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# Studies on isolation and nitrogen fixation ability of *Azospirillum* spp. isolated from Thanjavur district

**Kanimozhi.K. and Panneerselvam,A*.**

*Dept of Botany and Microbiology, A. V. V. M. Sri Pushpam College, Poondi, Thanjavur, Tamilnadu, India*

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## ABSTRACT

*Bacteria of the genus Azospirillum are considered, as plant growth promoting bacteria and stimulate plant growth. Azospirillum is a microaerophilic, plant growth. Promoting bacterium, grow well in semi-solid medium. In this study, Azospirillum strains were isolated from paddy rhizosphere soil of Thanjavur district. All 30 isolates were analyzed for its nitrogen fixing ability by using Microkjeldhal method. Thus the present study is focused on to identify the highest $N_2$ fixing of Azospirillum strain and compared to each other.*

**Key words:** $N_2$ fixation, *Azospirillum* sp., Microkjeldhal.

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## INTRODUCTION

In the current agriculture, nitrogen is a limiting nutrient for growth and yield of crops. Nitrogen ($N_2$) is found in the gaseous forms in atmospheric air and plants and animals do not get to use it in this form for their metabolism (Dobereiner, 1997). The plants get nitrogen, mainly from the application of nitrogen fertilizers, industrially synthesized from the atmospheric dinitrogen ($N_2$). This element, becomes available for the plants by the BNF, realized by some bacteria demominated diazotroph, which possesses an enzymatic apparatus capable to break the triple bond between two nitrogen atoms from the atmospheric nitrogen, forming ammonia that is similar to the industrial process (Dobereiner and Baldani, 1998; Okon and Vanderlegden, 1998; Victoria *et al.*, 1992).

Relative losses of nitrogen regularly occur from the soil through microbially mediated nitrification – denitrification processes in addition to leaching, volatilization, soil erosion and other natural processes. Admittedly, the increased nitrogen demand of the crop is met through

the application of mineral fertilizers, but biological nitrogen fixation by microorganisms has assumed greater significance as it is the major source of fixed nitrogen, about 17.2 x $10^7$ tons per year, on a global basis and it can substitute, in part at least, for chemical sources in satisfying the demand for nitrogen by plants. The limited availability, increasing production costs and procurement problems further limit fertilizer usage, particularly in farming systems operating in the developing world. These beneficial effects of nitrogen fixers make biological fertilizers more resourceful than chemical fertilizers.

Nitrogen fixation by *Azospirillum* sp. in association with grasses and other plants has been examined. The physiology of $N_2$ fixation and factors affecting the growth and nitrogenease activity of *Azospirillum* sp. has been shown that many organic acids and some monosaccharides and disaccharides support nitrogenase activity with *Azospirillum* sp. under microaerophilic conditions.

Biological $N_2$ fixation is gaining importance in rice ecosystem because of current concern on the environmental and soil health that are caused by the continuous use of nitrogenous fertilizers and the need for improved sustainable rice productivity. Thus biological fixation of atmospheric N, especially for low input agriculture.

We investigated the number of rhizosphere bacteria, nitrogenase activity during bacterial growth.

## MATERIALS AND METHODS

### Description of the study area

The present study focused on the area in and around Thanjavur district. The study area is situated in Tamilnadu state (Lat. 11°10′– 11° 30′ N and Long. 78°15′ – 78°30′ E) with the significant features of evergreen forests and also it was a less explored ecosystem for the investigation of *Azospirillum* population.

**Collection of soil samples** (Bashan and Wolowelsky, 1987)

For the enumeration of *Azospirillum*, soil samples were collected by aseptic manner at a depth of 5-10 cm according to the V - shaped method, at thirty different locations in and around the Thanjavur district. From each site, five samples were collected and pooled together and considered as one sample. The soil samples were brought to the laboratory and kept in the refrigerator for further process.

