Int J Pharm Bio Sci 2014 Oct; 5(4): (B) 153 - 163

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ISOLATION AND CHARACTERIZATION OF PGPR ISOLATES FROM RHIZOSPHERE SOILS OF GREENGRAM IN WARANGAL DISTRICT OF TELANGANA

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) stimulate plant growth and improve crop productivity by direct or indirect mechanisms. In our present study, a total of 120 bacterial strains were isolated from rhizosphere soils *of Vigna radiata L. Wilckzeck, from* Warangal Dist. , Telangana, India. Among them, 35 potential bacterial strains showing antagonistic and PGP activities were selected for characterization. Out of which six competent strains were selected which showed maximum plant growth promoting (PGP) activities and further subjected to the cell wall degrading enzyme production and seed germination test. All isolates exhibited production of indole acetic acid, whereas five isolates produced HCN and solubilized inorganic phosphate. Subsequently, to investigate *in vitro* test for all the six isolates the antagonistic activity against, *Fusarium oxysporum*, *Colletotrichum capsici*, *Rhizoctonia solani*, *Macrophomina phaseolena* spp. This result suggests that these PGPR isolates may be used as bio fertilizers as well as biocontrol agents to enhance the growth and productivity.

KEYWORDS: PGPR, Green gram, Plant growth promoting activities

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INTRODUCTION

Mung bean (*Vigna radiata*) is a food legume that is very rich in protein and essential amino acids with the exception of the sulphur amino acids, methionine and cysteine which may be nutritional limited. It is a good source of soluble carbohydrate, and contains a very high amount of crude fiber[1, 2]. However, this important food crop is beset by a number of bacterial diseases namely: Wilt, root rot, bacterial wilt and bacterial spot which results in yield loss. PGPR influence the plant growth in different ways by producing phytohormones like IAA and gibberellins, siderophore production, phosphate solubilization, synthesis of antibiotics, enzymes and/or antifungal compounds[3, 4.] PGPR may directly influence plant growth through enhanced provision of nutrients and the production of phytohormones and indirectly suppress deleterious rhizobacteria and pathogens to affect biological control by the production of antibiotics and iron chelating siderophores and the induction of plant resistance mechanisms[5]. Microorganisms are known to suppress soil borne plant diseases, promote plant growth and cause changes in texture of soil and vegetation. Plants are surrounded by diverse types of microbial organisms, some of which can contribute to biological control of plant diseases. In this regard, the use of PGPR is steadily increasing in agricultural crop yields and it is also an attractive way to replace chemical fertilizers and pesticides. Soil is the home (harbours) of innumerable forms of microorganisms. In the rhizosphere 2-5% of bacterial population is PGPR, they stimulate plant growth directly by nitrogen fixation, solubilization of nutrients, production of growth hormones, 1-amino-cyclopropane-1- carboxylate (ACC) deaminase and indirectly by antagonizing pathogenic fungi by production of siderophores, chitinase, β-1,3-glucanase, antibiotics, fluorescent pigments, and cyanide, PGPR have shown positive effects in plants on such parameters as germination percentage, tolerance to drought, weight of shoots and roots, yield and plant growth[6]. Production of indole acetic acid (IAA) by *Pseudomonas* and its role in the development of root system is also evidenced[7]. For many *Pseudomonas*, production of metabolites such as antibiotics, siderophores and hydrogen cyanide (HCN) is the primary mechanism of biocontrol[8]. In the current investigation, we focused on rhizobacteria isolation, characterization and screening for their biocontrol activity against fungal plant pathogens (*Fusarium oxysporum*, *Colletotrichum capsici*, *Rhizoctonia solani*, *Macrophomina Phaseolena* spp.) and also for their growth promoting efficacy.

MATERIALS AND METHODS

Collection of soil samples from green gram rhizosphere

The present investigation was under taken with an objective to select a promising native PGPR strains against pathogen of Green gram. Systematic surveys of Green gram growing areas of Warangal District, Telangana , India was under taken periodically to assess the prevalence of the disease during Rabhi and Kharif cropping seasons. The samples were placed in plastic bags and stored at 4°C in the Laboratory.

