

## Evaluation of Plant Growth Promoting Substance Production by *Azospirillum* Sp. Isolated from Rhizosphere of Chilli (*Capsicum annuum* L.)

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**Abstract:** Chilli (*Capsicum annuum* L.) is an important commercial spice cum vegetable crop of India. Chilli forms an essential ingredient of Indian curry. Chilli is a rich source of vitamin 'C' and vitamin 'A' with plenty of minerals. *Azospirillum* species are important plant growth promoting rhizobacteria (PGPR) used to enhance crop yield by direct and indirect mechanisms. In the present study, soil samples were collected from ten different locations. The *Azospirillum* isolates were screened for their efficiency by determining the ability to produce phytohormone Indole acetic acid (IAA), Nitrogen fixation and phosphorus solubilization. The isolates of *Azospirillum* were designated as Azs-1 to Azs-10. The highest IAA production was recorded by the isolate Azs-2 obtained from Sivapuri soil. The nitrogen fixation of *Azospirillum* isolate ranged from  $9.8 \pm 0.3$  to  $16.9 \pm 0.5$   $\mu\text{g}$  of nitrogen  $\text{g}^{-1}$  of malate utilized. Interestingly, the maximum nitrogen fixation  $16.9 \pm 0.5$   $\text{kg}$  of N  $\text{g}^{-1}$  of malate was also recorded by the isolate Azs-2.

**Key words:** *Azospirillum* • PGPR • Chilli • Indole Acetic Acid • Nitrogen Fixation and Phosphorus • Solubilization

### INTRODUCTION

Chilli belongs to the family Solanaceae, genus *Capsicum* and the two important cultivated species are *Capsicum annuum* and *Capsicum frutescens*. There is no spice probably to popular as chilli and no other spice has become such an indispensable ingredient of the daily food of majority people of the world [1]. The colour plays an important role in assessing the quality of chillies. The principal colouring matter is capsanthin, the carotenoid pigment which contributes about 35 per cent to the total pigments. Chilli is now gaining more importance in the global market because of its value added products like chilli powder, oleoresin, capsanthin and chilli oil etc. Chilli powder is the most important ground spice item exported from India.

Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, which enhance the growth of the plant either directly or indirectly. The direct mechanisms involve nitrogen fixation, phosphorus solubilization, HCN production, production of phytohormones such as auxins, cytokinins and

gibberellins and lowering of ethylene concentration [2]. Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas* and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus* and *Bradyrhizobium japonicum* have been shown to produce auxins which help in stimulating plant growth. There are many reports on plant growth promotion and yield enhancement by plant growth-promoting rhizobacteria. The mechanisms of plant growth promotion by PGPR include: the ability to produce phytohormones,  $\text{N}_2$  fixation, antagonism against phytopathogens and solubilisation of insoluble phosphates. It is also suggested that PGPR can also prevent the deleterious effects of stresses from the environment [3].

*Azospirillum* is a Gram negative, motile and produces beta hydroxyl granules. Since 1970's, *Azospirillum* strains have been isolated and used. This group of free living rhizobacteria encompasses ten species, each one classified according to its particular biochemical and molecular characteristics: *Azospirillum lipoferum* and *Azospirillum brasilense* *Azospirillum amazonense*,

*Azospirillum halopraeferens*, *Azospirillum irakense*, *Azospirillum largimobile*, *Azospirillum doebereineriae*, *Azospirillum oryzae*, *Azospirillum melinis* and recently *Azospirillum canadensis*. Although, *Azospirillum* was first isolated from cereals and most of its initial inoculation has been done on the main cereal crops and, there are more non-cereal species successfully inoculated with *Azospirillum* than cereals. *Azospirillum* appears that is not a plant specific bacterium and is a general root colonizer [4]. Members of the genus *Azospirillum* fix nitrogen under microaerophilic conditions and are frequently associated with root and rhizosphere of a large number of agriculturally important crops and cereals. It increases the yield is mainly attributed to improved root development due to the production of growth promoting substances and consequently increased rates of water and mineral uptake [5, 6].