**Isolation of *Azospirillum***

From the collected soil samples, 1 g was taken and serially diluted using sterile distilled water upto $10^{-8}$ dilutions. One ml of diluted sample from $10^{-6}$ to $10^{-8}$ dilutions was taken, and 0.1ml of aliquot was inoculated in test tube containing Nfb (Nitrogen free bromothymol) semisolid media. All the tubes were incubated at 32°C for 48 h and observed the growth by the formation of pellicles. The pellicles were streaked on Nfb solid media and incubated at 32°C for 24 h.

Morphologically divergent *Azospirillum* colonies (white, yellow and pink) were picked from the plates and streaked on basal minimal salt agar medium and incubated at 32°C for 24 h. After attained sufficient growth, all the isolates were preserved in a refrigerator for further

investigation. The stock cultures were sub cultured in fresh nutrient agar slants once in a month and maintained at refrigerated condition. The isolates were also streaked separately on Basal Minimal Salt and potato agar media separately and incubated at 32ºC for 48 h.

**Efficiency of $N_2$ fixation by micro-kjeldahl analysis (Bergerson, 1980)**

The efficiency of $N_2$ fixation of *Azospirillum* isolates were made in semisolid Nfb medium containing 0.05% of malate as carbon source. The isolates were inoculated in Nfb semisolid medium and incubated at 32°C. Triplicates were maintained in each isolates. The total amount of $N_2$ fixation was determined by a micro kjeldahl analysis.

**Sample preparation**

After 10 days of incubation, the media containing isolates were digested in the 100ml micro kjeldahl flask by adding the salt mixture (50:10:1 ratio of $K_2SO_4$, $CuSO_4$ and metallic selenium) and 3ml of concentrated $H_2SO_4$. After digestion, 100ml of distilled water was added and cooled.

**Distillation**

The digested samples were poured into the microkjeldahl distillation apparatus. For quick delivery, 10ml of the 40% NaOH was added in to the distillation apparatus. In a 12ml Erlen-Mayer flask, 10ml of 4% Boric acid reagent and 3 drops of mixed indicator were added. The flask was placed under the condenser of the distillation apparatus and the tip of the condenser outlet was beneath to the surface of the solution in the flask.

**Titration**

The solution, boric acid and mixed indicator containing the "distilled off" $NH_3$ was titrated against standard HCl.

**Calculation**

Percentage of $N_2$ in the sample

$$= \frac{\text{Sample titer} - \text{Blank titer}}{\text{Sample wt. in g X 1000}} \text{ X Normality of HCl X 14 X 100}$$

## RESULTS AND DISCUSSION

**Diversity of *Azospirillum***

For the isolation of *Azospirillum* spp., Nfb semisolid medium was used. After 24 h incubation, the Nfb semi-solid medium showed white colored pellicle (Fig 1 & 2). Appearance of pellicle formation on Nfb semi-solid medium indicated successful isolation of *Azospirillum*. The pellicles were transferred into Nfb plates. After 48 h white, merged colonies were observed on the medium. Typical white or pink, often wrinkled colonies were picked out and transferred into Nfb semi-solid medium. A total number of 30 morphologically distinct *Azospirillum* isolates were isolated and tabulated. For enumeration of population density, the number of colonies on the plates was counted in the range of 68 to 210 colonies. The highest population density was observed in sandy loamy soil at Thirubuvanam. The lowest population density was observed in sandy clay loamy soil at Thiruvalanjuli.

Fig:1 Pellicle formation of *Azospirillum* strain in Nfb semi-solid medium

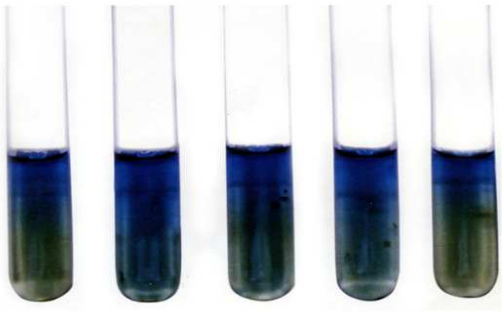
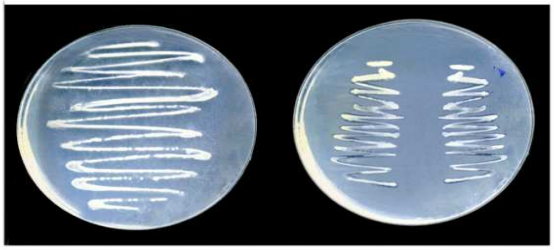


