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

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Abstract

Under present investigation 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. PGPR isolates are coded for *Azospirillum* spp. as AZS₁ to AZS₁₂, *Azotobacter* spp. as AZT₁ to AZT₁₂, *Pseudomonas* spp. as PSD₁ to PSD₁₂ and P-solubilizing bacteria spp. as PSB₁ to PSB₁₂. 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 C₂H₄/g/hr), mycorrhizal infection (80%), MPN of *Azospirillum* spp. (0.35 x 10⁵/g) and population of P-solubilization bacteria spp. (10.50 x CFU x 10⁵/g) were found maximum in isolates from Hazipur (diara belt) while population of *Azotobacter* spp. was maximum in Danapur (diara belt). Among the isolates, PSD₆ exhibited very high antifungal activity (% inhibition 83.83). Isolate AZT₄ showed maximum response against root and shoot length (6.75 and 4.72 cm) and very high response against Nitrogenase activity. *Azospirillum* spp. isolate AZS₆ showed best response against seed germination test and nitrogenase activity (114.48 mole C₂H₄/mg protein/hr) PSB₄ 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 (PSD₆, AZS₆ & AZT₄) 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: Suitable plant growth, rhizobacteria, maize based intercropping

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) [21] to describe a subset of rhizobacteria which induce increased plant growth after inoculation to seeds. Kloepper (1993) [22] and Cattelan *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) [42]. 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; Zhang *et al.* 1996, Glick and Bashan 1997, Ramamoorthy *et al.* 2002, Bashan, *et al.* 2004) [22, 44, 14, 45, 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) [27]; Cytokinins (Tien *et al.* 1979) [39], Ethylene (Arshad and Frankenberger 1991, Glick, 1995) [3, 13] and N₂-fixation by some of the strain (Boddy and Dobereiner, 1995, Mrkovacki and Milic, 2001; Salantur *et al.*, 2006) [6, 28, 46]. Indirect promotion like antagonism against phyto-Pathogenic microorganisms or deleterious bacteria (Kloepper 1993; Glick 1995; Lugtenberg *et al.* 1991) [22, 13, 26] and

solubilisation of mineral phosphate and other nutrients (Cattelan *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) ^[20]. Egamberdiyeva. D. (2007) ^[47] 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) ^[12] 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. 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 (0-15 cm) 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.

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 suspensions 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.

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. Pikovskay's 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.

Isolation of *Pseudomonas* isolates from King's B (KB) medium (King *et al.* 1954)

Proteose Peptone - 0.20 g

Glycerol - 0.10 g

K₂HPO₄ - 1.5 g

MgSO₄.7H₂O - 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 petriplate containing King's B media. These isolates were coded as PSD₁ to PSD₁₂. Triplicate petridishes for each sample were incubated at 28 ± 2 °C for 48 hr 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 °C throughout the experimentation. Aseptic condition were followed wherever needed.

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

Glucose - 10.0 g

Ca₃(PO₄)₂ - 5.0 g

(NH₄)₂SO₄ - 0.5 g

KCl - 0.2 g

MgSO₄.7H₂O - 0.1 g

MnSO₄ - Trace

FeSO₄ - 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 petriplate with Pikovskaya's media. Triplicate Petridishes for each sample were incubated for a week at 28 ± 2 °C. Presence of clear 'Halo' Zone around individual colonies were taken as criteria for solubilisation of Ca₃(PO₄)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 °C throughout the experimentation. Aseptic conditions were followed where ever needed. These isolates were coded as PSB₁ to PSB₁₂.

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

Sucrose - 20.0 g
 K₂HPO₄ - 1.0 g
 MgSO₄.7H₂O - 0.5 g
 NaCl - 0.5 g
 FeSO₄ - 0.1 g
 CaCO₃ - 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 were 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 °C in an incubator for 3-4 days. *Azotobacter* colonies appear as flat, soft, milky and mucoid on agar plates. 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 °C. Aseptic conditions were followed where ever needed.