## MATERIALS AND METHODS

**Details of Locations of Sample Collection:** Ten different places namely Keezhamanakudi, Sivapuri, Kavarapattu, Kothattai, Karikuppam, Pudhupettai, Periyapattu, Pudhuchathiram, Mutlur, Vallam were selected from Chidambaram Taluk, Cuddalore district for the collection of rhizosphere soil samples of Chili. The rhizosphere samples were used for isolation of *Azospirillum*.

**Isolation and Enumeration of *Azospirillum* Population in Rhizosphere Soils:** The *Azospirillum* strains were isolated from the different rhizosphere soils following the method of Dobereiner and Day [7]. Ten gram of rhizosphere soil sample was placed in 90 ml sterile water blank and mixed thoroughly by shaking which will provide  $10^{-1}$  dilution of the samples. The serial dilutions  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  were selected for enumeration. One ml of each suspension from  $10^{-4}$  dilution was transferred aseptically to each of the five test tube containing 10 ml semisolid NFB. Similarly, another five tubes each of media are inoculated with  $10^{-5}$  and  $10^{-6}$  dilution suspension respectively. The inoculated test tubes incubated at  $30 \pm 2^\circ\text{C}$  for one week period and undisturbed. After incubation, the tubes were observed for the growth of *Azospirillum*. Three replications were maintained for each dilution. The colonies were counted by using Colony counter. The total number of colonies in the original samples was using expressed as cfu/g. The *Azospirillum* growth as evidenced by the change of colour of the medium from greenish yellow to blue and the presence of

white dense subsurface pellicles. The pellicles were streaked on N free malic acid medium containing 0.3 %  $\text{NH}_4\text{Cl}$  and growth they were stored at  $4^\circ\text{C}$  until further use.

### Characterization of *Azospirillum* Isolates

**Production of Acid from Glucose:** The medium described by Krieg and Tarrant [8] was used to test the production of acid from glucose by *Azospirillum* strains. The medium was dispensed in 5 ml quantities in test tubes and sterilized. The tubes were inoculated with 0.1 ml of 24 h old culture of *Azospirillum* isolates and incubated for 96 h at  $30 \pm 2^\circ\text{C}$ . The change in colour of the broth to yellow indicated acid production.

**Utilization of Different Carbon Sources:** The ability of the *Azospirillum* isolates to utilize different carbon sources were tested in nitrogen free malate medium. The medium was prepared without bromothymol blue and malate. The various carbon sources viz., glucose, mannitol, succinate and  $\alpha$ -keto glutarate were incorporated separately at 1% level. After sterilization, the media were inoculated with 0.1 ml of 24 h old cultures of *Azospirillum* and incubated at  $30 \pm 2^\circ\text{C}$  and observed for growth.

**Requirement for Biotin:** The nitrogen free malate medium was used for assessing the requirement for biotin. Two sets of media were prepared with and without biotin ( $100 \mu\text{g L}^{-1}$ ). The cultures were grown for 48 h in glucose peptone broth [9], centrifuged and washed twice with sterile distilled water and resuspended in sterile distilled water to a uniform density. A quantity of 0.1 ml of this suspension was used to inoculate 5 ml volume of medium with and without biotin and incubated for 48 h at  $30 \pm 2^\circ\text{C}$  and the growth of the isolates was observed. For each set an uninoculated control was maintained.

**Quantitative Estimation of Indole Acetic Acid by *Azospirillum* Isolates:** The dinitrogen fixation of the *Azospirillum* isolates was determined by employing the Salper's reagent proposed by Gorden and Paleg [10].

**Determination of Dinitrogen Fixation of the *Azospirillum* Isolates:** The dinitrogen fixation of the *Azospirillum* isolates was determined by Microkjeldhal assay described by Humphries [11].