Fig:2 White colour *Azospirillum* colonies on BMS medium



The availability of selective media for isolating diazotrophs belonging to the genus *Azospirillum* and the case of its detection by characteristic features of sub-surface white pellicle formation in semi-solid agar medium has helped to isolate from the rhizosphere and root surface (Hegazi *et al.*, 1979; Baldani *et al.*, 1986; Ladha *et al.*, 1987; Sundaram *et al.*, 1988). Several isolates were obtained on semi-solid nitrogen free media from roots of Kallar grass (Bilal and Malik, 1987; Zafer *et al.*, 1987; Bilal *et al.*, 1990; Malik *et al.*, 1991). These isolates formed a fine sub-surface white pellicle in nitrogen-free malate medium within 24h, which gradually moved to the surface. *Azospirillum* species as described by Krieg and Dobereiner (1984) are curved plump rods, 0.8-1.0 x 25 µm in size, with PHB granules, which in $N_2$ fixing cells can reach 50% of the cell dry weight (Okon *et al.*, 1976). The cells are normally Gram negative but Gram variability has been observed in *A. brasilense* (Tarrand *et al.*, 1978). Cells are motile with a single polar flagellum. Numerous lateral flagella of shorter length are formed in *A. lipoferum* and *A. brasilense* on soft nutrient agar where swarming is observed (Hall and Krieg, 1983).

*Azospirillum* were identified by cultural techniques as described by Neyra and Dobereiner (1977). The purified isolates from paddy soils and roots of rice cultivars upon microscopic examinations revealed the characteristic rods with fat droplets and active spiral movements also important criteria for *Azospirillium* identification.

