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Screening of suitable plant growth promoting rhizobacteria in maize based intercropping system

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Abstract
Under present investigation 48 PGPR isolates were isolated from different rhizotic zones of maize based intercropping system by using different media from twelve different site of Bihar including diara belt. Laboratory and pot experiments were conducted at Deptt. Of Microbiology, F.B.S. & H, RAU, Pusa during Rabi 2008-09, PGPR isolates are coded for *Azospirillum* spp. as AZS_1 to AZS_{12}, *Azotobacter* spp. as AZT_1 to AZT_{12}, *Pseudomonas* spp. as PSD_1 to PSD_{12} and P-solubilizing bacteria spp. as PSB_1 to PSB_{12}. These isolates were screened on the basis of seed germination, production of IAA, P-solubilization activity, antifungal activity, and nitrogenase activity for the formulation of microbial consortium.

Nitrogenase activity (3.15 mole C_2H_4/g/hr), mycorrhizal infection (80%), MPN of *Azospirillum* spp. (0.35 x 10^5/g) and population of P-solubilization bacteria spp. (10.50 x CFU x 10^5/g) were found maximum in isolates from Hazipur (diara belt) while population of *Azotobacter* spp. was maximum in Danapur (diara belt). Among the isolates, PSD_6 exhibited very high antifungal activity (% inhibition 83.83). Isolate AZT_4 showed maximum response against root and shoot length (6.75 and 4.72 cm) and very high response against Nitrogenase activity. *Azospirillum* spp. isolate AZS_6 showed best response against seed germination test and nitrogenase activity (114.48 mole C_2H_4/mg protein/hr) PSB_4 isolate was identified for maximum response against seed germination test and also showed very high P-solubilization activity (75.67%).

Keeping in view of experimental findings, PGPRs of diara belt are extremely diversified and perform well in stress condition. They are also competitive in nature and efficient in nitrogen fixing, P-solubilization and producing plant growth hormones. Microbial consortium improves crop growth and increase biomass production. Hence, it may be concluded that PGPRs (PSD_6, AZS_6 & AZT_4 are best microbial Inoculant in all respect than that of others which is very significant not only in growth parameters but also in maintaining soil health for sustainable crop production.

Keywords: Screening, rhizobacteria, intercropping, maize, PGP

Introduction
The rhizobacteria that are beneficial to plant are called plant growth promoting rhizobacteria (PGPR). The term PGPR was first proposed by Kloepper and Schroth (1978) [20] to describe a subset of rhizobacteria which induce increased plant growth after inoculation to seeds. Kloepper (1993) [19] and Cattelen *et al*. (1999) [7] indicated that different strains of PGPR can increase crop yields, control root pathogens, increase resistance against foliar pathogens, promote legume nodulation and enhance seedling emergence. PGPR group of bacteria actively colonize plant roots and increase plant growth and yield (Wu *et al*. 2005) [45]. It may benefit the target plant by causing plant growth promoting and also a source of biological disease

control. Growth promoting activity has been reported in strains belonging to several genera, such as *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia serratia* and *Bacillus* (Kloepper 1993; Glick and Bashan 1997, Bashan, *et al*. 2004) [19, 10, 5]. Several mechanisms have been postulated to explain the role of PGPR as plant growth, stimulator which can be categorized as direct or indirect promotion. Direct promotion occurs due to ability of targeted strains to produce or change the concentration of phytohormones, like IAA (Mordukhova *et al*. 1991) [31]; Cytokinins (Tien *et al*. 1979) [42], Ethylene (Arshad and Frankenberger 1991, Glick, 1995) [3, 11] and N_2-fixation by some of the strain (Boddy and Dobereiner, 1995, Mrekovacki and Milic, 2001; Salantur *et al*., 2006) [6, 33]. Indirect promotion like antagonism against phyto-Pathogenic microorganisms or deleterious bacteria (Kloepper 1993; Glick 1995; Lutenberg *et al*. 1991) [19, 11, 28] and solubilisation of mineral phosphate and other nutrients (Cattelen *et al*. 1999) [7].

Numerous plant growth promoting rhizobacteria of the genus *Pseudomonas, Bacillus* (PSB), *Arthrobacter, Azospirillum, Klebsiella, Azotobacter* and *Enterobacter* have been isolated from the rhizosphere of various crops and have been evaluated for their synergistic effect on plant growth (Kloepper *et al*. 1992) [23]. Egamberdiyeva. D. (2007) studied the rhizosphere and phyllosphere bacteria isolated from wheat and peas and examined for their plant growth promoting properties. Bacterial strains were identified as *Pseudomonas, Bacillus* and *Microbacterium* species. After inoculation with effective bacterial strains, the root and shoot growth, and nodulation of peas increased. Gholami *et al*. (2009) [13] also reported that under *in vitro* condition seed treatment with PGPR strains improved seed germination, seedling vigour, seedling emergence and seedling stand over the control in maize.

In view the importance of PGPRs the present study has been taken up to explore the possibility of formulating microbial consortium from out of the microbial diversity in maize rhizosphere when grown either as a mono-crop or intercropped with others.

Materials and Methods

This chapter deals with the description of the material used and the methods or techniques adopted during the course of investigation.

1. Site selection

To study PGPR diversity and selection of suitable and efficient autochthonous PGPR, sites were selected from different location of fertilized and unfertilized maize field of Diara belt (Koshi and Ganga river belt) and fertilized field of maize based intercropping system.

Maize - without fertilizer application fields
Maize + Legume - Fertilizer application fields
Maize + Spices - Fertilizer application fields
Maize - Fertilizer application fields

Rhizosphere Soil (0-15cm) and plant sample were collected from above locations, and brought to laboratory. Bacteria were isolated from different rhizotic zones i.e. rhizosphere, rhizoplane, soil and roots.

