Asian Journal of Biological and Life Sciences | Original Research

Nitrogen fixation by the native *Azospirillum* spp. isolated from rhizosphere and non-rhizosphere of foxtail millet

Mokula Md. Raffi[1,2]*, P.B.B.N. Charyulu[1]

1 Department of Microbiology, Sri Krishnadevaraya University, Anantapur - 515003, India
2 Department of Biotechnology, Sri Krishnadevaraya University, Anantapur - 515003, India

E-mail : rafimokula@gmail.com

Submitted : 13.07.2012 Accepted :11.08.2012 Published : 31.12.2012

Abstract

Experiments were conducted to isolate *Azospirillum* spp. from the rhizosphere and non-rhizosphere soil of foxtail millet grown in native black loamy soils of Anantapur district. A total of 100 strains of *Azospirillum* were isolated and grown on semi-solid nitrogen-free bromothymol blue malate semisolid malate agar (Nfb), purified and tested for nitrogen fixing abilities, only 56 strains of *Azospirillum* spp. strains showed nitrogen fixing ability, out of which 20 strains fixed nitrogen efficiently. *Azospirillum* strains varied in their nitrogen-fixing abilities ranging from 2.95 to 12.4 mg g^{-1} malate. All the strains were identified as *Azospirillum lipoferum*. These strains when tested for IAA production, all produced IAA.

INTRODUCTION

The existence of life on earth is favoured by the cycling of biological elements where in the complex biological systems after their decay are converted into simpler forms. These cycles like carbon, sulphur, phosphorus and nitrogen are essential for biology and the role played by microorganisms in these cycles is important. Nitrogen is an essential and key element in improving crop productivity throughout the world. In recent years, chemical fertilizers are used to boost the agricultural production. About 42 million tons of fertilizer N is being used annually on global scale for the production of three major cereal crops viz., wheat, rice and maize. Crop plants are able to use about 50% of the applied fertilizer N while 25% is lost from the soil-plant system through leaching, volatilization and denitrification [1]. Even though molecular nitrogen is abundant in atmosphere, it is generally deficient in soils resulting in decreased yield of the crops. As a result of its role and its low supply, the management of nitrogen resources is an important aspect in agricultural productivity. The plants are unable to utilize molecular nitrogen, unless it is converted into the suitable form (ammonia). This ability of reduction of atmospheric nitrogen to ammonia, known as biological nitrogen fixation, is confined to microorganisms. The well known asymbiotic diazotrophic bacteria belong to the genera like *Acetobacter, Azotobacter, Azospirillum, Azoarcus, Burkholderia, Enterobacter, Herbaspirillum, Pseudomonas, Klebsiella* etc., which are able to exert positive effect on plants by producing and secreting plant growth regulators (PGRs) and/ or by supplying biologically fixed nitrogen [2] The nitrogen gain through non-symbiotic systems is low compared to symbiotic systems. However, the contribution of heterotrophic nitrogen fixers can be of considerable importance under conditions of high organic matter and moisture availability.

Azospirillum is an associative microaerophilic diazotroph isolated from the roots and above ground parts of a variety of crop plants [3,4,5]. The organisms are Gram negative curved rods of variable sizes which exhibit spirillar movement and polymorphism. The cells contain poly-β-hydroxy butyrate (PHB) granules and fat droplets. An extreme colonization by azospirilla to root hairs, epidermal cells, cortical intracellular spaces has also been reported [6]. It is also found to increase the formation of root hairs and lateral roots and results in higher biomass of plants. It also produces growth- promoting phytohormones and hence acts as plant growth-promoting rhizobacteria (PGPR), which colonize the rhizosphere of numerous crop plants in tropical and subtropical regions. Some of the investigations emphasize significant contribution of N with inoculation of diazotrophic bacteria like *Azospirillum* to foxtail millet [7,8]. Though there are several studies on nitrogen fixation, information on diazotrophs associated with foxtail millet is rather limited [9,10]. Hence, the present study was aimed at isolation of native *Azospirillum* spp. and to find nitrogen-fixing abilities and IAA production of the selected strains.

MATERIALS AND METHODS

Soil : Black soil of sandy loam type (pH-7.69, organic matter-1.24%, total N-0.21% and P-14.2 mg P/Kg) was collected from cultivated fields of Anantapur.