Isolation, screening and maintenance of bacteria

Bacteria were isolated from the rhizosphere, root samples were shaken vigorously to remove loosely adhering soil and 4.5 ml of sterile physiological water was added to 0.5 g of rhizospheric soil and the mixture was shaken at 120 rpm for 2 min. Serial ten-fold dilutions were prepared from the extract and 0.1 ml of each dilution was added onto King's B medium, supplemented with 100 µg/ml of cycloheximide to suppress fungi. The spread-plate cultures were incubated for 48 h at 25 ± 1°C. Ten to fifteen representative colonies, with different morphological appearances, were selected from the countable plates and re-streaked on a new plate containing the same media to obtain pure colonies. A total of 120 candidate isolates

obtained in this manner were maintained on nutrient agar slants.

Morphological characterization and microscopic observation

The selected bacterial isolates were examined for their morphological features. The morphological characteristics were examined on their respective agar plates. The pure cultures from the slants were placed on the agar plates. After the growth of colonies morphological characters of the colonies like the colour, shape, size, surface and gram staining etc. were recorded. All the 120 bacterial isolates were screened for their growth promoting activities like indole acetic acid (IAA) production, ammonia production, phosphate solubilization, HCN production, hydrolytic enzyme production such as catalase, proteases, lipases and amylases and their antifungal activity against the plant pathogen.

A. ***In vitro screening of multiple plant growth promoting activities of selected isolates***

1. Determination of IAA[9]

Luria Bertani broth medium (25 ml) amended with 50 µg/ml tryptophan was inoculated with the isolated bacteria. They were incubated for 24 h at 28°C on a rotary shaker. Cultures were centrifuged at 10,000 g for 15 min. 2 ml of supernatant was taken and 2 to 3 drops of ortho-phosphoric acid was added. 4 ml of Salkowski reagent was added and incubated for 25 min. at room temperature. Absorbance was read at 530 nm. auxin production was determined by using a standard graph.

2. Production of ammonia[10]

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube separately and incubated for 48-72 h at 28 ± 2°C. Nesseler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production.

3. Phosphate solubilizing activity [11]

The plates were prepared with Pikovskya's medium. The culture of six isolates were streaked on the plates and incubated in an incubator at 28°C for 7 days. The plates were then examined and data were recorded.

4. HCN production[12]

Hydrogen cyanide (HCN) production was evaluated by streaking the bacterial isolates on King's B agar medium amended with glycine. Whatman No.1 filter paper discs were dipped in 0.5% picric acid solution in 2% sodium carbonate solution the discs were placed in the lid of each petriplate. The plates were then sealed air-tight with Parafilm and incubated at 30°C for 48 h. A colour change of the filter paper from deep yellow to reddish-brown colour was considered as an indication of HCN production.

B. Other lytic enzymes

1. Catalase activity[13]

Catalase test was performed by taking a 3-4 drops of hydrogen peroxide (H_2O_2) were added to 48 h old bacterial colony which is grown on trypticase soya agar medium. The effervescence indicated catalase activity.

2. Production of caseinase (protease) [14]

The qualitative assay for protease production was performed on sterile skim milk agar plates (Panc. digest of casein 5.0g, Yeast extract 2.5g, Glucose 1.0g, Agar 15.0g, Distilled water 1000 ml, Skim milk 7% was added as inducer). Isolates were spot inoculated and followed by incubation at 30ºC and the zone of clearance around the colony indicating the enzymatic degradation of protease.

1. Production of lipase[15]

Bacteria were grown on nutrient agar amended with egg yolk. After 48 h of incubation the agar medium was flooded with a saturated aqueous solution of copper sulfate ($CuSo_4$) and kept for 10-15 min. The excess reagent was poured off. Formation of greenish blue colour zones around the colony indicated the production of lipase.

4. Production of amylase (starch hydrolysis) [16]

The bacterial isolates were spot inoculated on starch agar (Beef extract 3.0g, Peptone 5.0g, Soluble starch 2.0g, Agar 15.0g, Distilled water 1

lit.) medium plates and incubated at 30°C for 48 h. At the end of incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Iodine reacts with starch to form a blue colour compound. This blue colour fades rapidly. Hence the colour less zone surrounding colonies indicates the production of amylase.

***5. Production of cellulase*[17]**

Cellulase production was determined by using the method. M9 agar medium with yeast extract plates were inoculated with individual bacterial isolates and incubated for 3-5 days at 28°C. Bacterial growth surrounded by clear halos was considered as a positive indication of cellulose production.

***6. Production of Pectinase*[18]**

Pectin degrading enzymes were screened by using M9 agar medium amended with 4g of Pectin per liter. The plates were incubated for 2 days at 28±2°C. The appearance of a clear halo around colonies indicates pectinase production.

