International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Isolation and Characterization of *Azospirillum* sp. from Paddy Field Soil, Thanjavur District, Tamil Nadu

K. Kanimozhi[1], A. Panneerselvam[2]

PG and Research Department of Botany and Microbiology, A.V.V.M Sri Pushpam College (Autonomous), Poondi - 613 503, Thanjavur, Tamil Nadu, India

Abstract: ***The present study focused on isolation and characterization of Azospirillum sp. from paddy field soils of Thanjavur (Dt), Tamil Nadu. The physico-chemical characteristics of the soil samples were analysed by using standard methods. Totally nine native strains of Azospirillum were isolated. Among the nine isolated strains three strains were belonging to Azospirillum amazonense and six strains belonging to A. brasilense. Morphological and physiological properties were performed all the isolates. The effect of different concentration of NaCl and antibiotic sensitivity test also analyzed. All the isolates were possessed N_2 fixing capability, the highest level of nitrogen fixation was done by A.brasilense TPS05, which fixes nitrogen at a rate of 11.2 mg 'N'/g. Inoculation of A.brasilense TPS05 on paddy ADT 36 in pot culture produced the maximum number of tillers, roots, leaves, leaf length and breadth, shoot and root length and grain yield.***

Keywords: *Azospirillum* sp., Biofertilizer, Nitrogen fixation, Paddy and Pot culture

1. Introduction

Azospirillum is one of the best-studied plant growth-promoting rhizobacteria (PGPR) that are normally associated with grasses, rice, wheat and sugarcane (Bashan and De-Bashan, 2010; Babalola and Glick, 2012; Duca *et al*., 2014; Glick, 2014). Biofertilizers keep the soil environment rich in all kinds of micro and macro nutrients via nitrogen fixation, phosphate and potassium solubilisation or mineralization, release of plant growth regulating substances, production of antibiotics and biodegradation of organic matter in the soil (Sivakumar *et al*., 2013; Sinha *et al*., 2014) providing better nutrient uptake and increased tolerance towards drought and moisture stress.

Azospirillum under stress conditions enhance plant growth by fixing atmospheric Nitrogen and by the production of growth promoting substances and influencing root development, causing increased uptake of nutrients from the land, and inhibiting pathogenic fungi and bacteria in the rhizosphere (Hossain and Jahan, 2015). *Azospirillum* inoculation could significantly increase the growth in terms of height; number of leaf/plant; length and breadth of leaf; and fresh and dry weight/plant of rice plant (Hossain *et al*., 2015). In general, microbes commenly used as biofertilizers may be *Azotobacter*, *Azospirillum*, *Rhizobium* as nitrogen fixing soil bacteria (Verma *et al*., 2013, 2014; Verma *et al*., 2015; Verma *et al*., 2016a).

Azospirillum is known to fix a substantial amount of atmospheric nitrogen and supplies to the crop, enhances the fertilizer use efficiency, soil fertility grade and ensures partial saving of nitrogenous fertilizer. The ability of the *Azospirillum* to multiply in the rhizosphere of crop suggests its ability to get better the nutrient availability to the plants and can supplement the expensive inorganic and organic fertilizers. Despite the reductions in rice production and yield in the 2015/16 season, compared with the previous one, respectively 14.3 and 0.6% (CONAB, 2016). Hence the present research was designed to isolate and characterization of *Azospirillum* sp. from paddy field soil samples.

2. Materials and Methods

Description of the study area

The present study focused on the area of Thanjavur District. The study area is situated in Tamilnadu state with the significant features of granary of south 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 nine different locations of Aalathur, Budalur, Karuppur, Papanasam, Sethubavasathiram, Thiruppananthal, Thiruvaiyaru, Vadakkur and Vadakumangudi in 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.

Analysis of physico-chemical characteristics of the soil samples

Physico-chemical parameters such as soil texture, pH, bulk density, electrical conductivity, organic carbon, available nitrogen, available phosphorus, available potassium calcium , available micronutrients such as Zn, Cu, Mn, Bo, total nitrogen, total phosphorus and total potassium were analyzed by using standard methods.