Soil sampling

Roots of foxtail millet grown in pots (5 kg soil) along with the soil still adhering after gentle shaking and washing under tap water is considered rhizosphere soil and the same was used as the starting material for isolation of *Azospirillum* [11]. One gram portions of rhizosphere soil samples (roots and adhering soil) were ground in mortar with 10 ml of sterilized distilled water and ten-fold serial dilutions (up to 10^{-8}) of the rhizosphere samples were prepared aseptically. The black soil in pots alone without plants, served as the non-rhizosphere soil sample.

Isolation and enumeration of *Azospirillum* spp.

The suspensions (0.1 ml) of dilutions (10^{-3} to 10^{-8}) of the rhizosphere and the non-rhizosphere samples were inoculated into semi-solid nitrogen-free bromothymol blue malate (Nfb) medium [12] of the following composition (g/l) : DL-Malic acid 5.0, KOH 4.0, K_2HPO_4 0.5, $FeSO_4.7H_2O$ 0.05, $MnSO_4.H_2O$ 0.01, $MgSO_4.7H_2O$ 0.1, NaCl 0.02, $CaCl_2.2H_2O$ 0.01, $Na_2MoO_4.2H_2O$ 0.002, Bromothymol blue 2ml (0.5% alcoholic solution), Agar agar 1.75, Distilled water 1000 ml. The pH of the medium was adjusted to 6.8 using potassium hydroxide solution.

Agar was added after adjusting the pH and melted. 10 ml of the molten medium was dispersed into test tubes and sterilized by autoclaving at 121°C for 20 minutes. The tubes were inoculated with 0.5 ml of each of the dilutions (10^{-3} to 10^{-8}) of the rhizosphere and the non-rhizosphere soil samples. Five tubes were maintained for each dilution and incubated at 30°C for 72 h. *Azospirillum* spp. grew well on semi-solid Nfb malate medium forming a typical thin pellicle a few mm below the surface of the medium within 48 h. The test tubes with the pellicle were recorded as positive and MPN counts were calculated as per the table provided by Alexander (1965) [13].

Purification of *Azospirillum* spp.

The pellicles formed were transferred repeatedly (4-5 times) into fresh sterile-semi-solid Nfb malate medium and their purity was checked by microscopic observation for characteristic spiral movements and refractile PHB granules and by streaking on Nfb agar plates containing 50 mg of yeast extract per liter. Typical, small, dense and isolated single colonies, formed after one week, were picked up and transferred into fresh semi-solid Nfb malate medium. Formation of pellicle in this medium indicated successful isolation. Finally the cultures were purified by streaking on BMS medium [14] with the following composition (g/l): Potatoes 200, DL-Malic acid 2.5, KOH 2.0, Vitamin solution 1 ml, Agar agar 18.0, Distilled water 1000 ml, (10 mg biotin, 20 mg pyridoxine per 100 ml distilled water). Washed, peeled and sliced potatoes were boiled for 30 minutes in 1 litre of water and then filtered. Malic acid (2.5 g) was dissolved in 50 ml distilled water and two drops of bromothymol blue (0.5 alcoholic solution) was added. Potassium hydroxide solution was added until the malic acid solution turned green indicating neutral pH (pH 7.0). This solution together with sucrose, vitamin solution, and agar were added to potato filtrate. The final volume was made up to 1 liter with distilled water. The medium was sterilized by autoclaving at 121°C for 20 minutes. The sterilized medium was dispersed into previously sterilized Petri plates and streaked with the bacterial culture. After one week of incubation, typical, pink and wrinkled colonies were obtained. The colonies were transferred to Nfb malate agar slants containing 50 mg of yeast extract per liter for preservation and for further usage in experiments.

Estimation of nitrogen fixation

The amount of nitrogen fixed by each of the isolates of *Azospirillum* spp. was estimated by Kjeldahl method by means of an automatic nitrogen analyzer [15].

Azospirillum spp.