2. Isolation of Plant Growth Promoting Rhizobacteria (PGPR)

Different PGPR isolates were isolated by using selective or non-selective media. 10 g soil from the rhizosphere of host plant from selected site were taken and prepared 10 fold dilution series up to 10^{-6} by serial dilution method. 1 ml suspension from different dilutions (10^{-2} to 10^{-6}) were poured on plates containing respective media. The suspensions were spread on the plate by using sterilized spreader under aseptic condition.

2.1 Medium for PGPR

Different selective and non-selective media were used for isolation of Plant Growth Promoting Rhizobacteria (PGPR). Selective media used were King' B (KB) for *Pseudomonas* spp. Pikovskaya agar (PKV) for PSB, Jensen's medium (JEN) for *Azotobacter* spp., *Pseudomonas fluorescens* agar (PFA) for *Pseudomonas* spp. and N-free malate medium (Nfb) for *Azospirillum* spp. isolates and Non-selective media used for PGPR were nutrient agar (NA), Potato dextrose agar (PDA) and soil extract agar (SEA). Seeds of Maize CV Laxmi were obtained from All India Coordinated Maize Improvement Project (AICMIP), Department of Genetic and Plant Breeding, Tirhut College of Agriculture, Dholi, R.A.U., Pusa, Samastipur, Bihar.

2.1.a) Isolation of *Pseudomonas* isolates from King's B (KB) medium (King *et al*. 1954) [22]

Proteose Peptone - 0.20 g
Glycerol - 0.10 g
K_2HPO_4 - 1.5 g
$MgSO_4.7H_2O$ - 1.5 g
Agar - 0.15 g
pH - 7.2

Pseudomonas spp. isolates were isolated by serial dilution and plate count method. Ten grams of soil sample were transferred to 90 ml sterile distilled water in 250ml Erlenmeyer conical flask and mixed thoroughly by shaking the flask for 5 minutes. Serial dilutions of the suspension were prepared to obtained dilution upto 10^{-4}. One ml suspension from 10^{-4} dilution was plated in sterilized petri plate containing King's B media. These isolates were coded as PSD_1 to PSD_{12} (Table 2). Triplicate petri dishes for each sample were incubated at 28 $\pm$ 2 ^oC for 48hr and observe for yellow green pigment and fluorescence under U.V. light. All such colonies of bacteria were picked up and purified by streaking on solid media. Finally the pure isolates were grown on agar slants and maintained by sub-culturing and stored at 4 ^oC throughout the experimentation. Aseptic conditions were followed wherever needed.

Isolation of Phosphate Solubilizing Bacteria (PSB) from Pikovskayas medium, modified by (Sundara Rao and Sinha, 1963) [39]

Glucose - 10.0 g
$Ca_3(PO_4)_2$ - 5.0 g
$(NH_4)_2SO_4$ - 0.5 g
KCl - 0.2 g
$MgSO_4.7H_2O$ - 0.1 g
$MnSO_4$ - Trace
$FeSO_4$ - Trace
Yeast Extract - 0.5 g
Agar - 15.0 g
Distilled water - 1000 ml

Phosphate solubilizing bacteria isolates were isolated by serial dilution plating count method. Ten grams of soil sample was transferred to 90 ml sterile distilled water in 250 ml Erlenmeyer conical flask, and mixed thoroughly by shaking

the flask for 5 minutes. Serial dilution of the suspension was prepared to obtained dilution upto 10^{-4}. One ml suspension from 10^{-4} dilution was plated in sterilized petri plate with Pikovskaya's media. Triplicate Petri dishes for each sample were incubated for a week at 28 ± 2 ºC. Presence of clear 'Halo' Zone around individual colonies were taken as criteria for solubilisation of $Ca_3 (PO_4)_2$ by these isolates. All such colonies of bacteria were picked up and purified by streaking on solid media. Finally the pure isolates were grown on agar slants and maintained by sub-culturing and stored at 4 ºC throughout the experimentation. Aseptic conditions were followed where ever needed. These isolates were coded as PSB_1 to PSB_{12} (Table 2).

2.1.c) Isolation of *Azotobacter* spp. isolates from Jensen's medium

Sucrose	- 20.0 g
K_2HPO_4	- 1.0 g
$MgSO_4.7H_2O$	- 0.5 g
NaCl	- 0.5 g
$FeSO_4$	- 0.1 g
$CaCO_3$	- 2.0 g
Agar	- 15.0 g
Distilled water	- 1000 ml

Azotobacter spp. was isolated by serial dilution and plat count method. Ten grams of soil sample transferred to 95 mL sterile distilled water and mixed thoroughly by shaking the flask for 5 minutes. Serial dilutions of the suspension was made using sterile distilled water. One ml samples from the appropriate dilutions (10^{-2} to 10^{-6}) were spread evenly over cooled agar medium in petri-plates. The plates were incubated at 28 ± 2 ºC in an incubator for 3-4 days. *Azotobacter* colonies appear as flat, soft, milky and mucoid on agar plates (Table 3). All such colonies of bacteria were picked up and purified by streaking on solid media. Finally, the pure isolates were grown on agar slants and maintained by sub-culturing and stored at 4 ºC. Aseptic conditions were followed where ever needed.