Isolation of *Azospirillum*

The soil samples were collected from nine villages of Thanjavur district, Tamilnadu, South India for isolation of *Azospirillum.* From the collected soil samples, 1 g was taken

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and serially diluted using sterile distilled water up to10^{-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 blue) 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 32ºC for 24 h. After attained sufficient growth, all the isolates were preserved and used for further investigation.

Enumeration of *Azospirillum* isolates

For enumeration of *Azospirillum* cells, Nfb solid medium was used. After 48h of incubation the colonies in the Nfb plates were counted by using Quebec colony counter.

Population density was expressed in terms of Colony Forming Unit (CFU) per gram of soil with dilution factor

$$\text{Number of viable cells / ml} = \frac{\text{Number of colonies (average of three replicates)}}{\text{Amount of inoculum plated} \times \text{dilution}}$$

Morphological characteristics of *Azospirillum* isolates

Gram stain, size and motility of the bacterial isolates from a 24h old semi solid Nfb culture were examined microscopically.

Biochemical characteristics of *Azospirillum* isolates

Utilization of carbon sources, Indole test, Methyl red test, Voge's Proskauer test, Citrate utilization test, Starch hydrolysis test, Urease test were performed for the confirmation of the bacterial isolates according to the Bergey's Manual of Determinative Bacteriology (1994). Bacterial growth of various NaCl concentrations **(**1% to 6%) was determined as well as antibiotic sensitivity also done by using antibiotic discs such as ampicillin, chloromphenicl, streptomycin and erythromycin for comparison study.

Nitrogen fixation efficiency of *Azospirillum* by micro Kjeldahl analysis (Bergersen, 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 semi solid medium and incubated at 32°C. Triplicates were maintained in each isolates. The total amount of N_2 fixation was determined by micro kjeldahl analysis.

Sample preparation

After 10 days of incubation, the media containing isolates were digested in the 100ml micro kjeldahl flask by adding 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 micro kjeldahl distillation apparatus. For quick delivery, 10ml of the 40% NaOH was added in to the distillation apparatus. In a 12 ml 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

$$\%\ \text{of } N_2 \text{ in the sample} = \frac{\text{Sample titer - Blank titer}}{\text{Sample wt. in g} \times 1000} \times \text{Normality of HCl} \times 14 \times 100$$

Inoculation of *Azospirillum* isolates on paddy (ADT 36) in Pot culture

All the nine *Azospirillum* isolates were selected for pot culture studies. The nine isolates were inoculated individually into 250 ml Erlenmeyer flask containing 100 ml of Nfb broth and incubated at 32°C for 5 days with occasional shaking for proper aeration. After 5 days, the inoculum was transferred into 500 ml flask containing 250 ml of Nfb broth. Then the culture was diluted with water when the counts reached 10^{-7} in the medium. This liquid inoculum was used for the pot culture study. Disease free healthy paddy ADT 36 was selected for the pot culture experiment. Totally, 15 soil mud pots of 30 cm diameter were taken for the experiment. The collected soil samples were sterilized and each pot was filled with 5kg of sterile soil and poured water once in a day. Root dipping method (Islam and Bora, 1998) was adopted for the application of biofertilizer to the paddy plant. The diluted liquid culture was used to dip the roots of paddy seedlings and allowed for 10 min. Then the seedlings were transplanted into the pots.

Observation of parameters

The morphological assessment of growth (numbers of roots, root lengths, numbers of leaves, leaf lengths, leaf breath, shoot length, number of tillers and grains weight) were observed on 30th, 60th, and 90th day after transplantation of the paddy crops.

