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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF PGPB ORGANISMS FROM THE RHIZOSPHERE OF MAIZE (ZEA MAYS. L)

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ABSTRACT

PGPB (Plant growth promoting bacteria) are the group of bacteria which can able to enhance or influence the growth, development and yield of crop plants. The beneficial effect on the growth of crop plants actually due to 'N' fixation, Phosphorus solubilization and growth promoting substances production (IAA, GA_3, etc.). PGPB includes the vital 'N' fixing diazotrophs namely *Azospirillum* and a known endophytic diazotroph *Gluconacetobacter* and also phosphorus solubilizer namely *Bacillus* and *Pseudomonas*. In the present research about ten isolates of *Azospirillum*, *Bacillus* and *Pseudomonas* were isolated from ten different rhizosphere soil samples of maize from Salem District. These isolates were purified, characterized and identified as *Azospirillum brasilense, Bacillus megaterium* and *Pseudomonas fluorescens*. In addition the isolation of *Gluconacetobacter diazotrophicus* from the rhizosphere soil samples of maize ends in failure whereas the same organisms was successfully isolated from root extract of maize and purified and all these isolates were maintained for further study.

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INTRODUCTION

The world emerging population essentially requires an increase in the production of different food, fiber and cereal crops to meet out the food requirement for largely human growing population and animals. In the densely populated areas such as Asia, where there is little opportunity for the increased area production of crop plants. Hence, increase in production must be achieved through enhancement in agricultural productivity by employing latest technologies. The productivity of crop plants ensured by the application of higher amount of chemical fertilizers. The compounds of fertilizers are get entrance into the food chain through plant uptake from soil colloids as well the remaining amount in the form of residues causes soil pollution. In addition being the petroleum product the cost of raw materials to synthesis chemical fertilizer are costlier one, additionally industrial process require crores of investment, wastage of power and man power etc. Hence, to avoid above parameters, it is essential to renew biological alternatives to substitute nutrients for enhanced productivity by devoid of pollution. Cereal crops are the most important sources of carbohydrates containing energy rich food for more than one third of the world population. Plant productivity in agriculture soils is influenced by many abiotic and biotic factors.

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There is a thin layer of soil region immediately surrounding plant the roots that is an highly important and active area for intensive microbial activities, root activities and metabolism which is known as rhizosphere. The rhizosphere concept was first introduced by Lorez Hiltner in 1904, many studies have been reported that the soil environment attached to the root system is a range of microbial abundance and activity due to the presence of root exudates and rhizodeposits (Hiltner, 1904; Smalla *et al*., 2006; Hartmann *et al*., 2008). The rhizosphere provides organic substances and growth hormones to large amount and enhance the plant growth and development when, inputs of PGPB organism added to soil (Nihorimbere *et al*., 2011). Majority of microbial communities moves towards rhizosphere to have enhanced multiplication by utilizing nutrient food material and favourable shelter in the root region. Soil contains a broad diversity of microbes, which are highly intensive in nutrient-rich soil regions, including the top soil layer and the region around the plant root. Plants can benefit from soil microbes in lot of ways. Microorganisms are plays major role in agricultural crops in order to promote the circulation of plant nutrients and reduce the usage of chemical fertilizers through 'N' fixation 'P' solubiization stimulate plant growth by production of growth hormones, degrade pollutants, or biological control of plant pathogens. Plant growth-promoting bacteria (PGPB) are free-living, soil-borne bacteria, which, when applied to seeds or crops, improve the growth of

the plant or reduce the damages from soil-borne plant pathogens.

Nowadays microorganisms play an important role in agricultural system, especially the group of bacteria called plant growth promoting bacteria. PGPB are widely deliberate because of their potential for plant growth production under three characteristics. Firstly PGPB acting as biofertilizers (Vessey, 2003). Provide nitrogen *via* nitrogen fixation reaction, which can subsequently be used by the plants. Secondly, phytostimulators (Steenhoudt *et al*., 2006) can directly provide the growth of plant usually by the production of plant hormones. Finally, through biological control (Costa *et al*., 2007) are able to protect plant via root system from phyto pathogenic organisms.

Maize rhizosphere contains a high diversity of plant growth promoting bacteria. The rhizosphere is a region of intense microbial activity where root exudates allow the development of many rhizosphere communities. PGPB plant growth promoting bacterial strains having beneficial characters are directly increased different stage of plant growth: fixation of atmospheric nitrogen, solubilization phosphorous and production of siderophores. The root system plays a significant role in plant promotion because roots explore the soil for uptake of essential nutrition's. It is clear that PGPB can change the endogenous levels of phytohormones such as IAA, GA3 and other plant hormones. The possible alternatives inorganic fertilizer for promoting plant growth is plant associated nitrogen fixing bacteria, N_2 fixation and phosphate solubilization is most important for plant growth promoting activities. Several soil bacteria are able for the fixation of N_2 especially bacteria belonging to the genera like *Acetobacter*, *Arthrobacter*, *Azospirillum*, *Azotobacter, Herbaspirillum*, *Klebsiella* etc. Phosphate solubilizing bacteria especially *Pseudomonas* and *Bacillus* sp are able to change insoluble forms into soluble forms by secreting organic acids as formic acid, acidic acid, lactic acid propionic acid, fumaric acid and succinic acid, etc.

Biofertilizers have emerged as an important component of the nutrient provide system and hold a great promise to improve crop yields through environmentally better nutrient supplies.

The present research aimed to isolate microflora from the rhizosphere soil and root sample of maize and also to find out PGPB nature of the isolated bacterial organisms.