The ability of the isolates of *Azospirillum* spp., grown in nitrogen-free semi-solid malate medium to fix nitrogen, was estimated by an autoanalyzer. A loopful of the pure culture was inoculated into test tubes containing 10 ml of sterilized N-free semi-solid malate medium. Uninoculated tubes containing same medium served as control. All the tubes (3 replicates for each strain) were incubated at 30°C for 72 h. Triplicate samples were analyzed for total nitrogen. The amount of nitrogen fixed by each isolate was expressed as mg N fixed g^{-1} of malate after deducting the amount of nitrogen in control samples. The distillate was collected into 10 ml of 3% boric acid solution having bromocresol green and methyl red mixed indicator. Then the boric acid solution was titrated with 0.01 N sulphuric acid. The amount of total nitrogen in the sample was calculated by using the formula:

$$\text{Total Nitrogen} = (T\ B) \times N / S$$

Where, T = sample titre value, ml standard acid, B = Blank titre value, ml standard acid, S = sample weight in grams, N = normality of the standard acid.

Azospirillum strains

The identification of the strains was performed as per the procedures of Tarrand *et al.* (1978) [16].

Glucose as sole carbon source for growth

A loopful of the culture, grown in semi-solid Nfb malate medium, was inoculated into tubes containing semi-solid nitrogen-free glucose medium (malate replaced by 1% glucose which has been sterilized by filtration) and the tubes were incubated at 37°C. Within 3 days, the disc of growth formed in the depth of medium which migrated closer to the surface of the medium became dense in the culture tubes.

Biotin requirement for growth

To test the requirement of biotin for growth of the selected strains, the medium with the following composition was used (g/l): K_2HPO_4 0.5, Succinic acid 5.0, (Free acid), $FeSO_4.7H_2O$ 0.01, $Na_2MoO_4.2H_2O$ 0.002, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, $CaCl_2.2H_2O$ 0.026, $(NH_4)_2SO_4$ 1.0, Distilled water 1000 ml. The pH of the medium was adjusted to 7.0 with potassium hydroxide. Biotin (0.0001 g l^{-1}) was added to one portion. The biotin-free and biotin-containing media were sterilized in 10 ml in screw cap tubes by autoclaving at 15 lb for 20 min. Meanwhile the cultures were grown in MPPS broth [17] with the following composition (g/l): Peptone 5.0, Succinic acid 1.0 (Free acid), $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 1.0, $FeCl_3.6H_2O$ 0.002, $MnSO_4.H_2O$ 0.002, Distilled water 1000 ml. The pH was adjusted to 7.0 with potassium hydroxide. A loopful of the culture from MPPS broth was inoculated into 25 ml of one quarter strength nutrient broth and incubated at 37°C for 24 h. The cells were harvested by centrifugation, washed twice with 10 ml portion of sterile distilled water and suspended in water to a turbidity of 20 Klett units (blue filter, 16-mm tubes) 0.1 ml of this suspension was used to inoculate each 10 ml volume of the medium and incubated for 48 h at 37°C. The experiment was repeated to confirm the result.

Acidification of peptone-based glucose medium

The medium with the following composition was used (g/l): Peptone 2.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 1.0, $FeCl_3.6H_2O$ 0.002, $MnSo_4.H_2O$ 0.002, Bromothymol blue 0.025 (Dissolved in dilute KOH). The medium was made up to a volume of 950 ml, the pH was adjusted to 7.0 and sterilized by autoclaving at 15 lb for 20 min. After cooling, 50 ml of 20% (w/v) solution of glucose (sterilized by filtration) was added aseptically. The medium was inoculated with a loopful of 24 h culture from semi-solid Nfb malate medium. The tubes were incubated at 37°C for 96 h. The development of yellow colour was observed after the incubation period.

Indole Acetic Acid (IAA) test

IAA production test was carried out by the method described by Bric *et al.,* (1991) [18]. The medium used was Luria-Bertini agar medium of the following composition (g/l). Tryptone, 10, Yeast extract 5, NaCl 5, agar 20 in distilled water the pH was adjusted to 7.5 with 1N NaOH before autoclaving. The above medium was added with 5mM L-Tryptophan. The medium was poured into Petri plates and allowed to solidify. By means of a fine marker 20 squares were drawn on the plate so as to form a grid. Each square

Table 1. Nitrogen fixation by the isolates of *Azospirillum* from the rhizosphere and the non-rhizosphere soil samples of foxtail millet.