3. Results and Discussion

Physico-chemical properties of soil

In the present study, physico-chemical properties of soil samples from 9 different Taluk of Thanjavur district was summarized (Table 1). Out of 9 soil samples, 5 samples were sandy loam soil and 4 samples were sandy clay loamy soil. The maximum pH (7.40) of the soil was recorded at Sethupavasathiram and Budalur and minimum pH (6.80)

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was recorded at Vadakkumangudi. The bulk density of the soil was recorded as highest (1.610g/cm^3) at Budalur and lowest (1.230g/cm^3) at Vadakkumangudi. The maximum (37%) water holding capacity (WHC) of the soil was recorded at Thiruppananthal and minimum (24%) was recorded at Sethupavasathiram and Vadakkumangudi. The electrical conductivity of soil was recorded greatest (0.78) at Thiruppanthal and smallest (0.52) at Aalathur. The organic carbon content of the soil was recorded most (0.90%) at Budalur and least (0.65%) at Sethupavasathiram.

Table 1: Physico chemical Properties of soil samples from Thanjavur Dt

S.no	Sampling places	Texture	pH	Bulk density (g/cm^3)	Water holding capacity (%)	Electrical conductivity (dsm^{-1})	Organic carbon (%)
1	Aalathur	SCL	7.20	1.560	30	0.52	0.82
2	Budalur	SCL	7.40	1.610	33	0.72	0.90
3	Karuppur	SCL	7.30	1.270	31	0.67	0.72
4	Papanasam	SCL	6.90	1.310	32	0.69	0.75
5	Sethubavasathiram	SL	7.40	1.440	24	0.59	0.65
6	Thiruppananthal	SL	6.95	1.350	37	0.78	0.70
7	Thiruvaiyaru	SL	7.10	1.290	26	0.54	0.76
8	Vadakkumangudi	SL	6.80	1.230	24	0.59	0.74
9	Vadakkur	SL	7.10	1.520	25	0.81	0.69

SCL = Sandy clay Loam, SL = Sandy Loam

Nutrients status

In the present study showed, the salinity content was maximum recorded in Sethupavasathiram (5.2 psu) and minimum (4 psu) in Thirupananthal. The highest (195.4 kg/acre) available nitrogen was recorded at Budalur and lowest (135.3 kg/acre) was recorded at Vadakkur. The phosphorous content of the soil was recorded as maximum (8.5 kg/acre) at Aalathur and minimum (6.3 kg/acre) was recorded at Papanasam. The greatest potassium (235 kg/acre) content of the soil was recorded at Budalur and smallest (170 kg/acre) was recorded at Thirupananthal. The available micronutrients of the soil like Zn, Cu, Fe, Mn and Bo were evaluated. Zn content of the soil was recorded as maximum (2.90%) at Sethupavasathiram and minimum (1.65%) at Thiruppananthal. The Cu content of the soil was most (2.65%) at Papanasam and least (1.30%) at Sethupavasathiram soil. The Fe content of the soil was maximum (8.65%) at Vadakkumangudi and minimum (5.50%) at Sethupavasathiram soil. The Mn content of the soil was recorded maximum (5.10%) at Papanasam and minimum (2.10%) at Sethupavasathiram soil. The Bo content of the soil (0.580%) was recorded utmost at Thiruppananthal and least (0.410%) at Vadakkur soil.

Earlierly Rai *et al*., (2010) reported the growth of plants as high bulk density can reduce the root penetration in soil. Aziz *et al*., (2010) found that potassium (K) is an essential nutrient and plays an important role in the growth of plants, synthesis of amino acids and proteins. Velmurugan *et al*., (2012) reported that nitrogen is one of the necessary elements for the growth of plant.

Accordingly, Mandal *et al*., (2013) studied phosphorus (P) is a necessary element for maintaining a balance between the other plant nutrients and ensuring the normal growth of the crop. The content of P reported to show a positive correlation with mineral particles of the soil which differ in their susceptibility to cause release of P during weathering.

The present investigation revealed that, the total nitrogen content of soil was recorded maximum (1.95%) at Vadakkumangudi and minimum (1.20%) at Budalur. The highest (0.190%) phosphorus content of the soil was recorded at Sethupavasathiram and lowest (0.140%) was recorded at Thiruppanathal soil. The greatest (1.85%) potassium content of the soil was recorded at Aalathur and smallest (1.45%) at Vadakkur soil (Table 2).

Similarly, Senthil Kumar and Panneerselvam (2013) reported that totally 10 different paddy field soils were collected from in and around Thiruvarur district, Tamilnadu and their physico-chemical properties and population density of *Azospirillum* were analyzed.