2.1.d) Isolation of *Azospirillum* from root of maize Nitrogen free bromothymol blue medium (Dobereiner *et al.*, 1976) [8]

Malic acid	- 5.0 g
KOH	- 4.0 g
K_2HPO_4	- 0.5 g
$FeSO_4.7H_2O$	- 0.05 g
$MnSO_4. H_2O$	- 0.01 g
$MgSO_4.7H_2O$	- 0.1 g
NaCl	- 0.02 g
$CaCl_2$	- 0.01 g
Na_2MO_4	- 0.002 g
Distilled water	- 1000 ml
Bromothymol blue (0.5% alcoholic solution)	- 2.0 ml
Agar	- 1.75 g
pH adjusted to	- 6.5-7.0

For growing *Azospirillum* in liquid media, Nitrogen free bromothymol blue was used. Half centimetre long root pieces were surface sterilized in 70 per cent alcohol for 3-5 seconds. The root pieces were repeatedly washed in phosphate buffer (pH -7.0). The surface washed root were screw cap tube containing semi-solid, nitrogen free sodium malate medium. The tubes were incubated at 35 ºC for 4-5 days. Growth of *Azospirillum* was indicated by the formation of white pellicles 2-4 mm below the surface of the medium. The culture was purified by repeated transfer into semi-solid sodium malate medium in small screw-cap tubes. These isolates were codded as AZS_1 to AZS_{12} (Table 2).

Enumeration of *Azospirillum* by MPN method

The population of *Azospirillum* spp. in soil as well as root were enumerated employing MPN method. This method based upon the pattern of Positive and negative growth of *Azospirillum* in the tube inoculated with a consecutive series of dilutions of the soil/root samples. Based on the number of positive tube the population estimated with the help of MPN table (Okon *et al.* 1977) [34].

Calculation

$$\text{Population of } \textit{Azospirillum}\ g^{-1} \text{ of dry soil/or root} = \frac{\text{MPN index x Dilution factor}}{\text{Dry weight of the sample}}$$

Table 2: Isolates of PGPR from different locations under maize based intercropping system

Location		Maize based intercropping system	PGPR isolates and Coded Name			
			King' B Medium	NFB Medium	Jensen Medium	Pikovskaya Medium
			Pseudomonas spp. isolates	*Azotobacter* spp. isolates	*Azospirillum* spp. isolates	PSB isolates
1.	Saidpur	Maize	PSD_1	AZT_1	AZS_1	PSB_1
2.	Dholi farm	Maize	PSD_2	AZT_2	AZS_2	PSB_2
3.	Harpur	Maize+ Legume	PSD_3	AZT_3	AZS_3	PSB_3
4.	Danapur (Diara belt)	Maize + Legume	PSD_4	AZT_4	AZS_4	PSB_4
5.	Pusa farm	Maize	PSD_5	AZT_5	AZS_5	PSB_5
6.	Hazipur (Diara belt)	Maize	PSD_6	AZT_6	AZS_6	PSB_6
7.	Pusa farm	Maize+ Legume	PSD_7	AZT_7	AZS_7	PSB_7
8.	Babupur	Maize	PSD_8	AZT_8	AZS_8	PSB_8
9.	Kusiargaon	Maize + Spices	PSD_9	AZT_9	AZS_9	PSB_9
10	Mehsi	Maize	PSD_{10}	AZT_{10}	AZS_{10}	PSB_{10}
11.	Baisai	Maize + Spices	PSD_{11}	AZT_{11}	AZS_{11}	PSB_{11}
12.	Garhia	Maize + Spices	PSD_{12}	AZT_{12}	AZS_{12}	PSB_{12}

Morphological and biochemical characterisation of PGPR isolates

Standard method as describe by Vincent (1970) was employed for gram straining. Colony characters viz., colour form, elevation, margin, and surface texture and density form of growth in agar and surface growth in nutrient broth. The morphological studies of the bacteria were done according to the procedure given in Manual of Microbiological methods, Society of American Bacteriologists, (1957).

Biochemical tests (gram reaction, catalage and oxidase tests)

Biochemical test of PGPR isolates were performed according to procedure given in Manual of Microbiological method. Society of American Microbiologist (1957).

3. Characterization of PGPR

Pseudomonas spp. (PSD_1... PSD_{12}), *Azotobacter* spp. (AZT_1... AZT_{12}), *Azospirillum* spp. (AZS_1... AZS_{12}) and Phosphate solubilizing bacteria spp. (PSB_1... PSB_{12}) were screened for their plant growth promotion activities by bioassayed attributes like seed germination test, nitrogenase activity, production of IAA, Phosphate solubilizing and antifungal activity (Table 8, 11, 14 and 17).

3.1 Seed germination test

The selected Plant Growth Promoting Rhizobacteria isolates were bioassayed for their ability to promote/inhibit seedling growth using seed germination test (Shende *et al*. 1977). The seeds (cv. Laxmi) were surface sterilized with 0.1 per cent $HgCl_2$ for 3 minutes and then 70% ethyl alcohol for 5 min. and finally successive washed with sterile distilled water. All the surface sterilized seeds were presoaked in sterile distilled water over night prior to plating. Ten seeds were transferred aseptically on to 0.8% water agar.

3.2 Acetylene reduction activity

3.2.1 Acetylene reduction activity in Maize Roots

Procedure describe by Sindhu *et al*. (1986) [40] was followed for the measuring nitrogenase activity of freshly uprooted Maize roots. One gram of maize roots was placed in 15 ml capacity glass nursing tube/bottle, closed with suba seals. Ten per cent air in tubes were replaced by C_2H_2 (air: C_2H_2: 9:1). The tubes were incubated at 28 ºC for 1hr and C_2H_4 formed was determined by gas chromatograph (A-MIL NUCON Model 5700) using porapak-To columns (2m length), flame ionization detector (FID) oven temperature 110 ºC, detector 140 ºC and injector 130 ºC. Flow rate of carrier N_2 was maintained at 35 ml per min. Attenuation and sensitivity were adjusted to 32 and 100, respectively. Before analysis of test samples, a calibration curve using standard ethylene was prepared and a standard curve was plotted by taking volume of ethylene injected against area for ethylene in chromatogram. ARA was expressed as µ mole C_2H_4 h^{-1} g^{-1} fresh root.

$$\text{A.R.A.} = \frac{\text{Area of the peak x Volume of the flask x 0.006}}{\text{Volume of gas injected into c.c. x hrs of incubation x fresh weight of root sample}}$$

A.R.A. is expressed as n moles of ethylene produced g^{-1} fresh weight of root hr^{-1}.