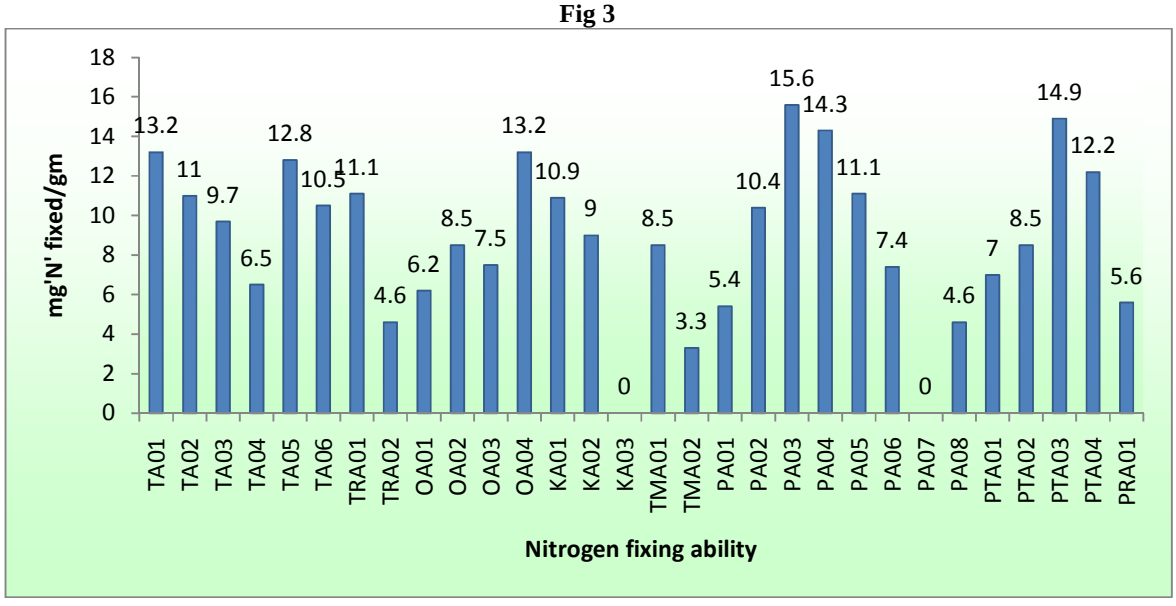
**Nitrogen fixing capacity**

Nitrogen is the most significant yield-limiting element factor in many agricultural production systems. It is known that in legumes, BNF by symbiotic bacteria provides a substantial amount of nitrogen required by the plant. When NF bacterium co-exists as an endophyte within non-legumes, the plant's total nitrogen content rises uniformly. Nitrogen accumulation in inoculated non-legumes may be the result of BNF (Boddey *et al.*, 1995; Elbeltagy *et al.*, 2001; Oliveira *et*

*al.*, 2002) or the increase in nitrogen uptake from the soil (Yanni *et al..*, 1997; Prayitno *et al.*, 1999). BNF is the process in which microorganisms, called diazotrophs, convert atmospheric nitrogen ($N_2$) into ammonia compounds that are assimilable by other organisms. The nitrogenase enzymatic complex, encoded by the *nif* HDK genes, catalyzes this reaction (Passaglia *et al.*, 1995). Although, *Azospirillum* spp. have the ability to fix nitrogen, freely living in the soil or in association with roots of economically important grasses, the positive effects of inoculation with *Azospirillum* are mainly derived from phytohormone production and from induced morphological changes in plant roots, resulting in enhanced mineral and water uptake (Burdman *et al.*, 1997 and 2000). The ability of an endophyte to fix atmospheric nitrogen within a host has been proved using different approaches: ARA, $^{15}N$ isotope dilution experiments, $^{15}N$ reduction assay or $^{15}N$ natural abundance assays (Dalton and Kramer, 2006).

Nitrogen fixation ability of the 30 isolates was measured by micro kjeldhal method. Among the 30 isolates tested, 28 isolates were able to fix nitrogen. The range of nitrogen fixing ability was from 3.3 to 15.6 mg 'N'/g. Among them, the maximum nitrogen fixing ability (15.6 mg'N'/g) was recorded from *A. brasilense* PA03 and minimum (3.3 mg 'N'/g) was recorded in *A. haloprаferens* TMA02. Among the 30 isolates, 10 isolates were fixed the highest amount of nitrogen such as 15.06, 14.09, 14.03, 13.02, 13.02, 12.08, 12.02, 11.01, 11.01 and 11.0 mg 'N' /kg by PA03, PTA03, PA04, TA01, OA04, TA05, PTA04, TRA01, PA05 and TA02 respectively. The two isolates namely KA03 and PA07 could not able to produce nitrogen (Fig. 3). The potential nitrogen producers were selected for pot culture experiment.

**Fig 3**




*Azospirillum* spp. could convert atmospheric nitrogen into ammonium under microaerophilic conditions at low nitrogen levels through the action of the nitrogenase complex. Most of the genetic and biochemical work on nitrogen fixation by *Azospirillum* has been carried out with *A. brasilense* (Hartmann and Baldani, 2006). The optimum level of dissolved oxygen concentration is in the range of 0.2 kPa , but species and strain dependent differences exist in oxygen tolerance (Hartmann *et al.*, 1985; Hartmann, 1988).

The present study has been measured nitrogen fixation ability of *Azospirillum* spp. by Microkjeldhal method in nitrogen-free semisolid medium with malate. Altogether, 30 isolates tested, only 28 isolates were able to fix nitrogen. Among them, only 10 isolates (PA03, PTA03, PA04, TA01, OA04, TA05, PTA04, TRA01, PA05 and TA02) were able to produce the highest amount of nitrogen (from 11.0 to 15.06 mg 'N'/kg). The two isolates namely KA03 and PA07 could not able to fix nitrogen. Okon *et al.* (1977) and Nelson and Knowles (1978) reported for *A. brasilense* about 5,000 nmol of $C_2H_4$ mg protein$^{-1}$ h$^{-1}$ in cell free extracts and in liquid malate medium. Haahtela *et al.* (1983) has also studied the maximum specific nitrogenase activity of *Escherichia agglomerans* was only 10% and of *Klebsiella penumoniae* and *A. lipoferum* 50% in nitrogen-free semisolid medium with malate or sucrose.