MATERIAL AND METHODS

Enumeration of PGPB microorganisms from the Rhizosphere soil samples of maize

Collection of sample

Soil samples were collected from depth of 6- 15 cm from the rhizosphere of maize plant root surroundings. Collected soil samples were stored in polythene bags aseptically and maintained at the laboratory for further study.

Isolation of *Azospirillum* isolates

Ten fold serial dilutions of each soil sample, ranging from 10^{-1} to 10^{-4} were made in mineral salts solution of Day and Dobereiner (1976). One ml of each dilution was inoculated in a set of five tubes containing 9 ml of nitrogen free semisolid malate medium (Day and Dobereiner, 1976). Atleast three consecutive dilutions were inoculated and tubes were incubated for three days at 30±2°C. Tubes showing sub – surface, thin pellicle growth were identified as positive tubes for *Azospirillum*. The MPN counts of *Azospirillum* were calculated on the basis of positive tubes by referring the MPN table (Cochran, 1950).

Purification of *Azospirillum* isolates

The inoculated test tubes were then incubated at 35°C for 3-5 days. After incubation, the test tubes were observed for the growth of *Azosipirillum*. *Azosipirillum* growth was observed by change in colour of the medium from greenish yellow to blue the presence of white dense subsurface pellicles. The pellicles were streaked on N-free malic acid medium containing 0.3% NH_4Cl and after growth they were stored at 4° C until further use.

Isolation of *Bacilllus* sp.

Maize rhizosphere soil samples of seven different soil types (Sandy loam, Clay loam, Clay, Sandy Clay, Loamy, loamy sand and Red soil) were collected from different locations of Salem district of Tamil Nadu. The phosphorous solubilizing microorganisms were isolated from these soil types by following the techniques of Dhingra and Sinclair (1985). Ten grams of rhizosphere soil sample was transferred to 100 ml of distilled water blank to get dilution of 10^{-1}. Similar dilutions were prepared till a dilution of 10^{-4} was obtained. One ml of 10^{-4} dilution was transferred to petriplates and the plates were poured with Pikovskaya's medium and incubated for 7 days. The plates were examined for hollow zones or zone of clearance around the colonies.

Purification of *Bacillus* sp.

From the isolated colonies of phosphobacteria, a loopful of culture was streaked in the petriplates containing Pikovskaya's medium. The streaked plates were incubated at room temperature for one week. Isolated single colony was taken and further purified by streaking in Pikovskaya's medium. The typical colonies were examined for morphology and microscopically and transferred to Pikovskaya's medium.

Isolation of *Pseudomonas*

Pseudomonas population was enumerated from rhizosphere soil sample of maize by serial dilution plate technique. The soil samples were serially diluted upto 10^{-4} dilution, one ml of aliquots of last dilution were plated in King's B agar medium (King's *et al*., 1954). Colonies were obtained after 48 hrs of incubation at room temperature were count and expressed as Cfu g^{-1} of oven dry soil.

Purification of *Pseudomonas sp*

Pseudomonas colonies obtained after 48 hours of incubation at room temperature. The colonies were purified by streaking a

significantly in Kings' B agar plate medium and colonies were examined microscopically and transferred to Kings' B agar slants and stored in refrigerator for further studies.

Isolation of *Gluconacetobacter* sp.

Gluconacetobacter cultures were isolated from the maize root samples following the methodology of Cavalcante and Dobereiner (1988). One gram of the maize samples (root) was washed thoroughly in running tap water placed in 70 per cent alcohol for 15 seconds and immediately washed in sterile distilled water for 3-4 times repeatedly. The surface sterilized samples were macerated in a sterile pestle and mortar. A drop of the suspension as such and 10^{-2} dilution were inoculated into various enrichment media *viz*., semisolid LGI medium and semisolid acetic LGI medium supplemented with yeast extract (20 mgl^{-1}) the tubes were incubated at room temperature without disturbance until the formation of sub surface pellicles.

Characterization and identification of plant growth promoting bacteria (PGPB) from the Rhizosphere soil of maize

Characterization of *Azospirillum* isolates

Microscopic observation of the wet mounts of the 72 hrs old Nfb medium cultures was carried out for the shape, motility and presence of poly β hydroxyl butyrate granules. Gram staining was carried out as per Hucker's 1927. Catalase activity was determined by observation of O_2 evaluation from cell suspensions following the addition of 0.5 mM H_2O_2 as described by Roth and Jensen (1967) subsurface pellicle formation in Nfb semisolid medium and pink colour colonies on BMS agar.

Production of acid from glucose (Krieg and Tarrand, 1978) of *Azospirillum* isolates

The ability to produce glucose by *Azospirillum* isolates was studied by following the method of Krieg and Tarand (1978). The medium was dispensed in 5 ml quantities in test tubes and sterilized. The tubes were inoculated with 0.1 ml of 24 hrs old cultures of *Azospirillum* isolates and incubated for 96 hrs at 30 ± 2°C. The change in colour of the broth from green to yellow indicated acid production.

Utilization of different carbon sources

The ability of the *Azospirillum* isolates to utilize different carbon sources were tested in Nfb medium. The medium was prepared without bromothymol blue and malate. The various carbon sources *viz.,* glucose, malate, lactate, mannitol, succinate and α-ketoglutarate where incorporated separately at 1 % level. After sterilization, the media inoculated with 0.1 ml of 24 hrs old cultures of *Azospirillum* and incubated at 30 ± 2°C and observed for growth.