Recently, Navarkhele (2015) found that, soil solution carries its nutrients such as Potassium (K), Phosphorus (P), and Nitrogen (N) that plant need in specific amount to grow and fight off diseases. Prameena Sheeja (2015) investigated that macro- and micronutrients in soils from Periyakottai (S1) and Thirumangalkkattai (S2) area of Tamil Nadu. The soil samples collected from Periyakoattai and Thirumangalkkattai in January 2015. The different physical parameters and macro elements were analyzed in laboratory are pH, EC, P and K.

Table 2: Macronutrients of soil sample from Thanjavur District

S.No	*Study site*	*Micro Nutrients (%)*		
		N	*P*	*K*
1.	Alathur	1.80	0.165	1.85
2.	Budalur	1.20	0.175	1.70
3.	Karuppur	1.75	0.160	1.55
4.	Papanasam	1.55	0.155	1.55
5.	Sethubavasathiram	1.85	0.190	1.60
6.	Thiruppananthal	1.60	0.140	1.65
7.	Thiruvaiyaru	1.45	0.170	1.65
8.	Vadakkumangudi	1.95	0.180	1.60
9.	Vadakkur	1.80	0.180	1.45

N=Nitrogen; P=Phosphorous; K=Potassium

Isolation of *Azospirillum*

In the present findings for, the isolation of *Azospirillum* Spp., Nfb semi-solid medium was used. After 24 h of incubation, the Nfb semi-solid medium showed white coloured pellicle. Appearance of pellicle formation on Nfb semi-solid medium indicated the successful isolation of *Azospirillum.* The pellicles were transferred in to Nfb plates.

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After 48 h of inoculation a white, merged colonies were observed on the medium. Typical white or pink, often wrinkled colonies were screened out and transferred into Nfb semi-solid medium. A total number of 4 morphologically distinct *Azospirillum* isolates were isolated and tabulated. The 9 isolates were belonged to 2 species. For enumeration of population density, the number of colonies on the plates was counted in the range of 69 to 105 colonies. The highest population density was observed in sandy loam soil at Thiruvaiyaru. The lowest population density was observed in sandy clay loamy soil at Karuppur.

Morphological characteristics of *Azospirillum* species

In microscopic observations the cell type and shape were observed using Gram staining technique. All the 9 isolates were Gram negative and rod shaped (TPA01, TBR02, TKK03, TPP04, TPS05, TTK06, TTT07, TTV08 and TOV09). The cell size was measured by micro metric method. The size of the cells was varied from 0.5µm to 1.5µm.

Biochemical characteristics of *Azospirillum* isolates

In the test of regarding carbon source utilization of *Azospirillum* isolates, all the isolates were utilized the following sugars as a carbon source namely fructose, sucrose and mannitol. All the 9 isolates produce indole and methyl red, utilized citrate, in starch hydrolization test, hydrolyzed and produced urease. All the 9 isolates were well grown in at 4% NaCl level.

Antibiotic sensitivity test on *Azospirillum* isolates

Among the 9 isolates tested for antibiotic sensitivity test, all the isolates were sensitive to Ampicillin and streptomycin (inhibitory zone 21mm to 17 mm) and resistant to Chloramphenicol and Erythromycin. Based on the morphological, cultural, physiological and biochemical characteristics, all the 9 isolates were identified up to species level as well as on the basis of sampling sites they were named as *Azospirillum amazonense* TPA01, TKK03 and TPP04 and *A. brasilence* TBR02, TPS05, TTT06, TTK07, TTV08 and TOV09.

Correspondingly, Ilyas *et al.*, (2012) studied the colony morphology, gram staining, and carbon/nitrogen utilization pattern the isolated strains from maize (*Zea mays* L.) were identified as members of genus *Azospirillum.*

Evidently, Sagadevan *et al.*, (2014) reported that *Azospirillum* strain was isolated from the rhizosphere soil of paddy field. The nitrogen fixing bacteria *Azospirillum* was confirmed by morphological, physical and cultural characteristics. The isolated strains were used for mass inoculum production and it is used to infer that the *Azospirillum* strains have better association and present in the rhizosphere and endorhizosphere of paddy field soil.