3.3 Procedure of IAA test

Inoculated loopful of culture in 5 ml of selected broth media (for Jensen' broth-*Azotobacter* isolates, N-free malate-*Azospirillum* isolates King's B-broth-*Pseudomonas* isolates, Pikovaskays broth-P-Solubilizing bacteria isolates were taken for IAA test. Sterilized tryptophan @ 100 µg mL^{-1} was amended each tube. These tubes were incubated for 5 days at 28 ºC on a rotating shaker. After that 1 ml aliquot was taken in an Eppendorf tube and centrifuge at 10,000 RPM for 10 minutes. Supernatant aliquot was transferred to clean glass tube and 4 ml of IAA reagent was added to it Samples were incubated for 30 minutes at room temperature to allow the colour (pink) to be developed.

Reagents

1. IAA Reagents

$FeCl_3$ (0.5 M)	- 15 ml
Conc. H_2SO_4 Sp.gr. (1.84)	- 300 ml
Distilled water	- 500 ml

2. IAA stock solution

IAA 100 µg/ml in 50% ethanol.

3.4 Phosphate Solubilisation test

All bacterial isolates were grown in Pikovskaya's broth for 24-48 hr. A loopful of active culture was inoculated in petri plate containing Pikovskaya agar. Plates were incubated for 48hr at 30 ± 2 ºC for growth. Presence of clear halo zone around individual colonies were taken as criteria for solubilisation of $Ca_3(PO_4)_2$ by these isolates. Potential solubilisations were selected on the basis of solubilisation index (SI), which was the ratio of solubilisation zone to growth zone.

Phosphorus solubilisation on solid medium was measured in terms of solubilisation efficiency (S.E.) as

$$\text{SE \%} = \frac{\text{(Z-C)}}{\text{C}} \text{ x } 100$$

Where,

Z = Solubilisation Zone

C = Colony diameter.

Screening of PSB isolates

100 ml of Pikovskaya's media with TCP as sole source of Phosphate was dispensed in 250 ml, Erlenmeyer conical flasks in duplicate and sterilized at 15 1b P.S.I. for 20 minutes. The flasks were inoculated with 1 ml coulture suspension containing 10^7 bacterial cells. The inoculated flask was incubated at 30 ± 2 ºC under aseptic condition for 24 hrs. Uninoculated control was also maintained for comparison. After 15 days of incubation, available P was determined by chlorostannous reduced molybdophosphoric blue method (Jackson 1973) [18].

3.5 Antifungal activity test

Antifungal activity was determined by dual culture technique. Maize rhizosphere soil was collected from the maize experimental plot for isolation of fungal pathogens. The fungal pathogens were isolated from their rhizospher soil

sample. The cultures were purified by sub-culturing and maintained in PDA slant. For assaying bacterial isolates (*Pseudomonas* isolate spp. *Azotobacter* isolates, *Azospirillum* isolates and *P.* solubilizing bactering isolate) respective sterilized bacterial medium were poured into sterilized petri plate for the growth of bacterium inoculums. Bacterial isolates were streaked on the respective medium for the growth at 30 ºC + 2 ºC for 42 hr. plug of the Pathogens taken from growing edge of the fungal culture was inoculated at the centre of petri plate and plates were incubated for 5-7 days at 28º ± 2 ºC. For each test three replicate plates were used. The inhibition zone of the fungal activity in individual plate was quantified.

Results were expressed as mean of percentage of inhibition of the growth of the pathogen ± standard error in presence of the bacterial isolates. The per cent inhibition was compared with control (Pathogen alone) using the formula:

$$I = \left(\frac{C-T}{C}\right) x\ 100$$

Where,
I = % inhibition
C = Radial growth of Pathogen in control
T = Radial growth of Pathogens in Treatment

Result and Discussion

Keeping in view the significance of Plant Growth Promoting Rhizobacteria (PGPR) diversity in maize based intercropping system microbial isolates were screened. Experiments were conducted in laboratory selection of suitable PGPR isolate.

1. Nitrogenase activity and Mycorrhizal infection (%) of plant samples collected from different maize cropping system of Bihar

Nitrogenase activity and Mycorrhizal infection (%) of roots maize collected from different locations of Bihar, has been presented in Table 4. Nitrogenase activity of root of maize varies from 3.15 mole C_2H_4 g^{-1} hr^{-1} to 1.98 mole C_2H_4 $g^{-1}hr^{-1}$.

Highest nitrogenase activity was recorded in maize plant roots collected from Hazipur, diara belt (3.15 n mole C_2H_4 $g^{-1}hr^{-1}$), followed by Maishi, Saharsa, (3.08 n mole C_2H_4 $g^{-1}hr^{-1}$), while lowest (1.98 n mole C_2H_4 g^{-1} hr^{-1}) in Baisai village of Purnia.

Similarly highest Mycorrhizal infection per cent (80%) was also recorded in maize roots collected from Hazipur, diara belt followed by Danapur, diara belt (77.00%) and Maihsi village, Saharsa (68.00%) while lowest was found (42%) in Pusa farm Samastipur.

The highest nitrogenase activity and mycorrhizal infection found in Hazipur, diara belt might be due to unique microbial diversity and high ability to survive and adaptability of plant growth promoting rhizobacteria in such stress condition. Range of nitrogenase activity of maize roots, variety Laxmi grown in saline calcareous soil of Bihar as recorded by Rai and Hunt (1993) [14] and Yadav, *et al*. (1992) was in the same range as recorded in the present investigation.