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
 K₂HPO₄ - 0.5 g
 FeSO₄.7H₂O - 0.05 g
 MnSO₄. H₂O - 0.01 g

MgSO₄.7H₂O - 0.1 g
 NaCl - 0.02 g
 CaCl₂ - 0.01 g
 Na₂MO₄ - 0.002 g
 Distilled water - 1000 ml
 Bromothymol blue - 2.0 ml (0.5% alcoholic solution)
 Agar - 1.75 g
 pH adjusted to - 6.5-7.0

For growing *Azospirillum* in liquid media, Nitrogen free bromothymol blue was used. Half centimeter 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 °C 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 coded as AZS₁ to AZS₁₂ (Table 1).

Enumeration of *Azospirillum* by MPN method

The population of *Azospirillum* spp. in soil as well as root was 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) [29].

Calculation

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

Table 1: 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
		<i>Pseudomonas</i> spp. isolates	<i>Azotobacter</i> spp. isolates	<i>Azospirillum</i> 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. Kusiagao	Maize + Spices	PSD ₉	AZT ₉	AZS ₉	PSB ₉
10. Maihsi	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 characterization of PGPR isolates

Standard method as describe by Vincent (1970) [60] 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).

Characterization of PGPR

Pseudomonas spp. (PSD₁... PSD₁₂), *Azotobacter* spp. (AZT₁ ... AZT₁₂), *Azospirillum* spp. (AZS₁ ... AZS₁₂) and Phosphate solubilizing bacteria spp. (PSB₁ ... PSB₁₂) were screened for their plant growth promotion activities by bio assayed attributes like seed germination test, nitrogenase activity, production of IAA, Phosphate solubilizing and antifungal activity.

Seed germination test

The selected Plant Growth Promoting Rhizobacteria isolates were bio assayed for their ability to promote/inhibit seedling growth using seed germination test (Shende *et al.*, 1977) [61]. The seeds (cv. Laxmi) were surface sterilized with 0.1 per cent HgCl₂ 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 pre soaked in sterile distilled water over night prior to plating. Ten seeds were transferred aseptically on to 0.8% water agar.

$$\text{ARA} = \frac{\text{Area of the peak} \times \text{Volume of the flask} \times 0.006}{\text{Volume of gas injected into cc} \times \text{hrs of incubation} \times \text{fresh weight of root sample}}$$

A.R.A. is expressed as n moles of ethylene produced g⁻¹ fresh weight of root hr⁻¹.

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- Solubling bacteria isolates were taken for IAA test. Sterilized tryptophane @ 100 µg mL⁻¹ was amended each tube. These tubes were incubated for 5 days at 28 °C 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 color (pink) to be developed.

Reagents

1. IAA Reagents

FeCl₃ (0.5 M) - 15 ml
Conc. H₂SO₄ Sp.gr.(1.84) - 300 ml
Distilled water - 500 ml

2. IAA stock solution

IAA 100 µg/ml in 50% ethanol.

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 48 hr at 30 ± 2 °C for growth. Presence of clear halo zone around individual colonies were taken as criteria for solubilisation of Ca₃(PO₄)₂ by these isolates. Potential solubilization 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

Acetylene reduction activity

Acetylene reduction activity in Maize Roots

Procedure describe by Sindhu *et al.*, (1986) [36] 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 sub seals. Ten per cent air in tubes were replaced by C₂H₂ (air: C₂H₂: 9:1). The tubes were incubated at 28 °C for 1hr and C₂H₄ formed was determined by gas chromatograph (A-MIL NUCON Model 5700) using porapak - To columns (2m length), flame ionization detector (FID) oven temperature 110 °C, detector 140 °C and injector 130 °C. Flow rate of carrier N₂ 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 C₂H₄ h⁻¹ g⁻¹ fresh root.

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

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 lb P.s.i. for 20 minutes. The flasks were inoculated with 1 ml culture suspension containing 10⁷ bacterial cells. The inoculated flask was incubated at 30 ± 2 °C 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 moly phosphoric blue method.