C. Antagonistic activities against plant pathogenic fungi

The antagonistic effects of all six bacterial isolates were tested against fungal pathogens of *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum capsici* and *Rhizoctonia solani*) For this the bacterial isolates were streaked at a distance of 3.5 cm from rim of individual petri plate containing potato dextrose agar (PDA) medium. 6 mm mycelial disc from a 7-day old PDA culture of fungal pathogens was then placed on the other side of the Petri dish and the plates were incubated at 28°C for 4-7 days[19].Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist from the fungal radius. The percent inhibition was calculated using the formula:

$$PI = \frac{(R-r)}{R} \times 100$$

Where' r ' is radial growth of the fungal colony opposite the bacterial colony and, R is the radial growth of the pathogen in control plate[20] .

Where, PI = Percent inhibition

R = Radial growth of pathogen in control plate

r = Radial growth of the fungal colony opposite the bacterial colony

D. Plant growth promotion ability of Vigna seeds in the laboratory

The experiment was conducted to assess the influence of selected efficient isolates on seed germination and tested for their plant growth promotion ability by the standard roll towel method[21] in growth chamber. Bacteria were grown in nutrient broth medium on a shaker (150 rpm) for 2 days and centrifuged at 10,000 rpm for 5 min. The pellet was mixed with sterile carboxy methyl cellulose (CMC) (HiMedia) suspension (1%). Tomato seeds were surface sterilized with 0.1% mercuric chloride for 5 min, rinsed with sterilized distilled water (SDW) and soaked in the bacterial suspension (3×108 cfu ml-1) using 1% carboxymethyl cellulose (CMC) for 24 hrs and the sterile blank nutrient broth served as control. Then the seeds were blot dried, placed in wet blotters and incubated in growth chamber maintained at 25±20°C and 95±3% RH. Each treatment was replicated four times with 20 seed per each. The percentage of germination was recorded on the 5th day. Ten seedlings were taken at random from each replication and length of root and shoot measured separately after 5 and 15days[22]. The seedling vigor index was calculated using the formula.

Germination Parameters

I. Germination Percentage (GP): The number of seeds germinated at the end of the experiment was considered and ex-pressed as percentage as described in the following equation[23, 24].

$$\text{Germination Percentage (GP)} = \frac{\text{Number of seeds germinated after 5 days}}{\text{Number of seeds tested}} \times 100$$

II. Germination Index (GI): It was calculated according to the following equation

$$\text{Germination Index (GI)} = \frac{\text{\% of germination in each treatment}}{\text{\% of germination in control}}$$

III. Seedling Vigor index (SVI): it was calculated using following equation[25].

Seedling Vigor index (SVI): (Average shoot length + average root length) X G.P

RESULTS

Isolation and characterization of bacterial isolates

A total of 120 bacteria was isolated from various varieties of plant rhizosphere soils from Warangal districts during 2011 to 2013(Table1). These isolates were evaluated for their antagonistic and plant growth-promoting traits. Six best potential bacterial strains showing antagonistic and PGP activities were selected for characterization. Among 6 isolates five were Gram positive and one was Gram negative.

TABLE 1
Selected Locations for isolation of PGPR

S.NO	Isolates	Location of rhizosphere soil	Variety of the crop
1	KG-2	Kesamudram	ML-267
2	TG-13	Thorrur	ML-267
3	MG-26	Maripeda	Local variety
4	YG-29	Yellampeta	ML-267
5	NG-35	Narasimhulapeta	ML-267
6	IG-42	Inugurthi	Local variety

Morphological characteristics and Microscopic observations of PGPR isolates

The morphological characteristics of PGPR isolates (KG-2, TG-13, MG-26, YG-29, NG-35, and IG-42) varied widely. All the isolates produced round shaped and raised colonies having rough surface with undulated to erosive margins /smooth shiny surface with smooth margin. No pigmentation was observed on NA media. Microscopic observations were performed to investigate the characteristics of PGPR isolates such as shape, gram reaction and motility. All isolates were rod shaped, motile. In gram reaction Five isolates were gram positive remaing One isolate shows gram negative reaction (Table2).