Similarly, Mozammel Hossain *et al.*, (2015) accounted that samples of rhizosphere soil, non-rhizosphere soil and roots of rice plants were collected from the particular locations of the 3 districts (Bogra, Naowgaon and Dinajpur) of North Bengal. Twenty different colonies of *Azospirillum* spp. were isolated.

Nitrogen fixing capacity of *Azospirillum* isolates

Nitrogen fixing ability of the 9 isolates was measured by micro kjeldhal method. Among the 9 isolates tested, all the isolates were found able to fix nitrogen. The range of nitrogen fixing ability was from 6.9 to 11.2 mg/g. Among the nine isolates tested, the maximum amount of nitrogen fixation was done by TPS05 isolate at a rate of 11.2 mg 'N'/g which was followed by TTK06 (10.7 mg 'N'/g) and TBR02 (10.3 mg 'N'/g) and minimum (6.9 mg 'N'/g) was recorded in TPA01 isolate (Fig -1).

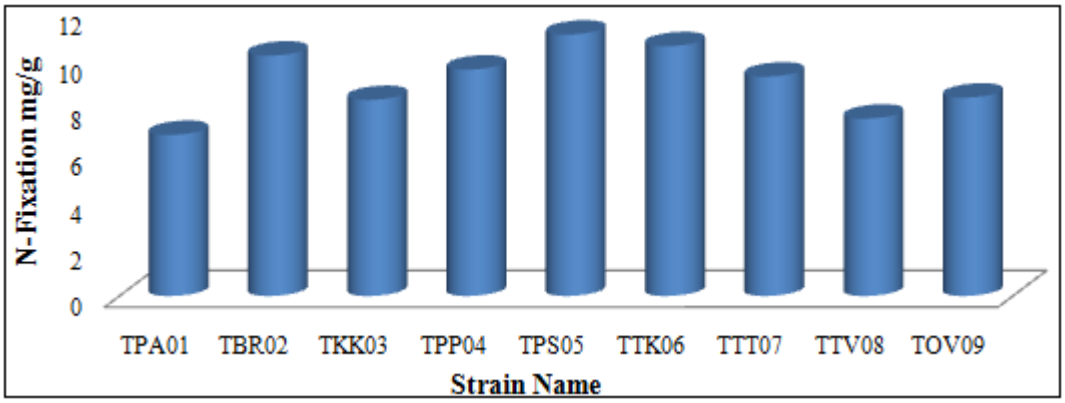

Figure 1: Nitrogen fixation ability of *Azospirillum* isolates

Likewise, *Azospirillum* is known to be a very active nitrogen fixer under laboratory as well as soil conditions providing fast growth, better health of the plant and higher yield (Kannan and Ponmurugan, 2010). Idress *et al.*, (2010) reported the occurrence of microorganisms is common in rice fields. Biological nitrogen fixation by microorganisms obviously plays the vital role in supplying nitrogen to rice plant, decreasing the requirement on chemical fertilizer in particular nitrogen by 20% - 50%.

Effect of *Azospirillum* isolates on the growth and yield of paddy ADT36 (transplanted plants) in pot culture

All the 9 isolates of *Azospirillum* were introduced in paddy crop ADT36 in pot culture to study the growth and yield trait. The following parameters such as number of roots, root length, number of leaf, leaf length, breadth of leaf, length of shoot, number of tillers and grains weight were observed (Plate -1).