Azospirillum isolate	mg N fixed g^{-1} malate
60 NR2	2.95 a
75 L1	4.05 b
75 C1	4.21 c
60 C1	4.26 d
60 C2	4.34 e
60 L2	4.56 f
60 L1	4.76 g
30 NR2	5.02 h
45 NR1	5.57 i
45 C3	6.02 j
45 L2	6.16 k
30 L2	6.58 l
30 C1	6.65 m
30 L1	7.14 n
45 L3	8.20 o
45 C2	9.30 p
45 L1	12.4 q
30 L2	6.02 j
30 C2	7.13 n
60 C3	6.62 m

Means (n=3) for each isolate followed by the same letter are not significantly different ($P \leq 0.05$) according to DMR test.

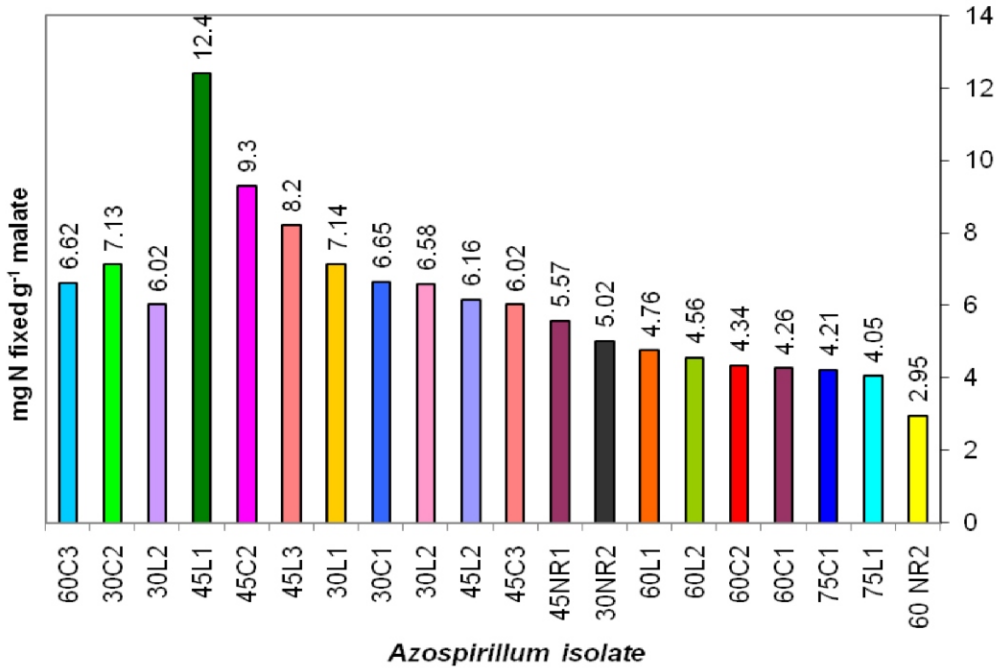

Fig. 1. DNitrogen fixation by the isolates of *Azospirillum* from the nonrhizosphere and the rhizosphere samples of foxtail millet.

Table 2. Biochemical tests of the selected isolates of Azospirillum spp.

Test	Glucose as sole carbon source for growth	Biotin requirement for growth	Acidification of peptone based glucose medium
60 NR2	+	+	+
75 L1	+	+	+
75 C1	+	+	+
60 C1	+	+	+
60 C2	+	+	+
60 L2	+	+	+
60 L1	+	+	+
30 NR2	+	+	+
45 NR1	+	+	+
45 C3	+	+	+
45 L2	+	+	+
30 L2	+	+	+
30 C1	+	+	+
30 L1	+	+	+
45 L3	+	+	+
45 C2	+	+	+
45 L1	+	+	+
30 L2	+	+	+
30 C2	+	+	+
60 C3	+	+	+

was inoculated with one strain of *Azospirillum* spp. The surface of the medium after inoculation was covered by nitrocellulose membrane without delay and incubated until the size of colonies reached 1-2 mm in diameter. Replicates were maintained through out the experiment. After incubation the membrane was gently removed from agar surface and was exposed to Van Urk Salkowski reagent containing 2% 0.5M FeCl in 35% perchloric acid [19] by overlaying on a reagent saturated filter paper at room temperature. The reaction was continued until a pink to red halo developed in the membrane surrounding the colony. Halo color and diameter were measured after 30min. Standard IAA concentrations applied directly onto nitrocellulose membrane and overlaid by Whatman filter paper saturated with Salkowski reagent. The color thus developed was used to compare the color developed by the bacterial strains.