Biotin Requirement

The nitrogen free malate (Nfb) medium was used for assessing the requirement for the biotin of *Azospirillum* isolates according to Reinhold *et al*. (1987). Two sets of media with and without biotin (100µg ml^{-1}) were prepared. Then the cultures were grown for 48 hrs in glucose peptone broth, centrifuged at 5000 x g for 20 min and washed twice with sterile distilled water and re- suspended in sterile distilled water to a uniform density of about 1 x 10^7 Cfu ml^{-1}. A quantity 0.1 ml of this suspension was used as inoculum to inoculate 10 ml volume of medium. For each set, a control was maintained without inoculation the test tubes were incubated at 37° C and observed for 48hrs. The biotin requirement was determined by presence or absence of growth.

Nitrate Reductase (NR) activity (Yordy and Ruoff, 1981)

Azospirillum cultures were grown in 10 ml of malate broth supplemented with 10 mM of sodium nitrate. This was incubated at 32°C for 5 days in shake culture condition. The broth was centrifuged and the supernatant was collected. To 10 ml of the supernatant, 0.3 ml of 1 % sulphanilamide, in 1.5 N hydrochloric acid and 0.2 ml of 0.002 % N (1-napthyl) ethylene diaminedihydrochloride were added. The appearance of pink colour indicated the presence of NR activity.

Nitrite Reductase (NiR) activity (Yordy and Ruoff, 1981)

For determining the activity of NiR, a loopful of the malate grown culture was transformed to 5 ml of medium containing 5 mM of sodium nitrate as a source of nitrogen and incubated at 32°C for 5 days. The broth was centrifuged and the supernatant was collected. To 1 ml of supernatant, 0.3 ml of 1 % sulphanilamide in 1.5 N HCl and 0.2 ml of 0.002 % N (1-napthyl) ethylene diaminedihydrochloride were added. The disappearance of pink colour indicated the presence of NiR activity.

Characterization of *Bacillus* isolates

The following characters were studied for each isolate.

Morphology

The morphology of each isolate was studied by observing sporulation and the size of the cell was measured by micrometry Gram's staining procedure was followed as described by Vincent (1970).

Acid production from glucose, mannitol, arabinose and xylose
Slants of the nutrient medium with different carbohydrates were inoculated with isolated phosphobacterial cultures and incubated for 7 days. A drop of culture to a spot plate was mixed with a drop of 0.04 % (w/v) alcoholic bromocresol purple and observed the change in colour of the indicator from purple to yellow as indicates acid production.

Hydrolysis of starch

Starch agar plates were prepared, streaked with suitable culture and incubated for 24 hrs at 37°C. After incubation, iodine solution was poured on to the plate. Appearance of clear zone around the culture streaked area indicated the utilization of starch.

Gelatin hydrolysis

Gelatin agar plates were prepared, streaked with suitable culture and incubated for 72 hrs at 37°C. After incubation, trichloroacetic acid solution was poured on to the plate. Appearance of clear zone around the culture streaked area indicates the utilization of gelatin.

Casein hydrolysis

It was performed by supplementing Nutrient agar medium with skimmed milk powder and the formation of clear zones adjacent to the bacterial growth was recorded as positive.

Catalase test

The colonies were transferred to a clean glass slide with the help of an applicator stick and a few drops of 3 % hydrogen peroxide were added. The production of gas bubbles was considered positive for the experiment.

Oxidase test (Collins and Lyne, 1970)

Small pieces of filter paper were soaked in 1 per cent aqueous tetra methyl-p-phenylene diamine and placed in a petridish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after ten seconds indicated positive oxidase test whereas development of a light violet colour indicated negative oxidase test.

Indole test

Tryptone broth was prepared poured into test tubes and sterilized. The medium was inoculated with a loopful of culture and incubated at 30 ± 2°C for 48 hrs mixed with 3 ml of Kovac's reagent, shaken well and allowed to stand for 5 min, the observations were recorded.

Methyl red test

The glucose phosphate broth was sterilized and inoculated with bacterial culture and incubated at room temperature for 48 hrs. Hence, few drops of methyl red indicator were added and observations were noted.

Urease test

Five ml medium for urease test was poured in pre – autoclaved test tubes. Two test tubes were taken for each strain and one for control. Test tubes were inoculated, except control, with fresh bacterial culture (24 hours incubation) and incubated at 37°C for seven days. A change in the colour of medium from yellow to pink is an indication of positive test.

Voges – proskauer test

The Voges – proskauer test was carried out by following the method described by Aneja (1993). The change in the colour is indication of positive VP test while no change in colouration is a negative test.

Citrate utilization test

The test was performed to find out the ability of test isolates to ferment citrate as the sole source of carbon. The test was performed on Simmon's citrate agar slants and a change in color of the medium from green to deep Prussian blue was considered as positive for the test.

Characterization of *Pseudomonas* isolates

The isolates were checked for fluorescent pigment productions were tested under UV light. The isolates streaked on agar slants incubated at 4°C and checked for their growth. Gram reaction, motility, hydrolysis of starch, hydrolysis of gelatin, Egg yolk reaction pigment production, casein hydrolysis, catalase, oxidase, indole, methyl, citrate and hydrogen sulfide (H_2S) were tested.

Hydrolysis of starch filter paper disks were dipped in a day old culture suspension and were placed on the petriplates containing starch agar medium then incubated for two days. The plates were then flooded with 1 per cent iodine solution. A colourless hale around the growth and blue colour in the rest of the plates showed utilization of starch by the microorganisms (Stolpe and Godkeri, 1981).

Hydrolysis of gelatin

Filter paper disks were dipped in 24 hrs old culture suspension and then placed in petriplates containing gelatin nutrient agar medium. The plates were incubated at 30°C for 2 days and then flooded with 12.5% $HgCl_2$solution. The development of yellow halo around the growth indicates utilization of gelatin (Stolpe and Godkeri, 1981).

