *A. brasilense* exhibited maximum nitrogen fixing ability at temperatures between 30° C and 35° C in nitrogen free semi solid malate medium. In this study, the activity of the selected strains was found to be optimum at 40° C (Table 3). The activities were low at 30° C and 45° C. The variations in activities at different temperatures were statistically significant. The result indicate that activity increases with the increase of temperature up to 40° C and declines at temperatures above 40° C. Khan *et al.* (2001) found that some thermophilic strains of *Azospirillum* exhibited higher growth and nitrogen fixation at 55° C than 35° C. The high temperature requirements of these organisms are of great ecological importance as in temperate regions

where, soil temperatures seldom reach 28° C for any significant period. In the tropics, however, optimal temperatures for nitrogenase activity of this system occur during the main growing season almost daily for most of the days (Day and Dobereiner, 1976). Nitrogen fixation by tropical *Azospirillum* strains was highly reduced when the strains was transferred from 36° C to 17° C (Day and Dobereiner, 1976). It shows that the strains are highly adaptive to their native environment. Eckert *et al.* (2001) reported that the best growth of *Azospirillum* spp. was observed at 30 °C and no growth occurred at 37 or 42 °C.

Table 3. Effect of pH on the activity of the reference and selected *Azospirillum* strains after 5 days

Strain	Evolved CO_2 (mg) at different temperature			
	30° C	35° C	40° C	45° C
F-1	5.50	8.80	31.21	5.50
F-2	10.60	37.40	42.90	24.20
F-3	6.60	8.80	26.62	6.60
F-4	1.10	8.80	27.50	8.80
F-5	3.30	9.90	33.00	9.90
F-6	1.10	7.70	22.00	6.60
Ab	8.80	24.20	38.50	23.10
LSD at 5% level	0.24	0.26	0.74	0.27

Ab: *Azospirillum brasilense* (ATCC NO. 29145)

Effect of salinity on the growth of the Azospirillum strains

Figure 1 shows the growth of the reference and selected strains at different concentration of NaCl. All of the strains showed best growth in the medium receiving no extra amount of NaCl. Growth of the strains decreased gradually with the increase of the concentration of NaCl. At NaCl concentration above 2.5%, the growth of all of the strains drastically reduced and became almost negligible at 4% NaCl. Salinity stress inhibits the growth and nitrogen fixation ability of the plant growth promoting rhizobacterium *Azospirillum* brasilense (Chowdhury *et al.*, 2007). Low concentration of NaCl produce an accelerating effect on the growth of bacteria (Salle, 1967). High concentrations of NaCl are generally inhibitory. Maximum count of *E. coli* was found at a concentration of 0.2 M (1.17%) (Salle, 1967). In addition to affecting osmotic pressure, high salt concentration tend to denature proteins and obligate hallophiles possess specialized enzymes that are in their active configuration only at high salt concentration (Atlas and Bartha, 1981). In the present investigation none of the strains preferred saline condition for proper growth. Similar results were reported by Khan and Akond (1996). They found that nitrogen fixation by five strains of *A. brasilense* gradually decreased with the increase in concentrations of NaCl in the medium. Tilak and Krishna Murti (1981) reported the isolation of *A. brasilense* from the root of salt tolerant varieties of barley. The seed inoculation with this organism could cause significant increase of yield of salt tolerant barley, grown in saline soil.

Nitrogen fixing potential of the type and selected Azospirillum strains

The nitrogen fixing potential of the reference and selected isolates of *Azospirillum* in the presence of carbon source malate is shown in Figure 2. All the selected strains could fix atmospheric nitrogen in semi solid nitrogen free malate medium (without bromothymol blue). Among the selected strains, F-2 fixed the highest amount of nitrogen followed by F-5. Strain F-3 fixed the least amount of nitrogen. The extent of fixation in all the 6 selected strains ranged from 10.80 to 13.19 mg nitrogen/g substrate. As per their nitrogen fixing capability the selected strains could arranged as F-2>F-5>F-6>F-4>F-1>F-3. The reference strain *Azospirillum brasilense* (ATCC NO. 29145) fixed 15.41 mg nitrogen/g substrate.

Microaerophilic condition is required for *in vitro* nitrogen fixation by *Azospirillum* (Subba Rao, 1982; Eckert *et al.,* 2001) and energy source is essential. Carbon substrate usually acts as source of energy. *Azospirillum* readily utilize organic acids like malate, succinate, pyruvate and lactate for its growth (Tarrand *et al.*, 1978; Malik *et al.,* 1997). In this study, malate was used as sole carbon source for the determination of nitrogen fixing potential of the selected strains. Values equivalent to the highest efficiencies of nitrogen fixation first reported by Dobereiner and Day (1976). 115 mg nitrogen/g lactate has not been reported in other studies. About *in vitro* nitrogen fixation, Okon *et al.* (1977) reported values of 20 to 24, Nelson and Knowles (1978) 4.7 to 28 and Lakshmi *et al.*, (1977) 12 to 36 mg nitrogen/g substrate. Lakshmi and Dhala (1984) reported that some aquatic isolates of *Azospirillum* had nitrogen fixing potential ranging from 3.08 to 11.9 mg nitrogen/100 ml culture. In the present investigation, the selected strains were found to fix nitrogen ranging from 10.80 to 13.16 mg nitrogen/g substrate (malate) (Figure 2). Khan and Hossain (1990) found that nitrogen fixation by ten strains of *Azospirillum* ranged from 2.9 to 7.3 mg nitrogen/50 ml culture. Khan and Akond (1996) however reported lower values, the amount of nitrogen fixed by their strains of *Azospirillum* ranged from 448 to 658 micro g nitrogen/25 ml culture. Khan *et al.* (2001) reported that the nitrogen fixing potentials of *Azospirillum* isolated from wheat fields of Dhaka ranged from 15.12 to 22.16 mg nitrogen/substrate. Khan *et al.* (2001) also reported that some thermophilic strains of *Azospirillum* isolated from Bangladesh soil could fix nitrogen well at 55° C and