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 rhizosphere 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* bacterium isolate) respective sterilized bacterial medium were poured into sterilized petriplate for the growth of bacterium inoculums. Bacterial isolates were streaked on the respective medium for the growth at 30 °C + 2 °C for 42 hr. plug of the Pathogens taken from growing edge of the fungal culture was inoculated at the centre of pteriplate and plates were incubated for 5-7 days at 28^o ± 2 °C. 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:

$$I = \left[\frac{C-T}{C} \right] \times 100$$

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.

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. Nitrogenase activity of root of maize varies from 3.15 mole C₂H₄ g⁻¹hr⁻¹ to 1.98 mole C₂H₄ g⁻¹hr⁻¹. Highest nitrogenase activity was recorded in maize plant roots collected from Hazipur, diara belt (3.15 n mole C₂H₄ g⁻¹hr⁻¹), followed by Maishi, Saharsa, (3.08 n mole C₂H₄ g⁻¹hr⁻¹), while lowest (1.98 n mole C₂H₄ g⁻¹hr⁻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) [33, 34] was in the same range as recorded in the present investigation.

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 Table1. Maximum MPN of *Azospirillum* spp. in root sample was recorded in Hazipur, diara belt (0.35 x 10⁵) followed by Danapur, diara belt (0.28 x 10⁵) while lowest was (0.07 x 10⁵) in Saidpur village of Samastipur

Maximum population dynamics of *Pseudomonas* spp. was recorded in Hazipurdiara belt (5.42 x 10⁵) followed by Danapur diara belt (4.84 x 10⁵ CFU), while lowest (1.64 x 10⁵ CFU) in Harpur village of Samastipur.

Similarly maximum population of P-solubilizing bacteria spp. was found in Danapur diara belt (11.00 x 10⁴ CFU) followed by Hazipur diara belt (10.50 x 10⁴ CFU), while lowest (3.2 x 10⁴ 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 10⁴ CFU) followed by

Hazipurdiara belt (7.4 x 10⁴ CFU), while lowest (4.20 x 10⁴ 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) [33, 34] and Yadav *et al.* (1991) [43], in roots of maize variety Laxmi grown in Saline calcareous soil of Bihar. Ramazan *et al.*, 2007 [35] reported that the density of N₂-fixing organisms ranged between 1.4 x 10⁵ CFU and 4.1 x 10⁶ CFU in soil.

Characterization of PGPR isolates

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 rhizosphere, rhizoplane and surface soil. These isolates were identified on the basis of cultural, morphological and some biochemical test as describe in Bergy's Manual of systematic. The colonies of *Pseudomonas* spp. isolates were mostly yellow, orange and green fluorescent. The green fluorescent colour forming isolates were very similar to *Pseudomonas fluorescence* (Rhodes, 1959) [62]. 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 specialized character of *Pseudomonas* groups as describe in Bergy's Manual of Systematic. 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.

Forbes *et al.* (1988) [10] similarly characterized 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.

Screening of *Pseudomonas* spp. isolates

Plant growth promoting activities of PGPR isolates has been presented in (Table 1). Twelve identified PGPR isolates (PSD₁.....PSD₁₂) 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.

Seed germination

Root length

Root length varies from 4.68 to 7.42 cm due to inoculation of *Pseudomonas* isolates, highest being due to PSD₆ (7.42 cm), which was at par with PSD₈ (7.14 cm) and PSD₁₀ (7.24) and significantly superior with UIC.

Shoot length

Shoot length varies from 3.54 to 5.60 cm due to inoculation of *Pseudomonas* isolates, highest being due to PSD₆ (5.60 cm), which was at par with PSD₈ (5.38 cm) and PSD₁₀ (5.46) and significantly superior with UIC.

Significant increased in root and shoot length might be due to vary high production of IAA. 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) [30].