Egg yolk reaction

Egg yolk reaction is carried out by the procedure described by Lelliott *et al*., (1966).

Pigment production

The *Pseudomonas* culture was plated in the fluorescent medium for checking fluorescent pigment production.

Casein hydrolysis

It was performed by supplementing nutrient agar medium with skim milk powder was prepared sterilized and was poured into pre – sterilized petriplates. The petriplates were kept inverted for 24 hrs and the bacterial cultures were inoculated by streaking the surface. It was incubated at 30°C for 48 hrs and the formation of clear zones adjacent to the bacteria growth was recorded as positive.

Catalase test

The colonies were transferred to a clean glass slide with the help of an applicator stick and a few drops of 3 % hydrogen peroxide were added. The production of gas bubbles was considered positive for the experiment.

Oxidase test (Collins and Lyne, 1970)

Small pieces of filter paper were soaked in 1 per cent aqueous tetra methyl-p-phenylene diamine and placed in a petridish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after ten seconds indicated positive oxidase test whereas development of a light violet colour indicated negative oxidase test.

Indole test

Tryptone broth was prepared poured into test tubes and sterilized. The medium was inoculated with a loopful of culture and incubated at 30 ± 2°C for 48 hrs mixed with 3 ml of Kovac's reagent, shaken well and allowed to stand for 5 min, the observations were recorded.

Methyl red test

The glucose phosphate broth was sterilized and inoculated with bacterial culture and incubated at room temperature for 48 hrs. Hence, few drops of methyl red indicator were added and observations were noted.

Citrate utilization test

The test was performed to find out the ability of test isolates to ferment citrate as the sole source of carbon. The test was performed on Simmon's citrate agar slants and a change in color of the medium from green to deep Prussian blue was considered as positive for the test.

Hydrogen sulfide (H_2S)

Sulfide indole motility agar medium was prepared poured into tubes and sterilized at 15lbs 15 min. The agar tubes were stabbed with sterile needle containing bacterial culture and kept at 30±2°C for 48 hrs. The tubes were examined for presence or absence of black precipitate along the line of inoculation and observations were noted.

Characterization of *G. diazotrophicus* isolates

All the isolated strains of *G. diazotrophicus* culture were grown in acetic LGI medium and single colony was streaked on acetic LGI agar slants and the young cultures at exponential phase i.e. on 7^{th} day were taken for further characterization.

Gram staining

Gram staining was carried out as per Huker's modified method (Rangaswami and Bagyaraj, 1933).

Motility

The presence of motility in the isolated cultures was observed by hanging drop technique using a cavity slide as described by Aneja (1993).

Oxidase test (Collins and Lyne, 1970)

Small pieces of filter paper were soaked in 1 per cent aqueous tetra methyl-p-phenylene diamine and placed in a petridish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after ten seconds indicated positive oxidase test whereas development of a light violet colour indicated negative oxidase test.

Nitrate Reductase test (Beishir, 1987)

Cultures were inoculated into the test tubes containing nutrient glucose broth with one per cent KNO_3 and incubated at 37 °C for 48 h. Test for the presence of nitrate reductase was carried out by adding 1 drop of sulfanilic acid and one drop alpha naphthylamine reagent to each of the nutrient broth cultures. Development of distinct red colour indicated positive test and no colour development indicated negative test.

Test for hydrogen sulphide formation (Beishir, 1987)

Peptone iron broths in tubes were incubated with cultures and incubated at 37°C for 48 hrs. black precipitation in the medium indicated hydrogen sulphide formation.

Catalase test (Rangaswami and Bagyaraj, 1993)

Loop of bacteria to be tested was taken from the solid medium and mixed with a drop of 3 per cent hydrogen peroxide on a glass slide. Catalase positive organisms showed bubbles of oxygen.

RESULTS AND DISCUSSION

In the present investigation soil samples were collected from 10 different location of Salem District, Tamil Nadu, *viz*., Veeraganoor, Gangavalli, Tharamangalam, Vazhappadi, Athur, Sivathapuram, Thalaivasal, Jalakandapuram, Puthiragoundampalayam and Pethanayakanpalayam were analyzed for physicochemical properties, finally the soil and root samples were used for the isolation of PGPB organisms namely *Azospirillum*, Phosphobacteria (*Bacillus*) *Pseudomonas* and *Gluconacetobacter*.

Determination of PGPB isolates from the rhizosphere soil of maize

In addition to the enumeration of microbial population the maize rhizosphere soil samples were further subjected to determine the population of certain important plant growth promoting bacteria (PGPB) *viz., Azospirillum, Bacillus* and *Pseudomonas* Table 1.

The results revealed that the maize rhizosphere soils harboured more of these PGPB to the level 10^{-4} Cfu/g of oven dry soil. The higher *Azospirillum* population of 6.66 x 10^{-4} Cfu/g and minimum population of 2.96 x x 10^{-4} Cfu/g were recorded in Sivathapuram and Vazhappadi locations respectively.

The soil samples collected from different location at Salem district recorded higher *Bacillus* population in Sivathapuram (12.6 x 10^{-4} Cfu/g) followed by Thalaivasal (10.8 x 10^{-4} Cfu/g). The lowest *Bacillus* populations of 6.30 x 10^{-4} Cfu/g of soil was recorded in Vazhappadi.