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TABLE 2
Morphological and Microscopic characters of PGPR isolates

S.No	Isolates	Motility	Shape	Gram stain	Colour	Surface	Margin	Pigmentation
1	KG-2	Motile	Bacilli	+	White	Smooth	Rough	Not showing
2	TG-13	Motile	Bacilli	+	White	Smooth	Rough	Not showing
3	MG-26	Motile	Bacilli	+	White	Smooth	Rough	Light brown
4	YG-29	Motile	Bacilli	-	White	Smooth	Rough	Grey
5	NG-35	Motile	Bacilli	+	White	Smooth	Rough	Not showing
6	IG-42	Motile	Bacilli	+	White	Smooth	Rough	Not showing

+ =Gram positive, - =Gram negative

Plant growth promoting activities of PGPR isolates and Production of secondary metabolites

The isolates showed varied levels of PGPR traits such as phosphate solubilization, IAA, ammonia, and HCN production (Table 3).

Phosphate solubilization

Five strains exerted ability for phosphate solubilization on Pikovskaya medium with different efficacy. Out of six 5 strains showed a maximum degree of phosphate solubilization of 52 %. The maximum phosphate solubilization was identified in IG-42 strain. The phosphate-solubilizing activity characterizes the microorganisms with the ability to produce and release metabolites such as organic acids that chelate the cations bound to phosphate, converting them into soluble forms.

IAA production

Auxin is the most investigated hormone among plant growth regulators. The most common, best characterized and physiologically most active auxin in plant is indole-3-acetic acid (IAA). IAA is known to stimulate both a rapid response (e.g. increased cell elongation) and a long-term response (e.g. cell division and differentiation) in plants. In our study, three bacterial isolates (KG-2, YG-29, IG-42,) were able to produce indole-3-acetic acid (IAA) growing in medium without the addition of tryptophan. Maximum IAA production was recorded in IG-42 strain as compared to other isolates.

HCN production

Ability for hydrogen cyanide synthesis was observed for selected isolates of 6 strains (KG-2, TG-13, MG-26, YG-29,) Hydrogen cyanide mediated antagonism was observed and the increased production of HCN by the efficient strain of *P. fluorescens* contributed to effective inhibition of mycelial growth of *Rhizoctonia solani in vitro* and appears to be a major factor in the control of soil-borne disease by *Pseudomonas fluorescens*

Ammonia

The production of ammonia observed in all isolates. The ammonia is useful for plant as directly or indirectly.

Other lytic enzymes

Proteolytic enzyme production was detected in the skim milk agar medium for strains- KG-2, TG-13, and IG-42. Strain TG-13, MG-26, IG-42, showed lipase activity. TG-13, MG- 26, YG-29, NG-35, and IG-42 showed cellulase activity. All isolates showed Pectinase activity. MG-26, YG-29, NG-35, IG-42, showed Amylase activity. TG-13, MG-26, YG-29, IG-42 showed Catalase activity. The present study revealed the production of mycolytic enzymes viz. cellulase, protease and lipase. Mycolytic enzymes produced by antagonistic microorganisms are very important in biocontrol technology. There are many reports on production of lytic enzymes by microorganisms.

TABLE 3
Lytic enzymes production and Plant growth promotion traits of selected isolates.

S.NO	IAA	Ammo nia	HCN	Phosphate solubilizati on	ENZYME PRODUCTION					
					Cellula se	Protea se	lipase	Pectina se	Amyl ase	Catala se
KG-2	+	+	+	-	-	+	-	++		-
TG-13	-	+	+	+	+	++	+	+	-	+
MG-26	-	+	+	+	++	-	++	++	-	+
YG-29	+	+	+	++	+	-		+	++	+
NG-35	-	+	-	+	+	-		+	+	-
IG-42	+	+	-	++	++	++	++	+	+	+

- = No production; + = Weak production; ++ = medium production; +++ = high production.

Antagonistic activity of selected strains

The selected strains are tested against four phyto pathogenic fungi how cause significant losses in agriculture and reduce crops and are often targeted in biological control. Several mechanisms have been proposed to explain the inhibition of phyto pathogenic fungi by *Bacillus and pseudomonas spp.* Including antibiotic production, hydrolytic enzymes synthesis, competition for nutrients, or a combination of these mechanisms in synergy. The twelve Bacterial strains have variable antagonistic activity of plant pathogenic fungi with PGI % target variables. In this present study revealed that, Opposition assay was used to determine the isolates that inhibit the growth of *C. capsici*, *R. solani*, *M.phaseolina*, *F. oxysporum*. All the selected strains show a good mycelial growth inhibition (> 70%) of the majority of targets In this study, among 12 PGPR isolates that significantly promoted plant growth. there were five isolates (TG-13, MG-26, YG-29, NG-35, IG-42.) that were able to inhibit *R. solani* and *Macrophomina* phaseolina ,four isolates (TG-13, YG-29, NG-35, IG-42) inhibited *C. capsici* . all the isolates were inhibit the *F. oxysporum.* The maximum inhibiton observed in isolate IG-42 (Table 4).