4.1.2 Population of different PGPR of root and soil samples collected from maize cropping system of Bihar

Root Colonization of *Azospirillum* spp., *Azotobacter* spp., *Pseudomonas* spp. and P-solubilizing bacteria spp. isolated from different locations of Bihar has been presented in Table 5. Maximum MPN of *Azospirillum* spp.in root sample was recorded in Hazipur, diara belt (0.35 x 10^5) followed by Danapur, diara belt (0.28 x 10^5) while lowest was (0.07 x 10^5) in Saidpur village of Samastipur.

Maximum population dynamics of *Pseudomonas* spp. was recorded in Hazipur diara belt (5.42 x 10^5) followed by Danapur diara belt (4.84 x 10^5 CFU), while lowest (1.64 x 10^5 CFU) in Harpur Village of Samastipur.

Similarly maximum population of P-solubilizing bacteria spp. was found in Danapur diara belt (11.00 x 10^4 CFU) followed by Hazipur diara belt (10.50 x 10^4 CFU), while lowest (3.2 x 10^4 CFU) in Saidpur village of Samastipur.

Similar trends were also found in case of *Azotobacter* spp. Population dynamics. Maximum population was found in Danapur diara belt (8.20 x 10^4 CFU) followed by Hazipur diara belt (7.4 x 10^4 CFU), while lowest (4.20 x 10^4 CFU) in Saidpur village of Samastipur. This may be due to congenial environment for these bacteria. Similar observations were also observed by Rai and Hunt (1993) [14] and Yadav *et al*. (1991) [46], in roots of maize variety Laxmi grown in Saline calcareous soil of Bihar. Ramazan *et al*. 2007 [15] reported that the density of N_2-fixing organisms ranged between 1.4 x 10^5 CFU and 4.1 x 10^6 CFU in soil.

3 Characterization of PGPR isolates

3.1 Morphological, cultural and biochemical characteristics of *Pseudomonas* isolates

Twelve isolates of *Pseudomonas* spp. were isolated from different maize and maize based intercropping system of different locations, from rhizospheric, rhizoplane and surface soil. These isolates were identified on the basis of cultural, morphological and some biochemical test as describe in Bergey's Manual of systematic Bacteriology (1984) Table 6. The colonies of *Pseudomonas* spp. isolates were mostly yellow, orange and green fluorescent. The green fluorescent colour forming isolates were very similar to *Pseudomonas fluorescens*. Six isolates were found round colonies and six-isolates formed circular colonies. The elevations of colonies of isolates were raised type. Isolates were mucoid on King's B agar and exhibited fluorescence under UV light. The green fluorescent colour of the colony is a specialised character of *Pseudomonas* groups as describe in Bergey's Manual of Systematic Bacteriology, (1984). All the young culture of the isolates in the present study showed Gram negative reaction. All the isolates were positive for catalase test, and oxidase test. (Table 7).

Similarly characterised bacterial strains on the basis of morphological, biochemical and physiological test, including pigment production, on nutrient agar medium, the Gram reaction, catalase and oxidase production.

3.1.1 Screening of *Pseudomonas* spp. isolates

Plant growth promoting activities of PGPR isolates has been presented in table 8. Twelve identified PGPR isolates (PSD_1.....PSD_{12}) were screened on the basis of morphological and biochemical test for their plant growth promotion activities like seed germination, production of IAA, P-solubilization ability, antifungal activity and nitrogenase activity.

3.1.1.1 Seed germination

3.1.1.1.a Root length

Root length varies from 4.68 to 7.42 cm due to inoculation of *Pseudomonas* isolates, highest being due to PSD_6 (7.42 cm), which was at par with PSD_8 (7.14 cm) and PSD_{10} (7.24) and significantly superior with UIC.

3.1.1.1.b Shoot length

Shoot length varies from 3.54 to 5.60 cm due to inoculation of *Pseudomonas* isolates, highest being due to PSD_6 (5.60 cm), which was at par with PSD_8 (5.38 cm) and PSD_{10} (5.46) and significantly superior with UIC.

Significant increased in root and shoot length might be due to vary high production of IAA (Table 8). Many plant associated bacteria have the ability to produced plant growth regulator, indole 3 acetic acid and IAA may influenced root and shoot length (Patten and Glick 2000) [36].

3.1.2 Production of IAA

Laboratory experiment was conducted to asses the potential of various *Pseudomonas* isolates (PSD_1 to PSD_{12}), for auxin biosynthesis (Table 8). Intensity of pink colour indicated the range of IAA production, like low, medium, high and very high. Among twelve isolates, seven (7) isolates under low (very light) categories of IAA producer. Isolates PSD_4, PDS_7 and PSD_8 cames under medium categories, Isolate PDS_{10} cames under high categories, while isolate PSD_6 come under very high categories.

IAA a member of the group of phytohormones is generally considered to be the most important native auxin. It may function as important signal molecules in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Khalid *et al.* 2004; Ahmad *et al.* 2005; Picard and Bosco, 2005) [21, 1, 37]. Similarly, Leinhos and Vacek (1994) [29] reported a production of 1.6 to 3.3 mg auxin ml^{-1} filtrate by *Pseudomonas* isolated from wheat. Furthermore, Prikryl *et al.* (1985) [35] have reported production of IAA and some other auxins in liquid culture of *Pseudomonas cepacia* and *P. fluorescens* isolated from maize rhizosphere.

