**International Journal of Biology, Pharmacy
and Allied Sciences (IJBPAS)**

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

**STUDY ON PLANT GROWTH PROMOTING ACTIVITIES OF *AZOSPIRILLUM* SPP.
ISOLATED FROM PADDY FIELDS OF THENI DISTRICT AND ITS BENEFICIARY
EFFECT ON PADDY GROWTH**

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Received 4th July 2019; Revised 8th Aug. 2019; Accepted 9th Sept. 2019; Available online 1st Jan. 2020

<https://doi.org/10.31032/IJBPAS/2020/9.1.4910>

ABSTRACT

About eight *Azospirillum* isolates were isolated and identified from paddy fields of Theni district. These isolates were studied for their nitrogen fixation ability and IAA production. Further pot experiments were conducted to confirm the efficiency of plant growth promoting activity of all *Azospirillum* strains on paddy. Various growth parameters such as root, shoot length and root and shoot weight were analyzed. Thus from the study results, it can be inferred that the efficient strain as bioinoculant increasing plant growth and development was found to be S2 strain *Azospirillum irakense* from Chinnamanur, followed by S7 strain *Azospirillum brasilense* from Kottur. Utilization of such strains could significantly improve the grain yield and bring out development of farmer friendly bioinoculants thereby reducing pollution.

Keywords: *Azospirillum*, nitrogen fixing bacteria, rice, Theni, paddy fields, PGPR

INTRODUCTION

In India, studies on *Azospirillum* were conducted by 1970s to reveal its population in the rice, sorghum, maize, bajra roots

(Laxmikumari *et al.*, 1976), interaction studies between leguminous and non-leguminous plants (cereals, plantation and

ornamental crops) and diazotrophic bacteria for nitrogen fixation process (Tilak *et al.*, 1981; Shah and Joshi, 1986; Subba Rao, 1986; Balasubramanian and Kumar, 1989; Subba Rao, 1993; Hemavathi, 1997; Gadagi *et al.*, 2002). There are many research reports suggesting increased crop production by using *Azospirillum* culture as inoculants for rice (Zaw LwinOo, 2010). Rice (*Oryza sativa* L.) has been feeding over billions of peoples in Asian countries and worldwide. Nitrogen, has been the major nutrient acting as key factor for highest yield, where 1 kg of nitrogen is required for 15-20 kg of grain. So, there is always a dependence of rice for crop sustainability in agriculture. With rise in pollution in hand, it is suitable to move to biofertilizers where soil microorganisms especially diazotrophs like *Azotobacter* and *Azospirillum* can be employed. *Azospirillum*, free living nitrogen fixing bacteria found growing in the rhizosphere region by root colonization. Phytohormones are hormones produced by plants necessary for their growth and development for important crop production. One of the important properties of PGPR organism while screening of biofertilizer from rhizosphere region of soil is indole acetic acid (IAA) production. Microbes at root surface utilize nutrients and using L- tryptophan act as precursor for IAA

production which is a secondary metabolite through different Trp-dependent pathways. IAA increases root uptake for plants, delay leaves abscission and induce flowering and fruiting (Zhao, 2010). Among various paddy field soil samples, a total of eight isolates were selected and identified based on their colony formation in BMS medium. At Chinnamanur and Royyanpatti paddy field soils, highest and lowest population density of *Azospirillum* sp was recorded. Based on the morphological, cultural, biochemical characteristics and 16srRNA sequencing study results, the isolates were identified as *Azospirillum lipoferum* (Erasakkanaickanur, Kuchanoor and Upparpatti), *Azospirillum irakense* (Chinnamanur and Veerapandi) and *Azospirillum brasilense* (Uthamapalayam, Royappanpatti and Kottur) in the studied paddy field areas of Theni district. In this paper we screened the rhizosphere strains for their nitrogen fixation and IAA production. With this research background, we are interested to study the effects of *Azospirillum* and various growth parameters of rice were assessed.

MATERIALS AND METHODS

In vitro nitrogen fixation

Qualitative estimation

About 1 ml of overnight broth culture of paddy field isolates from S1-S8 was

centrifuged at 8000 rpm for 5 min. Pellet was resuspended, washed thrice to remove medium traces and again resuspended with water. The suspension (5 µl) was spotted on medium (Walksman No.77 or N free malate) and plates were incubated at 28 + 2°C and observed for growth at 24 hrs interval for 5 days.