Production of IAA

Laboratory experiment was conducted to assess the potential of various *Pseudomonas* isolates (PSD₁ to PSD₁₂), for auxin biosynthesis. 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 PSD₄, PSD₇ and PSD₈ came under medium categories, Isolate PSD₁₀ came under high categories, while isolate PSD₆ came 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 (Mirza *et al.*, 2001; Khalid *et al.*, 2004; Ahmad *et al.*, 2005; Picard and Bosco, 2005 and Ashrafuzzaman *et al.*, 2009) [63, 18, 1, 31, 64]. Similarly, Leinhos and Vacek (1994) reported a production of 1.6 to 3.3 mg auxin ml⁻¹ filtrate by *Pseudomonas* isolated from wheat. Furthermore, Prikryl *et al.* (1985) have reported production of IAA and some other auxins in liquid culture of *Pseudomonas cepacia* and *P. fluorescens* isolated from maize rhizosphere.

P-solubilization test

Solubilization efficiency of *Pseudomonas* isolates has been presented in table 1. *Pseudomonas* isolates PSD₄ and PSD₆ 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) [11, 17, 37, 23].

Antifungal activity test

Antifungal activity test of different *Pseudomonas* isolates has been tested. Among the twelve *Pseudomonas* spp. isolates, two, isolates (PSD₈ and PSD₁₀) came under high (70-80%), two isolates come 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 *Rhizocotonia solani* in dual culture test was also observed by Reddy *et al.* 2007 [65]. Species of *Pseudomonas* are known to have a significant role in suppression of fungal pathogens apparently via the production of antibiotics (Hill *et al.* 1994, Dunne *et al.*, 1998 and Thomashow and Weller 1988) [15, 9, 66] and volatile compound.

Nitrogenase activity

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

Morphological cultural and biochemical characteristics of *Azotobacter* isolate (AZT₁ AZT₁₂)

Identified PGPR of *Azotobacter* isolates on the basis of colony morphology character and biochemical tests as describe in Bergy's Manual of systematic Bacteriology (1984) has been presented in table 1. 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 flate type. All the isolates in the present study showed Gram negative reaction. All the isolates were positive for catalase and oxidase test. Similar observation was also recorded by Forbes *et al.* 1988 [10] 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 °C on N-free basal medium.

Screening of *Azotobacter* isolates

Twelve isolates of *Azotobacter* (AZT₁....AZT₁₂) 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.

Seed germination

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

Root length

Root length of maize was increased significantly by *Azotobacter* isolates AZT₄ (6.75 cm), AZT₆ (6.48 cm), AZT₈ (6.00 cm), AZT₁₀ (5.84 cm), AZT₇ (5.80 cm), AZT₅ (5.60 cm), AZT₁₂ (4.75 cm) and AZT₂ (4.62 cm) in comparison to UIC (3.82 cm).

Shoot length

Azotobacter isolates caused significant increased in length of maize AZT₄ (4.72 cm), AZT₆ (4.54 cm), AZT₈ (4.24 cm), AZT₁₀ (4.10 cm), AZT₇ (4.10 cm), AZT₅ (3.92 cm), AZT₃ (3.64 cm) and AZT₉ (3.5 cm) in comparison 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) [30, 18, 1].

Production of IAA

Potential of various *Azotobacter* isolates for auxin biosynthesis has been identified. Intensity of pink colour indicated the range of IAA production like low, medium, high and very high among twelve isolates. Six isolates come under low ((+) very light) categories. Isolates AZT₅, AZT₈, AZT₁₀ and AZT₆ come under high categories (+++) while AZT₄ isolate comes under very high (++++) categories. Among them one isolate AZT₄ was found to be good producer of IAA. Production of IAA by *Azotobacter* has been reported by several workers (Ashrafuzzaman *et al.*, 2009; Picard and Bosco 2005) [64, 31]. 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 (Mirza *et al.*, 2001) [63].

P-solubilisation test

All the isolates of *Azotobacter* spp. showed negative response against P-solubilisation test.