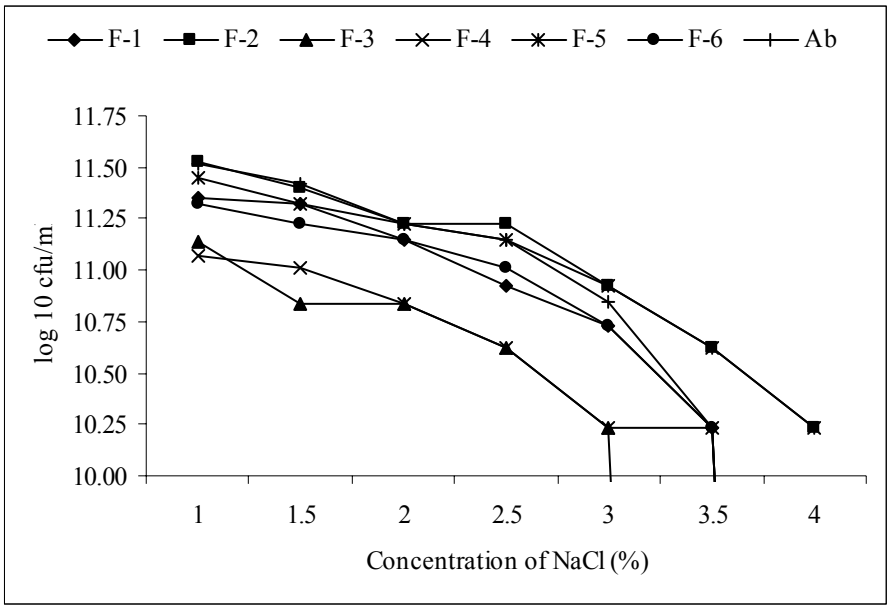

Fig. 1. Effect of salinity (NaCl) on the growth of the reference (Ab: *Azospirillum brasilense*, ATCC NO. 29145) and selected *Azospirillum* strains

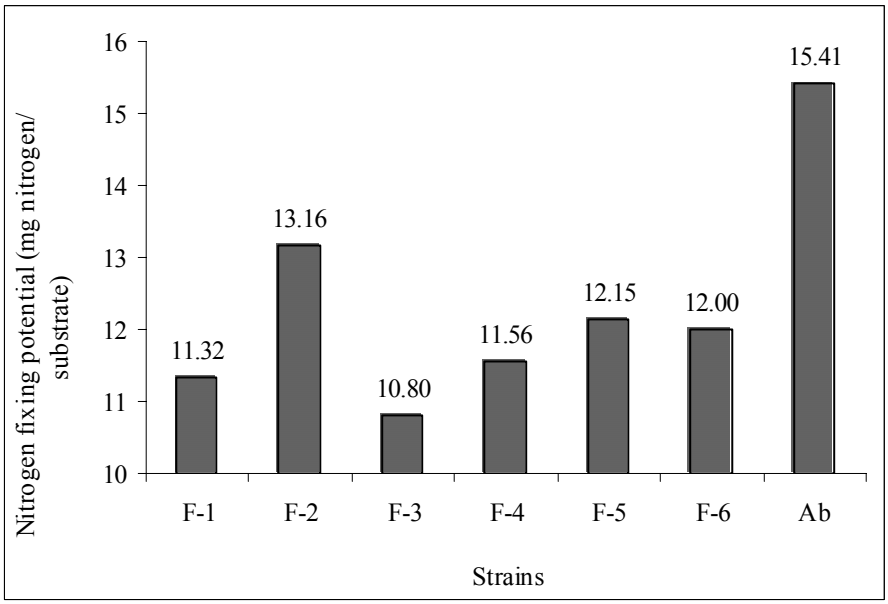

Fig. 2. Nitrogen fixing potential of the reference (Ab: *Azospirillum brasilense*, ATCC NO. 29145) and selected *Azospirillum* strains

the values ranged from 10.08 to 28.00 mg nitrogen/ g of substrate.

Conclusion

In the present investigation, a number of six *Azospirillum* spp. were used to evaluate for their nitrogen fixing potentiality and physiological characteristics. It was observed that among the 6 *Azospirillum* spp. F-2 were superior to other 5 strains. From this result, it can be concluded that F-2 including the other 5 *Azospirillum* spp. could be further studied by inoculating with grain crops to observe their ability to promote the growth of the plants by fixing atmospheric nitrogen.

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