Quantitative estimation

To 250 ml conical flasks, 100 ml of the medium (N free malate or Walksman No.77) was dispensed for all flasks and autoclaved. One ml of 24 hr old culture was inoculated to each flask. The flasks were incubated at 37°C for seven days. After seven days of incubation, the culture was homogenized and 10 ml was digested with 5

ml of concentrated H₂SO₄ along with 0.2 g digestion catalyst mixture K₂SO₄: CuSO₄: Selenium (100:10:1). After cooling, volume was made up to 10 ml with distilled water. Later, 10 ml of aliquot was transferred to microkjeldhal distillation unit, for which 20 ml of 40 per cent NaOH was added and distilled. Ammonia evolved was trapped in 4 per cent boric acid mixed indicator (Bromocresol green 0.066 g and methyl red 0.033 g in 100 ml methanol) till the solution turned from pink to green. It was titrated against 0.05 N H₂SO₄ and total nitrogen content of the culture was determined and results were expressed as mg N fixed per g of malate.

$$\text{Per cent} = \frac{\text{Titre value} \times 0.014 \times \text{N of H}_2\text{SO}_4 \times \text{vol. made}}{\text{N}} \times 100$$

Production of growth promoting substance by the PGPR

Test for IAA production

All the isolates were subjected to analysis for the production of IAA (Bric *et al.*, 1991). Luria agar supplemented with 0.5 mM tryptophan, sodium dodecyl sulphate and 1% glycerol was prepared and plated. The surface area of the agar medium was divided into squares of 2 cm × 2 cm by marking on the bottom of each plate. The

overnight grown culture of each isolate on Luria broth was spotted with sterile tooth picks in each square. The spotted plates were overlaid immediately with sterile Whatman No. 1 filter paper disc of the size of the inner dia of the petriplate. The plates were incubated until the colonies reached the size of 0.5 to 2 mm dia after incubation. The filter paper discs were removed from the plates and treated with Salkowaskis reagent (2% solution of 0.5 M FeCl₃ in 35% perchloric

acid) by soaking in the reagent taken in a petridish. The filter paper discs were observed for the development of pink colour and such isolates were taken as positive for IAA.

Quantitative estimation of IAA production in isolates by spectrophotometric method

A quantity of 0.5 ml of the sample was taken in a test tube and 1.5 ml of distilled water was added followed by a 4 ml of Sessler's reagent and incubated in darkness for 1 hr at 28°C. The intensity of pink colour developed was read in spectrophotometer at 540 nm. By referring to a standard graph prepared with chemical grade indole 3-acetic acid, the quantity of IAA in the sample was determined and expressed as µg per ml of culture filtrate (Sy *et al.*, 2001).

Preparations for Pot Experiment

The soil for pot experiment was collected from the paddy field at Chinnamanur at a depth of 15 cm, V-shaped method, dried at room temperature. The soil was sterilized for pot experiment by autoclaving and left in oven for a night and again autoclaved. About 5 kg of soil was used and sealed in pots before sowing. The paddy seeds were surface sterilized with 0.1%

HgCl₂ for 2 min, washed several times with sterile water.

Pot Experiment

After making the soil ready for potting, the seeds were sown (30 seeds per pot) and 8 strains of various areas S1-S8 was used as inoculums. The study inoculums was used at concentration of 10⁶ CFU/ml. Inoculation volume of one ml per seedlings was used for control and test pots from 10th day onwards at 10 day intervals until harvesting period. Filtered tap water was used for watering pots and no chemical fertilizer was applied during study period.

Growth Parameters

From each pot, random seedling was taken for each study on 30th, 60th, and 80th days of sowing and necessary measurements were taken. The crops after attaining maturity was harvested and fresh and dry weight of shoots and roots were recorded.

Statistical Analysis

The measured study parameters were used for analysis of variance and means of samples compared by least significance difference (LSD) using SPSS (Statistical Package for Social Science) software version 17.0.

Figure 1: Nitrogen fixing potential of paddy field isolates *Azospirillum* from Theni district

Figure 2: IAA production by paddy field isolates *Azospirillum* from Theni district

Figure 3: Effect of *Azospirillum* field isolates from Theni district on paddy plants root length

Figure 4: Effect of *Azospirillum* field isolates from Theni district on paddy plants shoot length

Figure 5: Effect of *Azospirillum* field isolates from Theni district on paddy plants shoot weight

Figure 6: Effect of *Azospirillum* field isolates from Theni district on paddy plants root weight

Table 1: Mean shoot and root lengths, fresh and dry weights of shoot and root of paddy plant at maturity stage (n=6)