RESULTS AND DISCUSSION

Nitrogen fixation by the isolates of *Azospirillum* spp.

The amount of nitrogen fixed by the pure culture isolates of *Azospirillum* spp. isolated from the non-rhizosphere and rhizosphere samples of Foxtail millet (Varieties Lepakshi and Chitra), was estimated by Kjeldahl method. All the 20 pure

culture isolates, obtained from the non-rhizosphere as well as rhizosphere sample, were found to be diazotrophic. The magnitude of Nitrogen fixed by the isolates varied significantly (Table-1 & Fig. 1). Wide variation in nitrogen-fixing abilities of 285 *Azospirillum* isolates was also observed [20]. *Azospirillum* isolates from different soils and cultivars of rice also exhibited such variations [21, 22]. It is interesting to note that the pure culture isolates obtained from the rhizosphere samples, of both the varieties exhibited higher nitrogen-fixing ability compared to the isolates obtained from the non-rhizosphere samples. Similar results were also reported by others [23]. Similarly, as reported else ware [24] the nitrogen fixation and pellicle formation were always greater in root samples than in the soil samples. Further, the isolates obtained from the rhizosphere samples of both varieties at 45 days possessed maximum nitrogen-fixing ability. Nitrogen fixation by the isolates ranged from 2.95 to 12.4 mg N/g of malate utilized. These values are in close agreement with those reported earlier in the literature. The amount of N fixed by five strains of *Azospirillum brasilense* ranged between 5.07 and 9.43 mg N fixed per g carbon source [25].

Identification of the two selected isolates of *Azospirillum* spp.

The efficient strains of *Azospirillum* spp. were identified as per the procedures described earlier. In the glucose utilization test where glucose was used instead of malate, both the isolates formed a veil of growth deep in the medium after inoculation and within three days the disc of growth migrated closer to the surface of the medium and became dense which is a characteristic feature of *A. lipoferum*. Both the strains grew well in the medium containing biotin and showed no growth in medium lacking biotin which is a positive test for *A. lipoferum.* Further, both the isolates grew well in the peptone based glucose medium which turned the medium yellow due to acidification after 96 h of incubation which is also a characteristic feature of *A. lipoferum* (Table-2). Hence the strains were identified as *A. lipoferum* based on the above tests [26].

Qualitative analysis of IAA produced

Azospirillum was considered as PGPR which promotes the plant growth not only due to its nitrogen-fixing ability, but also by producing plant growth promoting substances like IAA and others. 20 strains of *Azospirillum* spp. produced pink to red halo within the nitrocellulose membrane surrounding the colonies. The strains which failed to produce IAA gave no color on the membrane.

CONCLUSION

Findings of the present work revealed that the *Azospirillum* spp. varied in their nitrogen-fixing abilities and most of them produced IAA. All the isolates were identified as *Azospirillum lipoferum*.

REFERENCES

1. Saikia, S.P and V. Jain. 2007. Biological nitrogen fixation with non-legumes : An achievable target or a dogma? *Curr. Sci*., 92 : 317-322.

2. Bazzicalupo, M. and Y. Okon. 2000. Associative and endophytic symbiosis. In : *Nitrogen fixation: From molecules to crop productivity,* (Pedrosa *et al.,* Ed.), Kluwer Academic Publishers, Netherlands, pp. 409-410.

3. Agarwala, R. and K.V.B.R. Tilak. 1988. *Azospirillum* in leaves of *Eleusine coracana* and *Setaria italica. Zbl. Mikrobiol*., 143 : 533-537.

4. Indira, B.N. and D.J. Bagyaraj. 1997. Associative diazotrophs of sugarcane (*Saccharum officinarum* L.) cultivars. *Indian J. Microbiol.*, 37 : 95-98.

5. Singh, G. and K.V.B.R. Tilak. 1999. *Azospirillum*-current status. In: *Current Trends in Life Sciences*. *Agromicrobes*, 23 : 93-106.

6. Das, H.K. 1993. Molecular genetics of nitrogen fixation in azospirilla. *Proc. Indian Natn. Sci. Acad. Part B. Biol. Sci.*, 59 : 397-406.

7. Cohen, E., Y. Okon, J. Kigel, I. Nur and Y. Henis. 1980. Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen-fixing *Azospirillum* spp. *Plant Physiol*., 66 : 746-749.