3.1.3 P-solubilization test

Solubilization efficiency of *Pseudomonas* isolates has been presented in table 8. *Pseudomonas* isolates PSD_4 and PSD_6 were found to be low (<35%) solubilisation efficiency while rest of the isolates were negative response against P-solubilisation test. Kundu *et al*. (2002) found 16 per cent of the total bacterial population in rhizosphere were found to be phosphorus solubilizer. The result of present study is in conformity with the result obtained by several workers (Gaur and Pareek, 1972, Kavimandan and Gaur, 1971, Singh *et al*. 2002 and Kumar and Yadav, 2005) [16, 27, 41, 26].

3.1.4 Antifungal activity test

Antifungal activity test of different *Pseudomonas* isolates has been presented in Table 8. Among the twelve *Pseudomonas* spp. isolates, two, isolates (PSD_8 and PSD_{10}) cames under high (70-80%), two isolates comes under low (<60% of inhibition) antifungal activity. However, PSD6 showed very high antifungal activity (83.3% inhibition). *P. fluorescens* (Pf 003) by inhibition of mycelial growth of *Rhizoctonia solani* in dual culture test was also observed by Reddy *et al*. 2007. Species of *Pseudomonas* are known to have a significant role in suppression of fungal pathogens apparently via the production of antibiotics (Maumafer *et al*. 1991, Hill *et al*. 1994, Dunne *et al*. 1998 and Thomashow and Weller 1988) and volatile compound (Vioiscard *et al*. 1989).

3.1.5 Nitrogenase activity

Nitrogenase activity of all *Pseudomonas* isolates were found to be negative Table 8.

3.2 Morphological cultural and biochemical characteristics of *Azotobacter* isolate (AZT_1.... AZT_{12})

Identified PGPR of *Azotobacter* isolates on the basis of colony morphology character and biochemical tests as describe in Bergey's Manual of systematic Bacteriology (1984) has been presented in table 9. A unique differentiating character of *Azotobacter* is ability to form pigments. The pigment was mostly black, brown, black brown and light and yellow brown. The black brown pigment forming colour was very similar to *Azotobacter chroococcum*. Out of twelve, four isolates were circular, five were irregular form and two were round. The elevations of colonies of isolates were either raised type or flat type. All the isolates in the present study showed Gram negative reaction. All the isolates were positive for catalase and oxidase test (Table 10). Similar observation was also recorded by Forbes *et al*. 1988 who characterised bacterial strains on the basis of morphological, biochemical test and physiological test, including pigment production, on nutrient agar medium, the Gram reaction, catalase and oxidase production and growth at 36 ^oC on N-free basal medium.

3.2.1 Screening of *Azotobacter* isolates

Twelve isolates of *Azotobacter* (AZT_1....AZT_{12}) were screened on the basis of colony characters and biochemical test for their plant growth promotion activities like seed germination, production of IAA, P-solubilization test, antifungal activity and nitrogenase activity had been presented in Table (11).

3.2.1.1 Seed germination

Significant variation was observed among different *Azotobacter* isolates with regard to influence on root and shoot length after 72hr of incubation.

3.2.1.1a Root length

Root length of maize was increased significantly by *Azotobacter* isolates AZT_4 (6.75 cm), AZT_6 (6.48 cm), AZT_8 (6.00 cm), AZT_{10} (5.84 cm), AZT_7 (5.80 cm), AZT_5 (5.60 cm), AZT_{12} (4.75 cm) and AZT_2 (4.62 cm) incomprision to UIC (3.82 cm).

3.2.1.1b Shoot length

Azotobacter isolates caused significant increased in length of maize AZT_4 (4.72 cm), AZT_6 (4.54 cm), AZT_8 (4.24 cm), AZT_{10} (4.10 cm), AZT_7 (4.10 cm), AZT_5 (3.92 cm), AZT_3 (3.64 cm) and AZT_9 (3.5 cm) incomprision to UIC (2.66 cm).

Significant increases in root and shoot length over UIC might be due to synthesis of IAA by *Azotobacter* isolates (Pattern and Glick 2000; Aslantas *et al*. 2007, Khalid *et al*. 2004; Ahmad *et al*. 2005) [36, 21, 1].

3.2.1.2 Production of IAA

Potential of various *Azotobacter* isolates for auxin biosynthesis has been presented in Table 11. Intensity of pink colour indicated the range of IAA production like low, medium, high and very high among twelve isolates. Six isolates comes under low ((+) very light) categories. Isolates AZT_5, AZT_8, AZT_{10} and AZT_6 comes under high categories (+++) while AZT_4 isolate comes under very high (++++) categories. Among them one isolate AZT_4 was found to be good producer of IAA. Production of IAA by *Azotobacter* has been reported by several workers (Picard and Bosco 2005) [37]. It has been reported that IAA production by PGPR can varies among different species and strains and it also influenced by culture conditions and substrate availability.

3.2.1.3 P-solubilization test
All the isolates of *Azotobacter* spp. showed negative response against P-solubilisation test (Table 11).

3.2.1.4 Antifungal activity
All the isolates of *Azotobacter* spp. were found to negative response against Antifungal activity test (Table 11).

3.2.1.5 Nitrogenase activity
Nitrogenase activity of *Azotobacter* isolates has been presented in table 11. Nitrogenase activity of *Azotobacter* spp. Varies from 105.42 to 20.35 mole C_2H_4 mg^{-1} protein hr^{-1}. Highest nitrogenase activity was recorded in AZT_4 followed by AZT_6 and AZT_{10}. Nitrogenase activity has been categorized vary high (AZT_4), higher (AZT_6, AZT_8, AZT_9, AZT_{10}, AZT_{11} and AZT_{12}) and medium (AZT_2, AZT_3, AZT_5 and AZT_7). On the basis of above observations *Azotobacter* isolates AZT_4 performed the best in all different plant growth promoting attributes. The result of present investigation is in conformity with results of the several workers (Vasundhara *et al*. 2002 and Li and Mac Rae 1991) [44, 30].