TABLE 4
Antagonistic activities against plant pathogenic fungi

Isolates	Antagonistic activity			
	C. capsici	*R. solani*	*M.phaseolena*	*F.oxysporum*
KG-2	+++	-	-	+++
TG-13	-	+	+	+
MG-26	+	+	+	++
YG-29	+	++	++	+
NG-35	++++	+	+	++
IG-42	++	++++	++++	++++

- Indicates not showing, + Indicates low inhibition % , +++ Indicates Moderate inhibition % , + +++ indicates high inhibition

The assessment results stimulating germination ability, to root and the shoot length of green gram in the laboratory

The selected twelve isolates were then assessed to know their influence on the seed germination by measuring the shoot and root length. The growth promoting activity of 6 isolates of PGPR was tested for seed germination and seedling vigor by using seeds in roll towel methods. The data on seed germination and seedling vigor of Green gram seeds as influenced by seed bacterization with different rhizo bacterial isolates by roll towel method are given in (Table:5).

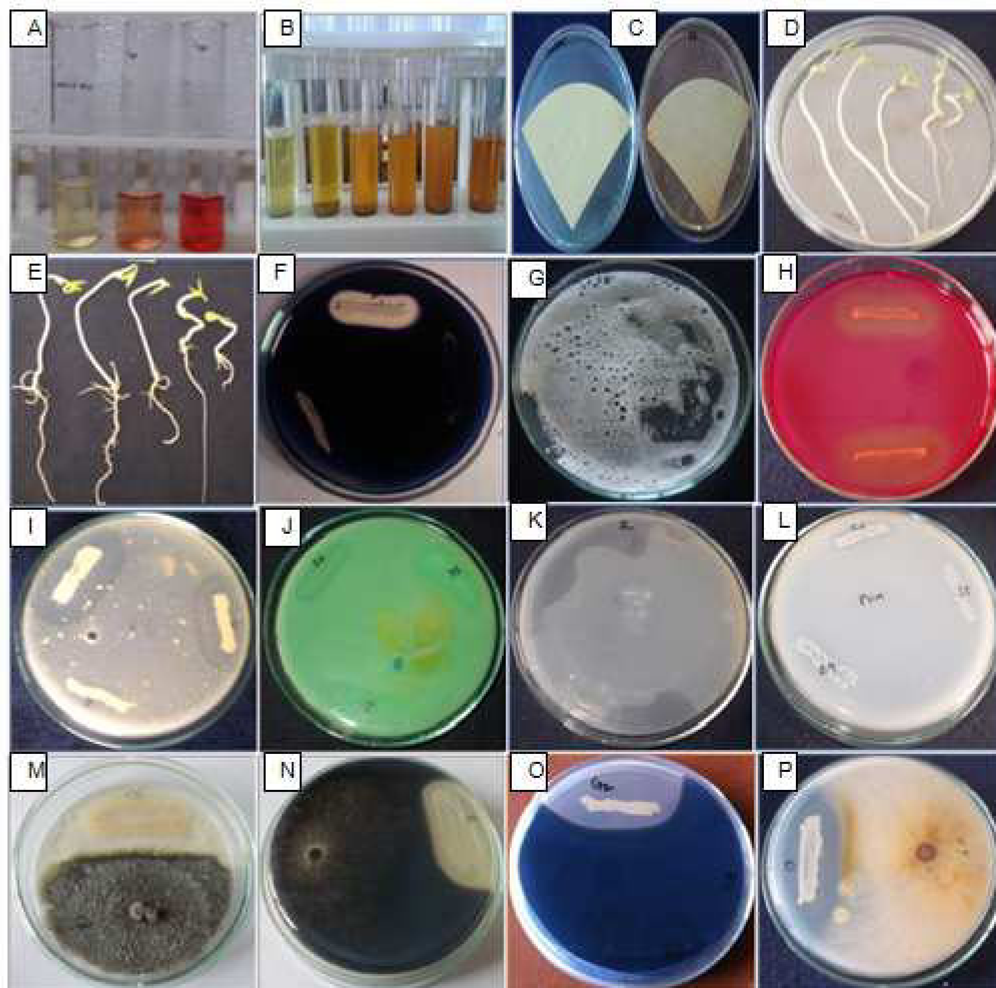
Table 5
Ability to stimulate germination root and shoot length of selected PGPR Strains