Table 1. Determination of species of PGPB isolates from the rhizosphere soil of maize

Location	Microbial population (CFU/g of soil)*			
	Azospirillum sp. ($x10^{-4}$)	*Bacillus* sp. ($x10^{-4}$)	*Pseudomonas* sp. ($x10^{-4}$)	*Gluconacetobacter* sp. ($x10^{-4}$)
Veeraganoor	4.60	8.33	5.36	-
Gangavalli	4.33	10.0	5.66	-
Tharamangalam	3.33	10.2	5.30	-
Vazhappadi	2.96	6.30	4.00	-
Athur	5.70	10.6	7.30	-
Sivathapuram	6.66	12.6	8.00	-
Thalaivasal	5.90	11.8	7.60	-
Jalakandapuram	4.60	9.33	5.60	-
Puthiragoundampalayam	3.66	9.01	5.26	-
Pethanayakanpalayam	3.43	7.33	4.30	-

*population on oven dry basis

Table 2. Isolation of *Gluconacetobacter diazotrophicus* isolates

S.No	Location	Rhizosphere soil of maize		Maize root		Name of the isolates
		LGI	ALGI	LGI	ALGI	
1	Veeraganoor	-	-	+	++	GdMRV-1
2	Gangavalli	-	-	+	++	GdMRG-2
3	Tharamangalam	-	-	+	++	GdMRT-3
4	Vazhappadi	-	-	+	++	GdMRV-4
5	Athur	-	-	+	++	GdMRA-5
6	Sivathapuram	-	-	+	++	GdMRS-6
7	Thalaivasal	-	-	+	++	GdMRT-7
8	Jalakandapuram	-	-	+	++	GdMRJ-8
9	Puthiragoundampalayam	-	-	+	++	GdMRP-9
10	Pethanayakanpalayam	-	-	+	++	GdMRP-10

+ - Moderate growth
++ - Good growth
(-) – No growth

The *Pseudomonas* population in maize rhizosphere soil samples ranged between 4.00 x 10^{-4} Cfu/g of soil and 8.00 x 10^{-4} Cfu/g of soil levels and the highest population of *Pseudomonas* and lowest were recorded in Sivathapuram and Vazhappadi locations respectively. The *Pseudomonas* population from other locations was recorded between these limits.

Maize rhizosphere soils collected from different locations of Salem district were studied for plant growth promoting bacterial diversity. The maize rhizosphere soil samples were accessed for the presence of certain important PGPB isolates namely *Azospirillum*, *Bacillus* and *Pseudomonas* population. The PGPB population ranged from 2.96 x 10^{-4} Cfu/g to 6.66 (*Azospirillum*), 6.30 x 10^{-4} Cfu/g to 12.6 x 10^{-4} Cfu/g of soil (*Bacillus*) and 4.00 x 10^{-4} Cfu/g to 8.00 x 10^{-4} Cfu/g of soil (*Pseudomonas*) were observed in the present investigation. The presents result in accordance with findings of Dang and Cao (2014). They was found nitrogen fixing bacterial population ranged from 5.54 to 6.99 Cfu $\log_{10}$/g soil and phosphorus solubilization of bacterial population ranged from 4.57 to 5.98 Cfu $\log_{10}$/g soil.

The occurrence of *Azospirillum* and *Bacillus* in different niches such as soils, roots and rhizosphere of various crop plants including maize has been reported by Bacon *et al* (2001); Bacilio – Jimenez *et al* (2001); Pinheiro (2002); Ramos *et al* (2002); Liu *et al* (2003); Yan *et al* (2003); Ashraf *et al* (2004); Mc Spadden Gardener, (2004); Nadeem *et al*, (2006); Palumbo *et al* (2007); Puente *et al* (2009). The occurrence of *Spirillum* in maize soils was first reported by Lakshmi kumara *et al*, (1976) in India.

Details of location and sources of *Gluconacetobacter* isolates

Gluconacetobacter was isolated from maize root samples collected from ten different locations (Veeraganoor, Gangavalli, Tharamangalam, Vazhappadi, Athur, Sivathapuram, Thalaivasal, Jalakandapuram, Puthiragoundampalayam and Pethanayakanpalayam, Salem District). The *G. diazotrophicus* were isolated from maize root samples. Ten isolates were obtained from maize root samples and the formation of yellow coloured sub surface pellicle on colourless semisolid LGI medium and typical heavy orange yellow coloured subsurface pellicle on acetic LGI semisolid medium were observed (Table 2).

Apart from the isolation of PGPB organisms namely *Azospirillum, Bacillus and Pseudomonas,* an attempt was made to isolate *Gluconacetobacter diazotrophicus* from the soil samples collected from ten different locations of Salem District, Tamilnadu.

In the present research an interesting finding was concluded i.e. there was no positive notification for the isolation of *Gluconacetobacter diazotrophicus from* all rhizosphere soil samples of maize and the present result showed zero per cent population of *Gluconacetobacter diazotrophicus.* Further *Gluconacetobacter diazotrophicus* was successfully isolated from the root samples of maize. Hence it is a concrete evident for the endophytic nature of *Gluconacetobacter diazotrophicus.*

The above results are in accordance with the findings of Dobereiner *et al*, (1988); Prabudoss and stella (2010); Muthukumarasamy *et al* (2005). The isolates were isolated

from all parts of sugarcane *viz*., root, stem, bud and leaf (Cavalcante and Dobereiner, 1988). More isolates were obtained when the macerates of surface sterilized root, stem, bud and leaves of sugarcane were used than the plant bits as such. This observation is substantiated by Dong *et al.* (1994) and James *et al.* (1994) and these observation illustrated the endophytic nature of the bacterium *G. diazotrophicus* (Reis *et al.* 1994; dos Santos *et al.,* 2010). It is the only nitrogen fixing bacterial endophyte and leading to designated as a model organism to evaluate the plant bacterial interactions in non-legumes (Dobbelaere *et al.,* 2003).