## RESULTS AND DISCUSSION

Ten different isolates of *Azospirillum* were isolated from the collected rhizosphere soils of Chili and maintained in the respective media for further studies. The isolates of *Azospirillum* were designated as Azs-1 to Azs-10 as shown in Table 1. The population of *Azospirillum* ranged from 2.0 to  $4.3 \times 10^5$  cfu g<sup>-1</sup> of soil and the soil samples collected from Kavaraipattu recorded the maximum occurrence. The lowest occurrence was observed with *Azospirillum* which ranged from 2.0 to  $310^5$  cfu g<sup>-1</sup> of soil in Mutlur (Figure 1).

It was pragmatic that all the ten isolates of *Azospirillum* Azs-1 to 10, formed characteristic, dense white sub surface pellicles on NFb semisolid medium. The isolates exhibited curved rod shaped cells, active spiral movement on wet microscopic mount and gram negative in reaction. In addition, the presence of poly-β-hydroxy butyrate was observed in all the isolates. *Azospirillum* isolates were further identified upto species level by performing some of the biochemical tests. Out of 10 isolates two isolates viz., Azs-4 and Azs-9 were *Azospirillum lipoferum* and the remaining 80% of the isolates were identified as *Azospirillum brasilense* (Table 2 and Table 3). The present study clearly revealed the predominance of *Azospirillum brasilense* in chili rhizosphere of Cuddalore district. Reetha [12] reported the predominance of *Azospirillum brasilense* in the rhizosphere of sunflower in Tamil Nadu.

Table 1: Designation of *Azospirillum* isolates from ten locations of Cuddalore District

Name of the location	<i>Azospirillum</i> isolates
Keezhamanakudi	Azs-1
Sivapuri	Azs-2
Kavaraipattu	Azs-3
Kothattai	Azs-4
Karikuppam	Azs-5
Pudhupettai	Azs-6
Periyapattu	Azs-7
Pudhuchathiram	Azs-8
Mutlur	Azs-9
Vallam	Azs-10

The *Azospirillum* isolates were screened for their efficiency to produce indole acetic acid and nitrogen fixing efficiency and the results are given in Table 4. All the isolates produced IAA under *in vitro* condition the amount of IAA produced ranged from 10.02 to 38.00 µg ml<sup>-1</sup> of culture filtrate. The highest IAA production was recorded by the isolate Azs-2 obtained from Sivapuri soil. The nitrogen fixation of *Azospirillum* isolate ranged from  $9.8 \pm 0.3$  to  $16.9 \pm 0.5$  µg of nitrogen g<sup>-1</sup> of malate utilized. Interestingly, the maximum nitrogen fixation  $16.9 \pm 0.5$  kg of N g<sup>-1</sup> of malate was also recorded by the isolate Azs-2. The *Azospirillum* isolates were screened for their efficiency to produce indole acetic acid and nitrogen fixing efficiency. All the isolates produced IAA under *in vitro* condition the amount of IAA produced ranged from 10.02 to 38.00 µg ml<sup>-1</sup> of culture filtrate. The highest IAA production was recorded by the isolate Azs-2 obtained from Sivapuri soil.

Table 2: General characteristics of *Azospirillum* isolates

Character studies	Reaction of Isolates									
	Azs-1	Azs-2	Azs-3	Azs-4	Azs-5	Azs-6	Azs-7	Azs-8	Azs-9	Azs-10
Subsurface pellicle formation in NFb medium	+	+	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+	+	+	+
Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Presence of PHB	+	+	+	+	+	+	+	+	+	+

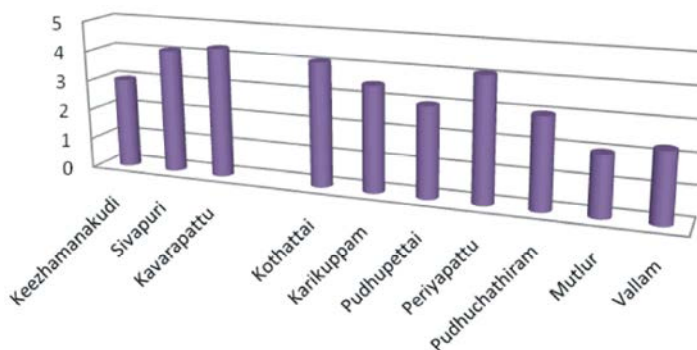


Fig. 1: Occurrence of *Azospirillum* organisms in the rhizosphere soil of chili

Table 3: Biochemical tests of species level identification of *Azospirillum* isolates