3.3 Morphological cultural and bio-chemical characteristics of *Azospirillum* isolates
Twelve PGPR isolates of *Azospirillum* spp. isolated from different locations were on the basis of cultural morphological and biochemical characters as describe in Bergey's Manual of systematic Bacteriology (1984) (Table 12). A characteristics growth of *Azospirillum* is indicated by the formation of white pellicles 2-4 cm below the surface of the medium. All the isolates showed white pellicle formation and after 72hr and colour changed to blue except AZS_6 (change blue to black). The colour change in tube blue to black in isolate AZS_6 was very close to *A. lipoferum.* All the isolates in the present study showed Gram negative reaction when subjected to differential staining. All the isolates were positive for catalase and oxidase test Table (13). Forbes *et al*. (1988) also characterised bacterial strains on the basis of morphological, biochemical test and physiological test, including pigment production, on nutrient agar medium, the Gram reaction, catalase and oxidase production and growth at 36 ^oC on N-free basal medium.

3.3.1 Screening of *Azospirillum* isolates
Twelve identified PGPR of *Azospirillum* spp. isolates (AZS_1... AZS_{12}) were screened on the basis of colony characters and bio-chemical tests for their plant growth promotion activities like seed germination, production of IAA, P-solubilization, antifungal activity and nitrogenase activity has been presented in Table 14.

3.3.1.1 Seed germination
Significant variations were observed among different isolates with regard to influence on root and shoot length after 72hr of incubation.

3.3.1.1a Root length
Root length of maize increased significantly in *Azospirillum* spp. isolates AZS_6 (6.84cm), AZS_{10} (6.68cm), AZS_8 (6.28 cm), AZS_4 (6.00cm), AZS_7 (5.95cm), AZS_5 (5.74cm), AZS_3 (5.48cm) and AZS_4 (5.35cm) incomprision to UIC (4.00cm).

3.3.1.1b Shoot length
Shoot length of maize increased significantly in *Azospirillum* spp. isolates AZS_6 (4.88 cm), AZS_{10} (4.66 cm), AZS_8 (4.66 cm), AZS_4 (4.3 cm), AZS_7 (4.28 cm), AZS_5 (4.0 cm), AZS_3 (3.84 cm) and AZS_9 (3.72 cm) over UIC.84 cm).
Among all the tested isolates of *Azospirillum* the highest value of root and shoot recorded in AZS_6, might be due to highest production of IAA (Table 14). Many plant associated bacteria have the ability to produce the plant growth regulator, indole 3 acetic acid and IAA may influence root and shoot length (Patten and Gilick 2000, Aslantas *et al*. 2007) [36, 4].

4.1.3.3.1.2 Production of IAA
Intensity of pink colour indicated the potential of various *Azospirillum* spp. isolates for auxin bio synthesis table 14. On the basis of intensity of Pink colour *Azospirillum* isolates have been categories very high (AZS_6), medium (AZS_4, AZS_8, AZS_{10}) and rest comes under low (AZS_1, AZS_2, AZS_3, AZS_7, AZS_{11} and AZS_{12}). Among them one isolate (AZS_6) was found to be good producer of IAA.
Production of IAA by *Azospirillum* has been reported by several workers (Picard and Bosco 2005) [37]. It has been reported that IAA production by PGPR can varies among different species and strains and it also influenced by culture conditions and substrate availability.

3.3.1.3 P-solubilization test
All the isolates of *Azospirillum* spp. were found to negative response against P-solubilisation Table 14.

3.3.1.4 Antifungal activity test
All the isolates of *Azospirillum* spp. were found to negative response against Antifungal activity test Table 14.

3.3.1.5 Nitrogenase activity
Nitrogenase activity of *Azospirillum* spp. varies from 114.48 to 18.48 n mole C_2H_4 mg^{-1} protein hr^{-1} (Table 14). Highest nitrogenase activity was recorded in AZS_6, followed by AZS_4 and AZS_{10}. Nitrogenase activity has been categorized very high (AZS_6), high (AZS_4, AZS_7, AZS_9 and AZS_{10}) medium (AZS_1, AZS_3, AZS_5, AZS_8, AZS_{11} and AZS_{12}) and low (AZS_2). On the basis of above observations *Azospirillum* isolate AZS_6 performed best in all different plant growth promoting attributes.
The result of the present investigation was conformity with result of the worker (Rai and Hunt 1993) [14].

3.4 Morphological cultural and bio-chemical characteristics of P-solubilizing isolates
Twelve PGPR isolates of P-solubilizing bacteria spp. isolated from different location were identified on the basis of cultural morphology and bio-chemical characters as describe in Bergey's manual of systematic Bacteriology (1984) Table 15. A characteristic growth of P-solubilizing bacteria were indicated by the presence of transparent clear 'Halo' zone around individual colonies. All the isolates showed 'Halo zone' around individual colony and positive for catalase and oxidase test. All the isolates in the present study showed Gram positive when subjected to differential staining (Table 16).
Based on results obtained with regard to extent and period of growth on Pikovskaya's medium, the isolates of P-solubilizing spp. were grouped in to poor, medium, high and very high growth, categories. Isolate PSB_4 come under very high growth after 72 hr.

3.4.1 Screening of P-solubilizing isolates

Twelve identified PGPR of P-solubilizing isolates (PSB_1..... PSB_{12}) were screened, on the basis of colony characters and bio-chemical test for their plant growth promotion activities like seed germination, production of IAA, P-solubilization, antifungal activity and nitrogenase activity Table (17).

3.4.1.1 Seed germination

Significant variations were observed among different PGPR isolates with regard to influence on root and shoot length after 72hr of incubation.