Characterization of plant growth promoting bacterial isolates

The characterization (morphological and physiological) of isolated PGPB bacterial strains was carried out on the basis of colony morphology (colour, shape and size), cell shape and motility. Ten isolates of each of *Azospirillum, Bacillus, Pseudomonas and Gluconacetobacter* were studied and based on the results obtained, the species of each genus was determined.

Phenotypic and biochemical Characterization of *Azospirillum*

The cultural characteristics of the purified bacterial strains were studied using light microscope. Six isolates (AZMRV-1, AZMRG-2, AZMRT-3, AZMRS-6, AZMRT-7 and AZMRP-10) were tentatively identified as *Azospirillum brasilense strains and four* isolates (AZMRV-4, AZMRA-5, AZMRJ-8 and AZMRP-9) *Azospirillum lipoferum* (Table 3). The characteristic dense white subsurface pellicles were observed in all the isolates and also change in colour from yellowish green to blue were noticed in nitrogen free malic acid medium (Nfb). The appearance of typical pink colour wrinkled colonies on Potato infusion agar confirmed the characteristics of *Azospirillum* sp. the characteristics, curved rod shaped cells and active spiral movement on the wet microscopic mount added additional confirmation of *Azospirillum* sp. all the isolates showed Gram negative reaction and the presence of PHB.

Production of acid from glucose

The production of acid from glucose was positive in four isolates and grouped as *A. lipoferum* and the remaining six isolates were negative for the acid production from glucose and tentatively grouped as *A. brasilense*.

Utilization of different carbon sources

PGPB were characterized on the basis of their morphological and cultural characteristics as well as ability to produce growth and productivity by utilize the different carbon sources.

In general, the *Azospirillum* isolates preferred either malate or succinate as sole source of carbon. In addition lactate, mannitol and ∝ketoglutarate were also utilized by the *Azospirillum* isolate to varied degrees. The specific characteristics *viz*., moderate utilization of lactate, mannitol and ∝ketoglutarate by *A. lipoferum* and moderate utilization of lactate and poor utilization of mannitol and ∝ketoglutarate by *A. brasilense* were observed.

Biotin requirement

Among the ten isolates of *Azospirillum*, four isolates required biotin for their growth and were grouped under *A. lipoferum* and the remaining six isolates were not required biotin for their growth and were grouped under *A. brsailense.* Hence, all the *Azospirillum* isolates were showed positive results for nitrite reductase activity and the *A. brasilense* negatively responded for nitrate reductase activity and the *A. lipoferum* were able to respond positive for nitrate reductase.

PGPB were characterized on the basis of their morphological and cultural characteristic as well as ability to enhance the plant growth and yield. Several factors *viz.,* root morphology, the stage of plant growth, root exudates and the physical and chemical properties of the soil are reported to influence the occurrence and distribution of microbial communities in the soil and rhizosphere. Previous isolations of nitrogen fixing bacteria have revealed a broad diversity of diazotrophs to inhabit the crop rhizosphere (Vessey, 2003; Haahtela *et al.,* 1981; Michiels *et al.,* 1989; Nosko *et al.,* 1994; Russel and Ifiorah, 1985).

Table 3. Phenotypic and Characterization of *Azospirillum* isolates of maize rhizosphere soil samples of Salem District

Isolates	Sub surface pellicle formation	Pink colonies BMS Agar	Cell lshape	Motility	Gram reaction	Presence of PHB	Acid production	Utilization of different carbon sources						Biotin requirement	Nitrite reductase activity	Nirate reductase activitv	Tentative species identification
								Malate	Succinate	Lactase	Glucose	Mannitol	α – keto-glutamate				
AZMRV-1	+	+	Curved rods	+	_	+	_	+++	+++	++	+	+	+	_	+	_	*A.brasilense*
AZMRG-2	+	+	Curved rods	+	_	+	_	+++	+++	++	+	+	+	_	+	_	*A.brasilense*
AZMRT-3	+	+	Curved rods	+	_	+	_	+++	+++	++	+	+	+	_	+	_	*A.brasilense*
AZMRV-4	+	+	Curved rods	+	_	+	+	+++	+++	++	+++	++	++	+	+	+	*A.lipoferum*
AZMRA-5	+	+	Curved rods	+	_	+	+	+++	+++	++	+++	++	++	+	+	+	*A.lipoferum*
AZMRS-6	+	+	Curved rods	+	_	+	_	+++	+++	++	+	+	+	_	+	_	*A.brasilense*
AZMRT-7	+	+	Curved rods	+	_	+	_	+++	+++	++	+	+	+	_	+	_	*A.brasilense*
AZMRJ-8	+	+	Curved rods	+	_	+	+	+++	+++	++	+++	++	++	+	+	+	*A.lipoferum*
AZMRP-9	+	+	Curved rods	+	_	+	+	+++	+++	++	+++	++	++	+	+	+	*A.lipoferum*
AZMRP-10	+	+	Curved rods	+	_	+	_	+++	+++	++	+	+	+	_	+	_	*A.brasilense*

In the present study ten different isolates from each PGPB organism *viz., Azospirillum, Bacillus and Pseudomonas* were obtained from maize rhizosphere soils collected from different locations of Salem district, Tamilnadu. Based on the morphological and biochemically characterized *Azospirillum* strains produced dense white subsurface pellicle were observed in all the isolates, the cells curved rod shaped and motile. All the isolates showed gram negative reaction and presence of PHB. Interestingly the biochemical testing showed positive trend in utilizing carbon sources such as, malate, succinate, lactase, glucose, mannitol and ∝ ketoglutamate. Among the ten isolates of *Azospirillum*, four isolates were required biotin for their growth which are placed under *A. lipoferum* and remaining six isolates were not required biotin for growth and are categorized under *A. brasilense*. All the *Azospirillum* isolates were showed positive trend for nitrite reductase activity and negatively responded for nitrate reductase activity. All the ten isolates were found to be members of the genus *Azospirillum* based on the formation of sub surface pellicle in Nfb semisolid medium, formation of pink colour colonies on BMS agar, cell shape, motility and Gram negative character and presence of PHB and molecular studies (Dobereiner, 1980). In C_4 plants, *Azospirillum lipoferum* was the predominant species and *Azospirillum brasilense* was the predominant species associated with C_3 plants in tropical conditions (Rocha *et al.,* 1981). No correlation could be observed in many cases between the preference of host phenotype and the species of *Azospirillum* (Del Gallo and Fendrick, 1994). Tarrand *et al.* (1978) classified the genus *Azospirillum* into two species based on the physiological and biochemical characters.