Isolates	Acid from glucose	Glucose	Succinate	Mannitol	$\alpha$ -keto glutarate	Biotin requirement	Identified species
Azs-1	-	+	+++	+++	+++	-	<i>A. brasilense</i>
Azs-2	-	++	++	+	++	-	<i>A. brasilense</i>
Azs-3	-	++	++	+	++	-	<i>A. brasilense</i>
Azs-4	+	+++	+++	++	++	+	<i>A. lipoferum</i>
Azs-5	-	++	+++	++	++	-	<i>A. brasilense</i>
Azs-6	-	++	+	++	+	-	<i>A. brasilense</i>
Azs-7	-	+	++	+++	+++	-	<i>A. brasilense</i>
Azs-8	-	+	+++	++	+	-	<i>A. brasilense</i>
Azs-9	+	+++	++	++	++	+	<i>A. lipoferum</i>
Azs-10	-	+	+++	+	+++	-	<i>A. brasilense</i>

Table 4: Efficiency of Nitrogen fixation by *Azospirillum* isolates

Isolates	IAA ( $\mu\text{g ml}^{-1}$ )	Nitrogen fixation ( $\mu\text{g of N g}^{-1}$ of malate)
Azs-1	22.75	13.5 $\pm$ 0.4
Azs-2	38.00	16.9 $\pm$ 0.5
Azs-3	24.02	16.0 $\pm$ 0.9
Azs-4	22.05	13.5 $\pm$ 0.9
Azs-5	26.00	12.5 $\pm$ 0.2
Azs-6	10.02	9.8 $\pm$ 0.3
Azs-7	16.35	16.3 $\pm$ 0.2
Azs-8	11.75	16.4 $\pm$ 0.2
Azs-9	19.25	13.6 $\pm$ 0.5
Azs-10	37.00	13.4 $\pm$ 0.5

The nitrogen fixation of *Azospirillum* isolate ranged from 9.8  $\pm$  0.3 to 16.9  $\pm$  0.5  $\mu\text{g}$  of nitrogen  $\text{g}^{-1}$  of malate utilized. Interestingly, the maximum nitrogen fixation 16.9  $\pm$  0.5  $\text{kg}$  of  $\text{N g}^{-1}$  of malate was also recorded by the isolate Azs-2. In this study, the *Azospirillum* isolates were evaluated for their efficiency in dinitrogen fixation and IAA production under in vitro condition. Interestingly the *Azospirillum* isolate Azs-2 could produce 38.00  $\mu\text{g ml}^{-1}$  IAA also could fix highest of 16.9  $\pm$  0.4  $\mu\text{g}$  of  $\text{ng}^{-1}$  of malate utilized. The growth and yield increase due to *Azospirillum* inoculation was accomplished by the increase in root volume, root length and higher lateral roots and also increased by mineral uptake and increased nitrogen fixation. Increased root biomass of the *Azospirillum* inoculated plant could be explained for the higher nutrient uptake and concomitant yield. Better response and best growth of *Azospirillum* inoculated sunflower were reported by Nalini [13].

### CONCLUSION

From the present study, it was concluded that the plant growth promoting rhizobacteria *Azospirillum* have the capacity to produce plant growth promoting substances and the isolate AZS-2 from Sivapuri was highly effective in the production of plant growth

promoting substances when compared to other *Azospirillum* isolates. Maximum nitrogen fixation 16.9 $\pm$ 0.5  $\text{kg}$  of  $\text{N g}^{-1}$  of malate was also recorded by the isolate Azs-2.

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