Antifungal activity

All the isolates of *Azotobacter* spp. were found to negative response against Antifungal activity test.

Nitrogenase activity

Nitrogenase activity of *Azotobacter* isolates has been presented in table. Nitrogenase activity of *Azotobacter* spp. varies from 105.42 to 20.35 mole C₂H₄ mg⁻¹ protein hr⁻¹. Highest nitrogenase activity was recorded in AZT₄ followed by AZT₆ and AZT₁₀. Nitrogenase activity has been categorized vary high (AZT₄), higher (AZT₆, AZT₈, AZT₉, AZT₁₀, AZT₁₁ and AZT₁₂) and medium (AZT₂, AZT₃, AZT₅ and AZT₇). On the basis of above observations *Azotobacter* isolates AZT₄ 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) [40, 25].

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 Bergy's Manual of systematic Bacteriology (1984). 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 72 hr and colour changed to blue except AZS₆ (change blue to black). The colour change in tube blue to black in isolate AZS₆ 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. Forbes *et al.* (1988) [10] also characterized 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 °C on N-free basal medium.

Screening of *Azospirillum* isolates

Twelve identified PGPR of *Azospirillum* spp. isolates (AZS₁ AZS₁₂) 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.

Seed germination

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

Root length

Root length of maize increased significantly in *Azospirillum* spp. isolates AZS₆ (6.84 cm), AZS₁₀ (6.68 cm), AZS₈ (6.28 cm), AZS₄ (6.00 cm), AZS₇ (5.95 cm), AZS₅ (5.74 cm), AZS₃ (5.48 cm) and AZS₄ (5.35 cm) in comparison to UIC (4.00 cm).

Shoot length

Shoot length of maize increased significantly in *Azospirillum* spp. isolates AZS₆ (4.88 cm), AZS₁₀ (4.66 cm), AZS₈ (4.66 cm), AZS₄ (4.3 cm), AZS₇ (4.28 cm), AZS₅ (4.0 cm), AZS₃ (3.84 cm) and AZS₉ (3.72 cm) over UIC.84 cm).

Among all the tested isolates of *Azospirillum* the highest value of root and shoot recorded in AZS₆, might be due to highest production of IAA. 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 Gillick 2000) [30].

Production of IAA

Intensity of pink colour indicated the potential of various *Azospirillum* spp. isolates for auxin bio synthesis. On the basis of intensity of Pink colour *Azospirillum* isolates have been categories very high (AZS₆), medium (AZS₄, AZS₈, AZS₁₀) and rest comes under low (AZS₁, AZS₂, AZS₃, AZS₇, AZS₁₁ and AZS₁₂). Among them one isolate (AZS₆) was found to be good producer of IAA.

Production of IAA by *Azospirillum* has been reported by several workers (Azhrufuzzaman *et al.*, 2009, Picard and Bosco 2005) [64, 31]. 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 (Mirza *et al.*, 2001) [63].

P-solubilisation test

All the isolates of *Azospirillum* spp. were found to negative response against P-solubilisation in table.

Antifungal activity test

All the isolates of *Azospirillum* spp. were found to negative response against Antifungal activity test in table.

Nitrogenase activity

Nitrogenase activity of *Azospirillum* spp. varies from 114.48 to 18.48 n moles C₂H₄ mg⁻¹ protein hr⁻¹. Highest nitrogenase activity was recorded in AZS₆, followed by AZS₄ and AZS₁₀. Nitrogenase activity has been categorized very high (AZS₆), high (AZS₄, AZS₇, AZS₉ and AZS₁₀) medium (AZS₁, AZS₃, AZS₅, AZS₈, AZS₁₁ and AZS₁₂) and low (AZS₂). On the basis of above observations *Azospirillum* isolate AZS₆ 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) [33, 34].

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 Bergy's manual of systematic Bacteriology (1984). A characteristic growth of P-solubilizing bacteria was 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.