Phenotypic and biochemical and Characterization of *Bacillus*

The isolates *viz.,* BMRV-1, BMRG-2, BMRV-4, BMRS-6 and BMRS-9 showed positive reaction for Gram reaction, Motility, spore forming, acid production from glucose hydrolysis of starch, hydrolysis of gelatin, catalase test, oxidase test, Inodole test, methyl red test and utilization of citrate test and negative for casein hydrolysis, urease test and voges – proskauker test which helped to characterize these isolates as *Bacillus megaterium.*

The isolates *viz.,* BMRT-3 and BMRT-7 showed positive for Gram reaction, Motility, spore forming, acid production from glucose, hydrolysis of starch, hydrolysis of gelatin, catalase test, oxidase test, methyl red test and voges – proskauker test and negative for casein hydrolysis, indole test, urease test and utilization of citrate based on the results these isolates tentatively characterized as *Bacillus polymyxa* (Table 4).

The isolates *viz.,* BMRA-5 and BMRJ-8 showed positive for Gram reaction, Motility, spore forming, acid production from glucose, hydrolysis of starch, hydrolysis of gelatin, catalase test, oxidase test, methyl red test and negative for casein hydrolysis, indole test, urease test voges proskauker and utilization of citrate and these character makes the isolate BMRA-5 and BMRJ-8 belongs to *Bacillus cereus*.

The isolate BMRP-10 showed positive for Gram reaction, Motility, spore forming, acid production from glucose hydrolysis of starch, hydrolysis of gelatin, catalase test, oxidase test, methyl red test voges proskauker test and utilization of citrate test and negatively correlated to casein hydrolysis, indole test and urease test which helped to characterize these isolates as *Bacillus subtilis*.

In the present study, the ten *Bacillus* isolates were *isolated* from maize rhizosphere soil and morphological and biochemical studies showed they belongs to as *Bacillus megaterium, Bacillus polymyxa, Bacillus cereus* and *Bacillus subtilis*. Four different *Bacillus* strains obtained from the seven different soil types from ten different locations of Salem district Tamilnadu. Among the ten isolates of *Bacillus,* five isolates belonged to *Bacillus megaterium,* two isolates belonged to *B. polymyxa*, two isolates belonged to *B. cereus* and one isolates belonged to *B. subtilis*. Similar results were already reported by Sheerin (1995) isolated three phosphate solubilizing bacterial strains from maize rhizosphere. Bacteria belonging to genera *Bacillus*, *Pseudomonas*, *Serratia*, *Enterobacter*, etc., are reported to solublize the insoluble phosphatic compounds and aid in plant growth (Rodriguez and Fraga, 1999).

Table 4. Phenotypic and biochemical Characterization of *Bacillus* isolates from maize Rhizosphere soil samples of Salem District

S.No	Name of the isolates	Gram reaction	Motility	Spore staining	Acid production from glucose	Hydrolysis of starch	Hydrolysis of gelatin	Casein hydrolysis	Catalase test	Oxidase test	Indole test	Methyl red test	Urease test	Voges-proskauker test	Citrate utilization test	Probable of identification
1	BMRV-1	+ve	+	+	+	+	+	_	+	+	+	+	_	_	+	*Bacillus megaterium*
2	BMRG-2	+	+	+	+	+	+	_	+	+	+	+	_	_	+	*B. megaterium*
3	BMRT-3	+	+	+	+	+	+	_	+	+	_	+	_	+	_	*B. polymyxa*
4	BMRV-4	+	+	+	+	+	+	_	+	+	+	+	_	_	+	*B. megaterium*
5	BMRA-5	+	+	+	+	+	+	_	+	+	_	+	_	_	_	*B. cereus*
6	BMRS-6	+	+	+	+	+	+	_	+	+	+	+	_	_	+	*B. megaterium*
7	BMRT-7	+	+	+	+	+	+	_	+	+	_	+	_	+	_	*B. polymyxa*
8	BMRJ-8	+	+	+	+	+	+	_	+	+	_	+	_	_	_	*B. cereus*
9	BMRS-9	+	+	+	+	+	+	_	+	+	+	+	_	_	+	*B. megaterium*
10	BMRP-10	+	+	+	+	+	+	_	+	+	_	+	_	+	+	*B. subtilis*

Phenotypic and biochemical Characterization of *Pseudomonas*

In morphological characteristic studies such as motility, and fluorescent pigment production and physiological characters such as catalase activity, hydrolysis of starch, hydrolysis of gelatin, Egg yolk reaction and casein hydrolysis and biochemical test of the different *Pseudomonas* isolates were studied and presented in Table 5.