Isolate no	Germination %	Shoot length(cm)	Root length(cm)	Vigor index
KG-2	**80**	**14.32**	**7.26**	**2**
TG-13	**70**	**13.9**	**6.92**	**1.75**
MG-26	**60**	**13.28**	**8.52**	**1.50**
YG-29	**40**	**8.64**	**5.32**	**0**
NG-35	**70**	**11.56**	**10.5**	**1.75**
IG-42	**40**	**16.02**	**9.46**	**2**

DISCUSSION

Plant rhizosphere is known to be preferred ecological niche for various types of soil micro organisms due to rich nutrient availability. It has been assumed that inoculation with diazotrophic bacteria like Rhizobium, Azatobactor, and Azospirillum enhanced the plant growth. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities[26, 27]. In the present study, isolation of bacterial cultures from rhizosphere soil samples of green gram from different regions of Warangal district. The rhizosphere soil supported a total of 120 PGPR isolates with varied characteristics. Among 120 isolates 35 bacterial strains were selected out of which 6 potential strains KG-2, TG-13,MG-26,YG-29NG-35,IG-42 were selected. The most important trait of PGPR is the production of ammonia that indirectly influences the plant growth. All the selected isolates were positive for ammonia production and also produced a significant amount of IAA. Phosphate solublization in the plate-based assay showed a clear halo zone around the colony. HCN production has been proposed as a defense regulatory against phyto pathogens was already reported [28]. Bacterial strains showing lytic enzyme activity must be highly resistant to environmental, mechanical and chemical stress. Antagonistic activity of the bacterial isolates was evaluated in terms of inhibition zone diameter as an indicator of the reduction in the growth of green gram pathogenic fungi. Bacterial plant growth promotion is a well established and complex phenomenon that is often achieved by the activities of more than one PGP trait exhibited by plant associated bacteria. In our study, 55% of the isolates exhibited more than 2 PGP traits. A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield contribute to the protection of plant against certain pathogens and pests [29, 30, 31, 32, 33]. Selected 6 bacterial isolates were evaluated for their PGP activity on green gram seedling on the basis of seed germination parameters like seed emergence, shoot and root length elongation and vigor index (VI). PGPR seed inoculation significantly enhanced seed germination and seed vigor of green gram.

Figure1
Antagonism, growth promoting properties and Extracellular enzyme activity of Greengram rhizobacterial isolates



A. IAA Production 1- Control, 2- High Production, 3- Low Production; B. Ammonia Production 1- Control, 2- Low Production, 3 & 4 -High Production, 5 & 6-Moderate Production; C. HCN production; D&E. Seed germination; F. Amylase activity; G. Catalase activity; H. Cellulase activity; I. Protease activity; J. Lipase activity; K. Pectinase activity; L. Phosphate solubilization; Antifungal activity M. Colletotrichum capscici / G57 N. Macrophomina phaseolena / G-2 O. Rhizoctonia solani / G-2, P. Fusarium oxysporum / G-13.

CONCLUSION

Currently our research effort is to help the poor farmers in this study, we focus on role of the rhizobacteria in plant growth promotion. A pool of rhizobacteria was screened *in-vitro* and their plant growth promoting properties. To evaluate the influence of the most promising bacterial strains on plant growth, bacterized tomato seed was planted in paper towel method[34]. The potential of these has been analyzed, that is to provide data that affect of growth parameters in the green gram. Based on results we have suggested that the strains of PGPR potential and biocontrol ability which can be used as biofertilizers as well as biocontrol agents. The differences in plant growth promotion among the isolates are attributed to their individual competencies. Future studies are required to prove the nature of these isolates and to harness their potential as a bio-inoculants in agriculture.

ACKNOWLEDGMENT

We are gratefully acknowledging the UGC-Major Research Project New Delhi, for providing financial support for this work. We are thankful to Head department of botany, OsmaniaUniversity, Hyderabad for providing necessary laboratory facilities.

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