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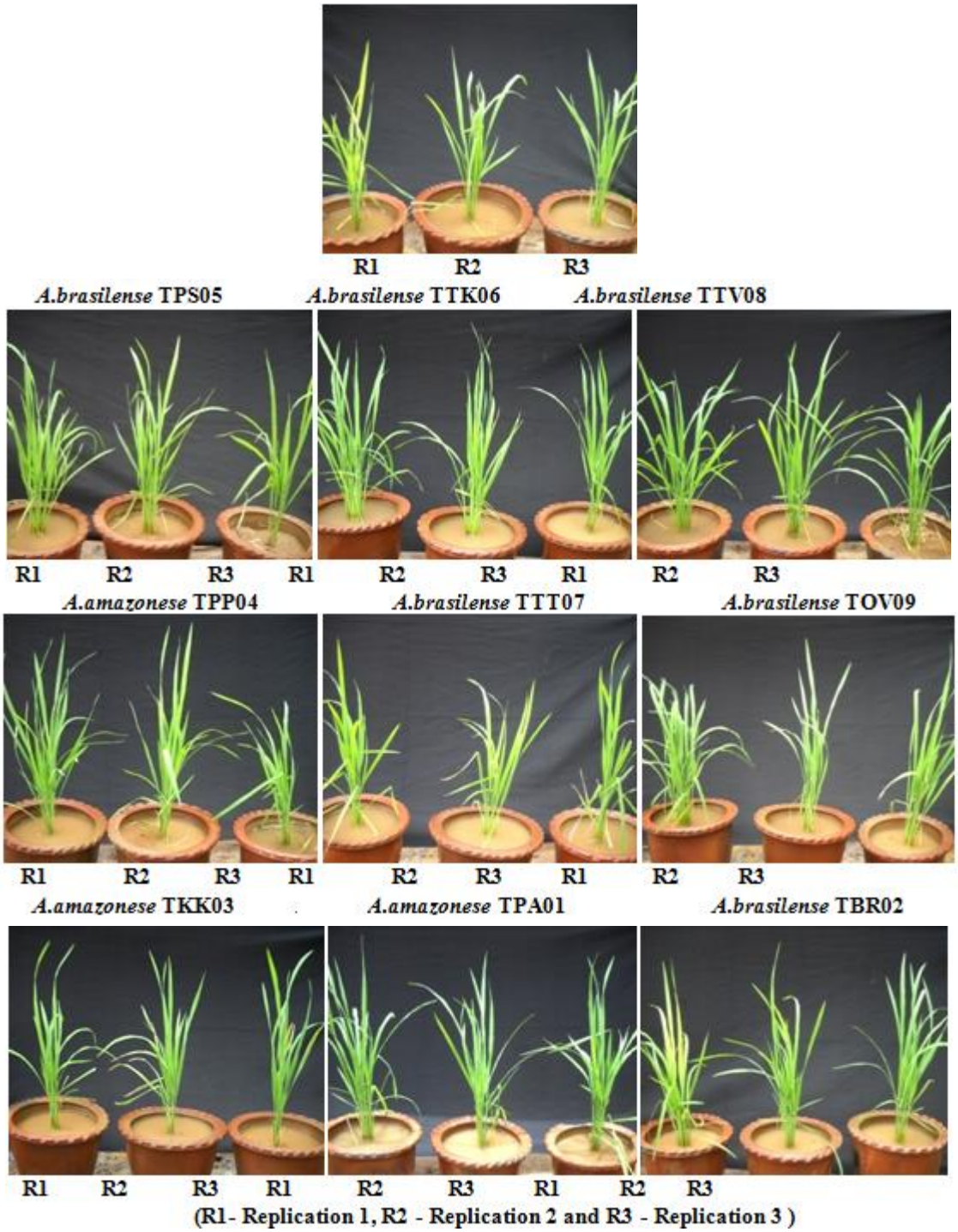
Plate- 1. Growth pattern rice (ADT 36) with *Azospirillum* strains (Pot culture method)



(R1- Replication 1, R2 - Replication 2 and R3 - Replication 3)

Number of roots

After 30 days of transplantation, the numbers of roots in *Azospirillum* isolates inoculated plants as well as in the control were studied on the 30th day of transplantation. The control plants produced minimum number (21.66 ± 0.57) of roots than the treated plants. Among the treated plants, the maximum numbers of roots (32.65 ± 1.15) were recorded in *A. brasilense* TPS05 and the minimum (22.63 ± 0.53) was recorded in *A. brasilense* TBR02. Similarly, on 60th and 90th day, the maximum number of roots (58.66 ± 1.14, 81.00 ± 1) was recorded respectively in *A. brasilense* TPS05 and the minimum (52.30 ± 1.50) *A. brasilense* TBR02 and 72 ± 1.00) *A. basilence* TBR02 were recorded 60th and 90th respectively. The control plants were produced the minimum number of roots on 60th and 90th days. On the whole, *A. brasilense* TPS05 treated plants were gradually produced the maximum number of roots than the other isolates treated plants.