Treatments	Mean length $\pm$ SD (cm)		Mean weight $\pm$ SD (g)			
	Shoot	Root	Fresh Shoot	Fresh root	Dry shoot	Dry root
Control	71.23 $\pm$ 10.32	9.4 $\pm$ 1.02	2.54 $\pm$ 1.67	0.12 $\pm$ 0.040	0.823 $\pm$ 0.32	0.045 $\pm$ 0.032
S1-Erasakkanai	88.03 $\pm$ 8.45	15.43 $\pm$ 4.89	4.78 $\pm$ 2.3	0.35 $\pm$ 1.21	1.326 $\pm$ 0.32	0.092 $\pm$ 0.04
S2- Chinnamanur	103.78 $\pm$ 12.54	19.63 $\pm$ 1.34	6.5 $\pm$ 3.72	0.521 $\pm$ 0.67	1.542 $\pm$ 0.22	0.145 $\pm$ 0.05
S3- Uthamapalayam	87.33 $\pm$ 10.11	15.48 $\pm$ 2.76	4.83 $\pm$ 3.52	0.427 $\pm$ 0.54	1.214 $\pm$ 0.43	0.137 $\pm$ 0.03
S4- Kuchanoor	90.55 $\pm$ 11.28	14.34 $\pm$ 2.3	5.32 $\pm$ 1.44	0.378 $\pm$ 0.43	1.382 $\pm$ 0.15	0.097 $\pm$ 0.06
S5- Royappanpatti	88.44 $\pm$ 11.27	12.43 $\pm$ 3.6	4.92 $\pm$ 1.73	0.459 $\pm$ 0.62	1.498 $\pm$ 0.32	0.103 $\pm$ 0.05
S6- Veerapandi	94.22 $\pm$ 12.37	13.89 $\pm$ 2.48	5.2 $\pm$ 2.4	0.389 $\pm$ 0.87	1.435 $\pm$ 0.29	0.093 $\pm$ 0.06
S7- Kottur	100.87 $\pm$ 8.22	16.67 $\pm$ 0.7	5.7 $\pm$ 3.2	0.516 $\pm$ 0.28	1.490 $\pm$ 0.05	0.124 $\pm$ 0.07
S8- Upparpatti	85.24 $\pm$ 10.36	14.45 $\pm$ 2.12	4.76 $\pm$ 2.56	0.423 $\pm$ 0.93	1.389 $\pm$ 0.03	0.104 $\pm$ 0.03

DAS = Days after sowing; Control = Dilute nutrient broth without inoculum

RESULTS AND DISCUSSION

Nitrogen fixation of *Azospirillum* isolates

IAA is an important auxin, production of which varies among organisms and species, highly influenced by media constituents and growth conditions. All the eight isolates were checked for in vitro nitrogen fixation (**Figure 1**). The **Figure 1** shows that the paddy field isolate, S2 at Chinnamanur showed nitrogen fixation potential of 18.2 mg N/g malate utilized, when compared with reference strain of *Azospirillumbrasilense* value of 19.76 mg N/g malate utilized. The lowest amount of nitrogen was fixed by paddy field isolate S6 from Veerapandi of 9.63 mg N/g malate utilized (**Figure 1**). Thus the values of nitrogen fixation of paddy field isolates from Theni district ranged from 9.63 to 19.76 mg N/g malate utilized. In a similar study on rice plants in acid sulphate soils in Kerala, 11 to 21 mg of Nitrogen was fixed per gram malate by 50 *Azospirillum* strains

(Sivaprasad *et al.*, 2003). In a study among various diazotrophic bacteria from different crops, *Azospirillum* isolates produced better nitrogen fixation in range of 2.55 to 22.4 mg per gram of 'C' source (Naikar, 2003). Also in a study at Zanzibar and Tanzania, *Azospirillum* strains isolated from rice roots showed 5.9 – 76.4 n mole C₂H₂ reduced/h/mg protein (Yasmin *et al.*, 2004). Various study by researchers shows nitrogen fixation in cultivated crops inoculated with *Azospirillum* as follows : high (values between 60-120 %) (Hegazi *et al.*, 1981; Kapulniket *al.*, 1981; Subbarao, 1981; Baldani *et al.*, 1983); moderate (20-60 %) (Boutonet *al.*, 1979; Tyler *et al.*, 1979; Okon *et al.*, 1981); and low (<20 %) (Albrecht *et al.*, 1982; Reynders and Vlassak, 1982). The overall nitrogen fixing potential of *Azospirillum* spp. has been reported to be between 1.6 and 23.96 mg per g of carbon source (Purushothaman *et al.*, 1988).

Production of plant growth promoting substances (PGPR) of paddy field isolates from Theni district

IAA production by *Azospirillum*

The figure shows that the paddy field isolate, S2 at Chinnamanur showed IAA production potential of 28.83 µg per 50 ml of medium, when compared with reference strain of *Azospirillum brasiliense* value of 31.84 µg per 50 ml of medium. The lowest amount of IAA production by paddy field isolate S6 from Veerapandi of 13.42 µg per 50 ml of medium (**Figure 2**). Thus the values of IAA production of paddy field isolates from Theni district ranged from 13.42 to 28.83 µg per 50 ml of medium. Similar study results have been reported for *Azospirillum* isolates to produce IAA from range of 2.56 µg per ml and 29.91 µg per ml (Hernandez *et al.*, 1996; Malik *et al.*, 1997; Dayamani, 2003). Veena (1999) reported that among the different groups of organisms isolated from sorghum rhizosphere, *Azospirillum* isolates produced maximum amounts of IAA and GA (16.20 – 40.21 µg IAA and 1.64 – 4.17 µg GA/25 ml broth). Naikar (2003) have reported that the range of IAA produced by isolated endophytic diazotrophs was 7.2 to 974.4 µg per L. in a study at Thailand, 59 isolates of *Azospirillum* produced IAA in the range of

10 to 69 mg per L (Meunchang W *et al.*, 2006).