8. Kapulnik, Y., Y. Okon, L. Kigel, I. Nur and Y. Henis. 1981. Effect of temperature, nitrogen fertilization, and plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (Strain Cd). *Plant Physiol*., 68 : 340-343.

9. Bashan, Y. and G. Holguin. 1997. *Azospirillum* - plant relationships: Advances (1990-1996). *Canad. J. Microbiol.*, 43 : 103-121.

10. Bashan, Y., G. Holguin and L.E. de-Bashan. 2004. *Azospirillum*-plant relationships: Physiological, molecular, agricultural and environmentaladvances (1997-2003). *Canad. J. Microbiol.*, 50 : 521-577.

11. Subba Rao, N.S. 1986. Soil microorganisms and plant growth. Oxford & IBH Publishing Co., New Delhi, pp. 137-144.

12. Dobereiner, J. and J.M. Day. 1976. Associative symbiosis in tropical grasses : Characterization of microorganisms and dinitrogen fixing sites In : *Proc. First Inter. Symp. on N_2 fixation* (W.E. Newton and C.J. Nyman, Eds.), Washington University Press, Pullman, WA. pp. 518-538.

13. Alexander, M. 1965. Most probable number method for microbial population. In : *Methods of Soil Analysis. Part II. Chemical and Biological properties.* (C.A. Black, Ed.), American society for Agronomy, Madison. pp. 1467-1472.

14. Baldani, V.L.D. and J. Dobereiner. 1980. Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem*., 12 : 33-439.

15. Jackson, M.L. 1973. *Soil Chemical Analysis*. Prentice-Hall of India Ltd, New Delhi.

16. Tarrand, J.F., N.R. Krieg and J. Dobereiner. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of new genus, *Azospirillum* gen. Nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov and *Azospirillum brasilense*. sp. nov. *Canad. J. Microbiol*., 24 : 967-980.

17. Caraway, B.H. and N.R. Krieg. 1974. Aerotaxis in *Spirillum volutans. Canad. J. Microbiol.,* 29 : 1367-1377.

18. Bric, J.M., R.M. Bostok and S.A. Silverstone, 1991. Rapid insitu assay of indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol*. 57: 535-538.

19. Salkowski,E., 1985. "Uber das Verhalten der Skatolcarbonsa" Ure im organismus. *Z. Physiol. Chem*. 9:23-33.

20. Han, S.O. and P. B. New. 1998. Variation in nitrogen fixing ability among natural isolates if *Azospirillum. Microb. Ecol.*, 36 : 193-201.

21. Nayak, D.N., J.K. Ladha and I. Watanabe. 1986. The fate of marker *Azospirillum lipoferum* inoculated into rice and its effects on growth yield and N_2 fixation of plants studied by acetylene reduction, $^{15}N_2$ feeding and ^{15}N dilute techniques. *Biol. Fertil. Soils*, 2 : 7-14.

22. Charyulu, P.B.B.N., D.N. Nayak and V.R. Rao. 1981. $^{15}N_2$ incorporation by rhizosphere soil : influence of rice variety, organic matter and combined nitrogen. *Plant Soil*, 59 : 399-405.

23. Sreenivasulu, G., K.V. Bhaskara Rao and P.B.B.N. Charyulu. 2001. Diazotrophic bacteria from the rhizosphere of foxtail millet [*Setaria italica* (L.) Beauv.] and their screening by spermosphere model. *Indian J. Microbiol.,* 41 : 23-26.

24. Bhaskara Rao, K.V. and P.B.B.N. Charyulu. 2006. Growth and yield of foxtail millet [*Setaria italica* (L.) Beauv.] : Effects of nitrogen fertilization and inoculation with *Azospirillum lipoferum*. *Asian J. Microbiol. Biotechnol. Environ. Sci.*, 8 : 307-314.

26. Venkateswarlu, B. and A.V. Rao. 1983. Response of pearlmillet to inoculation with different strains of *Azospirillum brasilense. Plant Soil*, 74 : 379-386.

27. Krieg, N.R. and J. Dobereiner. 1986. The genus *Azospirillum.* In : *Bergey 's manual of systematic bacteriology.* (N.R. Krieg and J.G. Holt Eds.) Vol. I. Williams and Wilkins Co., Baltimore, MD. pp. 96-104.