Based on results obtained with regard to extent and period of growth on Pikovaskay's medium, the isolates of P-solubilizing spp. were grouped in to poor, medium, high and very high growth, categories. Isolate PSB₄ come under very high growth after 72 hr.

Screening of P-solubilizing isolates

Twelve identified PGPR of P-solubilizing isolates (PSB₁..... PSB₁₂) 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.

Seed germination

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

Root length

Root length of maize increased significantly in P-solubilizing bacteria isolates PSB₄ (6.40 cm) PSB₆ (6.38 cm), PSB₈ (5.74 cm), PSB₁₀ (5.62 cm) PSB₇ (5.44 cm) PSB₅ (5.32 cm), PSB₃ (4.84 cm), PSB₉ (4.78 cm) and PSB₁₂ (4.52 cm) over UIC (3.40 cm).

Shoot length

Shoot length of maize increased significantly in P-solubilizing bacteria isolates PSB₄ (4.46 cm) PSB₆ (4.48 cm), PSB₈ (4.02 cm), PSB₁₀ (3.94 cm) PSB₇ (3.8 cm) PSB₅ (3.72 cm), PSB₃ (3.38 cm), PSB₉ (3.34 cm) and PSB₁₂ (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 PSB₄.

Production of IAA

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

P-solubilisation test

Phosphate solubilisation efficiency (%) of different isolates ranged from 39.33 to 75.63%, highest being due to PSB₄ (75.67%) followed by PSB₉ (70.00%), PSB₁₀ (69.33%) and lowest in UIC (39.33%).

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, Khundu *et al.* 2002, Anu and Kundu, 2005, Kumar and Yadav 2005) ^[11, 67, 2, 23].

Antifungal activity

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

Nitrogenase activity

All the isolates were found to negative response against nitrogenase activity. On the basis of above observations PSB₄ 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, and microbial population on post-harvest soil were under taken. Selected four best isolates of PGPR *Pseudomonas* (PSD₆), P-

solubilizer (PSB₄), *Azospirillum* (AZS₆) and *Azotobacter* (AZT₄) 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 randomized 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.

- i. 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 AZS₁ to AZS₁₂, AZT₁ to AZT₁₂, PSD₁ to PSD₁₂ and PSB₁ to PSB₁₂ respectively.
- ii. Highest Nitrogenase activity 3.15 mole C₂H₄g⁻¹hr⁻¹), Mycorrhizal infection (80%), Maximum MPN of *Azospirillum* spp. (0.35 x 10⁵) and maximum population of P-solubilizing bacteria (10.50 x 10⁴ CFU) were recorded in sample collected from Hazipur (diara belt), while maximum population of *Azotobacter* spp. (8.20 x 10⁴ CFU) and *Pseudomonas* spp. (5.42 x 10⁵ CFU) were recorded in Danapur (diara belt).
- iii. On the basis of colony character and biochemical test *Pseudomonas* isolates coded as PSD₁ to PSD₁₂ were mostly yellow to green fluorescent colonies, circular to round form and raised type. Isolate PSD₆ was identified as fast growing up to 72 hr on King's medium.
- iv. All the *Pseudomonas* isolates were Gram negative and gave positive response against catalase and oxidase test.
- v. *Pseudomonas* isolate PSD₆ 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),
- vi. 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 C₂H₄/mg protein/hr) *Azotobacter* isolate AZT₄ was selected out of twelve isolates (AZT₁.....AZT₁₂).
- vii. 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.
- viii. 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 C₂H₄/mg protein/hr.) *Azospirillum* isolate AZS₆ was selected out of twelve isolates (AZS₁ AZS₁₂).
- ix. All the isolates of P-solubilizing bacteria (PSB₁ to PSB₁₂) showed transparent 'Halozone' around individual colony. These isolates were gram negative, rod shaped and showed positive response against gram reaction, catalase and oxidase test.
- x. 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 PSB₄ was selected out of twelve isolates (PSB₁..... PSB₁₂).

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