The variability of the nitrogenase activity of *Azospirillum* has been observed previously *in vitro* by Han and New (1998), with ARA varying from 0 to 155 nmol of $C_2H_4$ mg protein-1h-1, in pure cultures of *A. lipoferum* and *A. brasilense* obtained from soils of different regions. In another study, the nitrogenase activity of *Azospirillum* isolates ranged from 17.6 to 49.6 nmol C2H4ml$^{-1}$h$^{-1}$ being higher in cultures of *A. brasilense* than for *A. lipoferum* isolates (Mascarua – Esparza *et al.*, 1998). In the present study, it was showed that large differences in the nitrogen fixing of *Azospirillum* isolates. (0 to 15.6 mg N' fixed / g))

Acetylene reduction by *S. lipoferum* with glucose, in combination with a small amount of carbon starter compound such as malate or yeast extract, has reported by Day and Dobereiner (1976). Growth and activity probably stopped after the carbon starter compound was depleted, and no further increase in absorbance was observed during 2 weeks of incubation (Okon *et al.*, 1976). Schroder (1932) has been reported good growth on glucose for 3 of 12 isolates of *S. lipoferum.* In the present study, among the 30 isolates, only 10 isolates showed good growth and acetylene reduction with malate as sole carbon source.

Moreover, consistent variation in $N_2$ fixation by *Azospirillum* strains isolated from 20 rice cultivars was observed over two cropping seasons (Nayak and Rao, 1980). Nayak and Rao (1977) observed the variation in $N_2$ fixation by *Spirillum* sp. obtained from roots of the same rice cultivar exposed to various concentrations of combined N. $N_2$ fixation in cultures of *Azospirillum* isolated from rice cultivars varied uniformly irrespective of the growth stage of the plant. Albrecht *et al.* (1977) found that variation in temperature and light had only a small influence on $N_2$ fixation in *Spirillum – Zea mays* association. Day and Dobereiner (1976) and Okon *et al.* (1976) reported a maximum pH of 7.8 for optimum nitrogenase activity in *S. lipoferum*. The decline in nitrogenase activity could be caused either by exhaustion of nutritions in the medium or by the pH rising above the optimum for nitrogenase activity also reported by Eskew *et al.* (1977). Organic acids are the main energy sources required for nitrogen fixation. It has been previously demonstrated that some members of the genus *Azospirillum* are capable of autotrophic growth through hydrogen oxidation and $CO_2$ assimilation with ribulose-1,5-bisphosphate carboxylase (Tilak *et al.*, 1986). The marked effect of temperature may be caused by the high temperature (33-36$^{o}$C) required for optimal growth and $N_2$ fixation by *A. brasilense* (Neyra and Dobereiner, 1977). Higher temperature increased acetylene reduction in the *Setaria – A. brasilense* association also reported by Kapulnik *et al.* (1981a). The nitrogenase activity of *A. brasilense* is sensitive to salt stress. The effect of NaCl stress on acetylene reducing activity is more pronounced on nitrogenase biosynthesis than on nitrogenase activity.

In the present study, $N_2$ fixing efficiency of *Azospirillum* isolates were positively correlated with the grain yield which were showed by increasing the plant growth parameters such as number of roots, length of root, number of leaf, length of leaf, breadth of leaf, length of shoot, number of tillers and grains weight. Piao *et al*., (2005) reported that ARA of free – living $N_2$ – fixing bacteria were found to be positively correlate with the ARA of the *rhizosphere* soil from which the bacteria were originally isolated suggesting that the $N_2$ – fixing bacteria interacting with rice roots during rice growth. This is important because such $N_2$ – fixing bacteria may respond rice roots when inoculated back to the soils.

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