Table 5. Phenotypic and biochemical Characterization of *Pseudomonas* isolates from maize Rhizosphere soil samples of Salem district

S. No	Name of the isolates	Gram reaction	Motility	Hydrolysis of starch	Hydrolysis of gelatin	Egg yolk reaction	Pigment production	Casein hydrolysis	Catalase test	Oxidase test	Indole test	Methyl red test	Citrate test	H_2S production	Probable of identification
1	PMRV-1	–	+	–	+	–	+	+	+	+	–	–	+	–	*Pseudomonas fluorescens*
2	PMRG-2	–	+	–	–	–	+	+	+	+	–	–	+	–	*P. putida*
3	PMRT-3	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. striata*
4	PMRV-4	–	+	–	–	–	+	+	+	+	–	–	+	–	*P. putida*
5	PMRA-5	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. fluorescens*
6	PMRS-6	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. fluorescens*
7	PMRT-7	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. fluorescens*
8	PMRJ-8	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. striata*
9	PMRP-9	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. fluorescens*
10	PMRP-10	–	+	–	+	–	+	+	+	+	–	–	+	–	*P. fluorescens*

Table 6. Characterization of *Gluconacetobacter* isolates from root sample of maize

S.No.	Name of the isolates	Gram reaction	Shape	Nitrate reductase activity	Motility	Catalase activity	Oxidase activity	H_2S formation
1	GdMRV-1	Negative	Rod shaped	–	+	+	+	+
2	GdMRG-2	Negative	Rod shaped	–	+	+	+	+
3	GdMRT-3	Negative	Rod shaped	–	+	+	+	+
4	GdMRV-4	Negative	Rod shaped	–	+	+	+	+
5	GdMRA-5	Negative	Rod shaped	–	+	+	+	+
6	GdMRS-6	Negative	Rod shaped	–	+	+	+	+
7	GdMRT-7	Negative	Rod shaped	–	+	+	+	+
8	GdMRJ-8	Negative	Rod shaped	–	+	+	+	+
9	GdMRP-9	Negative	Rod shaped	–	+	+	+	+
10	GdMRP-10	Negative	Rod shaped	–	+	+	+	+

The results showed that all the isolates were gram negative, motile and produced fluorescent pigment. In biochemical characterization all the strains positively responded for casein hydrolysis, catalase test, oxidase test and citrate utilization test and negative for hydrolysis of starch, Egg yolk reaction, indole, methyl red and H_2S production. Six strains positively responded for gelatin hydrolysis they belonged to *Pseudomonas fluorescens* while the *P. striata* strains also positively responded to gelatin hydrolysis and remaining two isolates were negatively responded and belonged to *P. putida*.

The study revealed that the universal occurrence of plant growth promoting bacteria with mederate to very good load as influenced by soil environmental conditions, root exudates and rhizodeposits in the rhizosphere of maize samples collected from different locations from Salem district, Tamil Nadu. The present study clearly revealed that the ten different isolates of *Pseudomonas* obtained from maize rhizosphere soils, six isolates belonged to *P. fluorescens*, two isolates belonged to *P. striata* and two isolates belonged to *P. putida* in the maize rhizosphere soil samples.

The present study showed similar trends to the findings i.e. presence of *Pseudomonas* and *Bacillus* species in the rhizosphere will highly influence the phosphorous availability to the host plants (Kannaiyan, 2000b and Mishra *et al* 2011). Pazhaniraja and Prabudoss, (2014) observed effective influence of PGPB organisms *Azospirillum*, *Pseudomonas*, *Bacillus* and *Gluconacetobacter* on the growth, development and yield of sugarcane – *Saccharum officinarum*.

Characterization of *Gluconacetobacter* isolates

The ten *G. diazotrophicus* isolates were confirmed by performing characterization tests *viz.,* gram reaction, motility,

catalase activity, oxidase activity, nitrate reductase activity, hydrogen sulphide formation and growth under different conditions (Table 6).

Gram reaction

All the ten isolates were gram negative since they developed red coloured cells after gram reaction. When observed under phase contrast microscope, they were rod shaped with rounded ends and red in colour.

Motility

All the ten isolates showed movement when observed in Hanging Drop Technique using a cavity slide.

Oxidase test

A deep violet colour was developed by all ten isolates as a result of presence of oxidase enzyme.

Catalase test

Catalase activity was positive in all the ten isolates were produced bubbles of oxygen with 3 per cent hydrogen peroxide solution.

Hydrogen sulphide formation

Development of black precipitate was recorded by all the ten isolates and it confirmed the formation of hydrogen sulphide. All the ten isolates of *G. diazotrophicus* produced yellow to dark yellowish orange coloured sub surface pellicles on semisolid LGI and acetic LGI medium. They developed brown pigmented colonies on potato infusion agar medium. This was in accordance with the findings of Cavalcante and Dobereiner (1988). No nitrate reductase activity was observed in the isolates (Boddey *et al.,* 1995). The isolates were gram negative and rod shaped as described by Cavalcante and Dobereiner (1988). The tested isolates were motile, catalase and oxidase positive activities exhibiting the reactions. All the isolates produced hydrogen sulphide. The isolates formed smooth, initially small white colonies, which became yellow, orange and finally dark orange on LGI and acetic LGI plates. The diameter was around 2-3 mm, but in potato infusion agar plates diameter was 4-5mm and colonies were chocolate brown with light coloured margins (Cavalcante and Dobereiner, 1988; Rojas and Mellado, 2003).

The yellow colour of the isolate is due to assimilation of bromothymol blue in the medium and strong acid production (Boddey *et al.,* 1991; Sathyan and Thangaraju, 2003). The natural occurrence and colony characters were extensively studied in India by Muthukumarasamy *et al.* (2005).

Future study

The successful isolation of PGPB organisms from rhizosphere and root of maize creates interest to find out their plant growth promoting nature by in open environment as bioinoculants.

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