Pot experiment results

The *Azospirillum* species isolated and identified from various paddy fields of Theni district applied as bioinoculant on paddy pot experiment revealed consistent growth and development. The study parameters namely root length, shoot length, shoot and root weight are noted and presented in **Figures 3, 4, 5, 6** respectively. In all the study parameters concerning plant growth, there was significant increase in all treatments over control.

Root length

With respect to plant root, the control plant produced minimum root length (5.33 ± 1.14) than treated plants after 30 days of transplantation. In treated plants from S1-S8, maximum root length (6.57 ± 2.5) in S2 strain *Azospirillum irakense* isolated from Chinnamanur and minimum root length (4.23 ± 2.3) at S5 *Azospirillum brasiliense*. In study results after 60th and 90th days, highest root length was recorded by S2 strain *Azospirillum irakense* isolated from Chinnamanur (14.22 ± 2.73 , 17.89 ± 3.9) and S7 strain *Azospirillum brasiliense* isolated from Kottur (13.55 ± 1.38 and 16.33 ± 4.1) respectively (**Figure 3**).

Shoot length

The shoot length of control paddy plants was found to be 37.23 ± 5.86 , 68.62 ± 3.81 and 71.45 ± 8.77 respectively at 30, 60 and 80th DAS. With reference to treated plants with various *Azospirillum* isolates revealed that maximum shoot length was recorded with S2 strain *Azospirillum irakense* isolated from Chinnamanur (54.86 ± 5.23 , 80.75 ± 7.15 and 88.36 ± 7.54) and minimum root length by S5 *Azospirillum brasilense* from Royappanpatti. (46.55 ± 5.78 , 77.64 ± 6.65 and 80.22 ± 6.48) respectively for 30th, 60th and 80th DAS (Figure 4).

Shoot weight

The control plants had shoot weight of 0.11 ± 0.034 , 1.37 ± 0.8 and $2.16 \pm 1.1.2$ g respectively at 30th, 60th and 80th DAS. The shoot weight of S2 strain *Azospirillum irakense* isolated from Chinnamanur had high values at 5.73 ± 1.1 at 80th DAS. Lowest shoot weight was observed for S8 strain *Azospirillum lipoferum* isolated from Upparpetti (Figure 5).

Root weight

The root weight of control plants had values of 0.02 ± 0.02 , 0.05 ± 0.03 and 0.12 ± 0.05 respectively. The values were range of 0.01 to 0.05 ; 0.04 to 0.13 and 0.12 to 0.32 for 30th, 60th and 80th DAS (Figure 6).

Yield of grains

The maximum yield gain was recorded at 90th day of transplantation (60.5g) by S2 strain *Azospirillum irakense* isolated from Chinnamanur and the minimum (51.67 g) grain weight was recorded in S5 *Azospirillum brasilense* from Royappanpatti. And control plants showed value of 50.23 g. Study by various researchers reported to increase the root and shoot length in rice plants through *Azospirillum* inoculation (Mubassara *et al.*, 2008; Salamone *et al.*, 2010; Isawa *et al.*, 2010) leading to enhanced growth of paddy plants (Prajapati *et al.*, 2008; Kannan and Ponmurugan, 2010; Bao *et al.*, 2013).

CONCLUSION

Screening the rhizosphere soil for diazotrophic bacteria and IAA producers can give beneficial organisms for high crop production. *Azospirillum irakense* from Chinnamanur resulted in highest numbers of roots, shoots and weight increased substantially when compared to other isolates. Thus the strain *Azospirillum irakense* can have potential for higher rice production, being ecofriendly to farmers. Further field experiments are to be carried out to bring it as a commercial biofertilizer in agriculture to benefit farmers. This study is of importance to develop much effective

biofertilizers necessary for the hour in pollution scenario also bringing about high crop production.

ACKNOWLEDGEMENT

The author Uma devi acknowledges the support and encouragement from the Head, Department of Microbiology and Biochemistry, Principal and management of Nadar Saraswathi College of Arts and Science, Theni. She also acknowledges the paddy farmers at various study areas of Theni district for support towards study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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