Root length

On the 90th day of transplantation, among the treated plants, the maximum root length (22.46 ± 0.19) was recorded in *A. brasilense* TPS05 treated plants and the minimum (18.43 ± 0.19) was recorded in *A. amazonense* TPA01 treated plants. The control plants produced the minimum root length (17.9 ± 0.1).

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Number of leaves
On the 90th day of transplantation, among the treated plants the maximum numbers of leaves (26.65 ± 0.57) were recorded in *A. brasilense* TPS05 and the minimum (17.33 ± 0.56) was recorded in *A. amazonense* TPA01 treated plants. The control plants produced minimum number of leaves (13.65 ± 0.56).

Leaf length
On the 90th day the maximum leaf length (55.3 ± 0.52) was recorded in *A. brasilense* TPS05 and the minimum was recorded (47 ± 0.29) in *A. amazonense* TPA01 treated plants. The control plant produced minimum leaf length (46 ± 0.1) than *Azospirillum* isolates treated plants.

Leaf breath
On the 90th day the maximum leaf breath (1.66 ± 0.1) was recorded in *A. brasilense* TPS05 and the minimum (1.55 ± 0.05) was recorded in *A. brasilense* TTK06 treated plants. The control plants produced the minimum leaf breath (1.30 ± 0.05).

Shoot length
On the 90th day the maximum shoot length (52.52 ± 0.24) was observed in *A. brasilense* TPS05 and the minimum (38.4 ± 0.2) was recorded *in A. amazonense* TPA01 treated plants. The control plant produced the minimum shoot length (37.26 ± 0.35).

Number of tillers
On the 90th day the maximum numbers of tillers (23.65 ± 0.57) were recorded in *A. brasilense* TPS05 and the minimum (17.5 ± 1) was observed in *A. brasilence* TOV09 treated plants. The control plant produced the minimum number of tillers (16 ± 0.56).

Yield of grains
On the 90th day of transplantation of the maximum (59.5g) grain weight was recorded in *A. brasilense* TPS05and the minimum (55.3 g) grain weight was recorded in *A. amazonense* TPA01. The control plants produced the minimum grain weight (55.1g) than the treated plants.

Additionally, Araújo *et al*., (2010) reported that faster germination reduces the period of heterotrophism and reduces the chances of attack by soil pathogens. Rice physiologically responded to *Azospirillum* inoculation increasing its aerial biomass (Salamone *et al*., 2010).

Similarly, Isawa *et al*., (2010) showed that inoculation of rice seeds with *Azospirillum* sp. isolate B510 significantly enhanced the growth of newly generated leaves and shoot biomass under greenhouse conditions. Rice seedlings inoculated with *Azospirillum* sp. B510 also significantly increased growth in terms of tiller numbers and shoot length in paddy field (Bao *et al*., 2013).

4. Conclusion

The present studies specify the isolated strain *Azospirillum brasilense* TPSO5 was suitable for inoculating on paddy (ADT 36). *A. brasilense* TPS05 strain produced the highest number of tillers, roots, leaves, leaf length and breadth, shoot and root length and grain yield when compared to other isolates. *A. brasilense* TPS05 have high potential for getting higher rice production. Therefore this strain suggest for large scale field application, which may reduce production cost.

5. Acknowledgement

The authors acknowledge the University Grants Commission (SERO) Hyderabad for the financial support (MRP) and the management, for the permission.

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Occurrence and microbiological characteristics of *Azospirillum* strains associated with leguminous and non-leguminous plants in Al Akdar eco-region, Libya, American-Eurasian *J.Agric. & Environ. Sci*., 8(6), 2010, 617-625.

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