4.1.3.4.1.1a Root length

Root length of maize increased significantly in P-solubilizing bacteria isolates PSB_4 (6.40 cm) PSB_6 (6.38 cm), PSB_8 (5.74 cm), PSB_{10} (5.62 cm) PSB_7 (5.44 cm) PSB_5 (5.32 cm), PSB_3 (4.84 cm), PSB_9 (4.78 cm) and PSB_{12} (4.52 cm) over UIC (3.40 cm).

3.4.1.1b Shoot length

Shoot length of maize increased significantly in P-solubilizing bacteria isolates PSB_4 (4.46 cm) PSB_6 (4.48 cm), PSB_8 (4.02 cm), PSB_{10} (3.94 cm) PSB_7 (3.8 cm) PSB_5 (3.72 cm), PSB_3 (3.38 cm), PSB_9 (3.34 cm) and PSB_{12} (3.16 cm) over UIC (2.38 cm). Among the tested P-solubilizing isolates, the highest value of root and shoot length were recorded in PSB_4.

3.4.1.2 Production of IAA

All the isolates were found to negative response against production of IAA Table 17.

3.4.1.3 P-solubilization test

Phosphate solubilization efficiency (%) of different isolates ranged from 39.33 to 75.63%, highest being due to PSB_4 (75.67%) followed by PSB_9 (70.00%), PSB_{10} (69.33%) and lowest in UIC (39.33%) Table 17.

The production of Organic acids by Phosphate solubilizing micro-organism play major role in transformation of inorganic P-compound in the medium has been reported by several workers (Gaur and Pareek 1972, Anu and Kundu, 2005, Kumr and Yadav 2005) [16, 2, 26].

3.4.1.4 Antifungal activity

All the isolates were found to negative response against antifungal activity Table 17.

3.4.1.5 Nitrogenase activity

All the isolates were found to negative response against nitrogenase activity.

On the basis of above observations PSB_4 isolate performed best in all different plant growth promoting attributes.

Summary and Conclusions

The present investigation was under taken to isolate plant growth promoting rhizobacteria (PGPR) from twelve different diversified area of Bihar including, diara belt (Koshi and Ganga), where microbial diversity was maximum under maize based inter cropping system. Efforts were also made to screen the PGPRs for their plant growth promoting attributes like seed germination, production of I.A.A., P-solubilizing activity, antifungal activity and nitrogenase activity. The insertion of isolates for the formulation of microbial consortium and its effect on plant physiological parameters, total biomass production, nutrients uptake, microbial population on post-harvest soil were under taken. Selected four best isolates of PGPR *Pseudomonas* (PSD_6), P-solubilizer (PSB_4), *Azospirillum* (AZS_6) and *Azotobacter* (AZT_4) were examined alone or in the possible combinations with other isolates. The pot experiment was conducted at the Department of Microbiology, F.B.S. & H., R.A.U., Pusa in completely randomised design (CRD) with three replications during Rabi 2008-09 or a very little work on this aspect has been done in Bihar state.

A brief summary of the result on each aspect has been presented here in following paragraphs.

1. A total of 48 plant growth promoting rhizobacteria (PGPR) were isolated from different rhizotic zones of maize based inter cropping system using selective and non-selective media from twelve different area of Bihar including diara belt. Out of these twelve isolates each of *Azospirillum*, *Azotobacter*, *Pseudomonas* and P-solubilizing bacteria were coded as AZS_1 to AZS_{12}, AZT_1 to AZT_{12}, PSD_1 to PSD_{12} and PSB_1 to PSB_{12} respectively.
2. Highest nitrogenase activity 3.15 mole $C_2H_4g^{-1}hr^{-1}$), Mycorrhizal infection (80%), Maximum MPN of *Azospirillum* spp. (0.35×10^5) and maximum population of P-solubilizing bacteria (10.50×10^4 CFU) were recorded in sample collected from Hazipur (diara belt), while maximum population of *Azotobacter* spp. (8.20×10^4 CFU) and *Pseudomonas* spp. (5.42×10^5 CFU) were recorded in Danapur (diara belt).
3. On the basis of colony character and biochemical test *Pseudomonas* isolates coded as PSD1 to PSD_{12} were mostly yellow to green fluorescent colonies, circular to round form and raised type. Isolate PSD_6 was identified as fast growing up to 72hr on King's medium.
4. All the *Pseudomonas* isolates were Gram negative and gave positive response against catalase and oxidase test.
5. *Pseudomonas isolate* PSD_6 has been selected on the basis of seed germination root length (7.42 cm) and shoot length (3.54 cm), antifungal activity (83.83% inhibition),
6. On the basis of screening test with regard to seed germination (root length 6.75 cm and shoot length 4.72 cm) and nitrogenase activity (105.42 mole C_2H_4/mg protein/hr) *Azotobacter* isolate AZT_4 was selected out of twelve isolates (AZT_1.....AZT_{12}).
7. All the *Azospirillum* isolates showed white pellicle formation in nitrogen free bromothymol blue media. These isolates were gram negative, spiral shaped, and positive against catalase and oxidase test.
8. On the basis of screening test with regard to seed germination root length (6.84 cm) and shoot length (4.88 cm) and nitrogenase activity (114.48 mole C_2H_4/mg protein/hr) *Azospirillum* isolate AZS_6 was selected out of twelve isolates (AZS_1... AZS_{12}).
9. All the isolates of P-solubilizing bacteria (PSB_1 to PSB_{12}) showed transparent 'Halo zone' around individual colony. These isolates were gram negative, rod shaped and showed positive response against gram reaction, catalase and oxidase test.
10. On the basis of screening test with regard to seed germination root length (6.40 cm) and shoot length (4.48 cm) and solubilizing efficiency (75.67%) P-solubilizing bacteria PSB_4 was selected out of twelve isolates (PSB_1..... PSB_{12}).

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