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# Isolation of Plant growth promoting rhizo bacteria from rhizosphere soils of green gram , biochemical characterization and screening for antifungal activity against pathogenic fungi

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***ABSTRACT:*** *Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms. In our present study, 180 PGPR strains were isolated from the rhizosphere soils of green gram and screened for their antifungal activity against Macrophomina phaseolina, Colletotrichum capscici, Rhizoctonia solani, Fusarium oxysporum. 20 antagonistic isolates were tested for their Plant growth promoting (PGP) traits, seed germination ability, extra cellular enzyme production, salt and temperature tolerance. Six isolates were most effective, which may be useful as biofertilizers, they enhanced the growth of green gram due to the production of ammonia, IAA, HCN, phosphate solubilization, and also having antifungal activity against phyto pathogenic fungi.*

***KEY WORDS:*** *PGPR, Green gram, antifungal activity, Extra cellular enzyme activity*

## I. INTRODUCTION

A group of rhizosphere bacteria that exert beneficial effect on plant growth is referred as PGPR(Kloepper et al., 2004). PGPR may induce plant growth promotion by direct or indirect modes of action (Beauchamp, 1993; Kloepper, 1993; Kapulnik, 1996; Lazarovits and Nowak, 1997). Based on their activities Somers et al. (2004) classified PGPR as biofertilizers, phytostimulators, rhizoremediators and biopesticides. Bashan and Holguin (1998) proposed the division of PGPR into two classes: biocontrol-PGPB and PGPB. Furthermore, in most studied cases, a single PGPR will often reveal multiple modes of action including biological control (Kloepper, 2003; Vessey, 2003).

Green gram is one of the important pulse crops in India. It has been reported that Green gram has been cultivated in India since ancient times**.** Green gram is a protein rich staple food. It contains about 25 percent protein, which is almost three times that of cereals. It supplies protein necessity of vegetarian inhabitants of the country. In addition to being an important source of human food and animal feed, Green gram also plays an important role in nourishing soil fertility by getting better soil physical properties and fixing atmospheric nitrogen. It is a drought resistant crop and suitable for dry land farming and mostly used as an intercrop with other crops. (Pandey ,P.H.(1988).

## II. MATERIALS AND METHODS

**Isolation of Rhizobacteria:** Green gram rhizosphere soil samples were collected during seedling stage, vegetative stage and flowering stage were randomly collected from three agricultural fields in triplicates in the winter season. By using serial dilution plate method the rhizosphere soil samples were processed within 24 h for isolating the most predominant PGPR**.** Soil dilution plate method was adapted for isolating microorganisms from rhizosphere.

**Screening of PGPR for Antagonism assay against phyto pathogenic fungi in dual culture plate method:**
All the 180 PGPR isolates were screened for antagonism against Colletotrichum capscici, Rhizoctonia solani, Macrophomina phaseolina and Fusarium oxysporum using potato dextrose agar (PDA) medium by placing the pathogen and bioagent in opposite direction and allowing them to grow for 7 days. The percent of inhibition on growth of the test pathogen was calculated by using the formula described by Rabindran and Vidyasekaran, (1996), which is I= C – T/ C× 100 (I = Per cent inhibition, C = Radial growth of the pathogen in control, T = Radial growth of pathogen in treatment).

**Characterization of PGPR:**

**Production of ammonia:** It was done by growing the isolates in peptone water (Dye 1962) at 30°C for 4 days. 1ml of Nessler's reagent was added to each tube. Development of faint yellow color indicates small amounts of ammonia and deep yellow to brownish color indicates maximum amount of ammonia production. (Cappucino & Sherman, 1992)

**Determination of IAA:** Isolates were inoculated in Luria Bertani (LB) broth medium (25ml) was enriched with 50 µ/ml tryptophan, incubated for 24h at 28°C on rotary shaker. Cultures were centrifuged at 10,000 rpm for 15 min. 2 drops of ortho phosphoric acid and 4ml of Salkowsky reagent were mixed with supernatant (2ml) and incubated for 25 min at room temperature, development of pink colour indicates IAA production and the quantitative estimation of IAA is performed by using a standard graph (Gordon A.S and Weber 1951).

**HCN Production:** The isolates were streaked on King's B medium amended with 4.4 g/l glycine (Bakker and Skipper 1987). Whatman No.1 filter paper discs were dipped in 0.5% picric acid in 2% sodium carbonate solution. The discs were placed in the lid of each petriplate and sealed. Plates were incubated for 4 days at 28+2C. Colour change of the filter paper from deep yellow to orange and orange to brown indicates the production of HCN.

**Extra cellular enzyme production:**

**Phosphate solubilization:** Isolates were streaked on Pikovaskya's (Pikovaskya, 1948) agar medium and incubated for 6 days at $28^{o}$C. Plates were observed for clearing zones around the bacterial colony indicates Phosphate solubilizing activity(Gaur, 1990).

**Catalase activity:** Catalase test was performed by adding three to four drops of $H_2O_2$ on bacterial culture which was grown for 48hr on trypticase soy agar medium. The effervescence indicates Catalase activity (Schaad, 1992).

**Protease activity:** Protease activity was determined by clear zone in protease medium (Chaiharn, 2008).

**Lipase activity:** Bacterial cultures were grown on nutrient agar amended with egg yolk (Omidvari M, 2008). After 24hr of incubation clear zones around the colony indicates positive for lesithinase activity. The plates were flooded with saturated $CuSO_4$ solution and dried at $37^{0}$C for 20min and the appearance of blue greenish color on the surface around the colony indicates lipase activity (Cowan, 1974).

**Amylase activity:** Amylase activity was indicated by clear zone around the bacterial colony on starch agar medium. After 72 to 96 hr of incubation. the plates were flooded with Iodine solution for 1min and pour off the excess iodine solution, the appearance of clear zone surrounding the colony indicates positive for starch hydrolysis test (Collins, C. H 1995).

**Chitinase activity:** Colloidal chitin was prepared from chitin following (Rodriguez-Kabana (1983) method. The isolates were grown on chitin medium for 4 days at 28+2C. Clear zones were observed around the bacterial colonies indicating the utilization of colloidal chitin.

**Cellulose activity:** Cellulose degrading enzymes were screened by using M9 medium amended with 10 g of cellulose and 1.2 g of Yeast extract per liter. The plates were incubated for 2 days at 28+2°C , halo zone around the colony indicates cellulose activity. ( Miller and Samanta et al).

**Pectinase activity:** Pectin degrading enzymes were screened by using M9 medium amended with 4g of Pectin per liter. The plates were incubated for 2 days at 28+2°C. The appearance of clear halo around colonies indicates pectinase production (Fogarty WM, Kelly CT 1982).

**Gelatinase test:** Gelatinase test was performed by inoculating the culture into gelatin tubes and incubated for 4 to 7 days. Refrigerate the tubes for half an hour, if gelatinase is present, the liquid medium will fail to solidify upon refrigeration (Blazevic and Ederer, 1975).

**Salt tolerance:** Isolates were tested for their salt tolerance ability by spot inoculating the isolates on nutrient agar plates containing different concentrations of NaCl ( 2 %, 4%, 6%, 8% and 10%), incubated at 32°C for 5 days. Observe the growth .

**Growth at different temperatures:** Isolates were tested for their temperature tolerance ability by streaking the isolates on nutrient agar plate and incubated at $10^{0,}$ $20^{0,}$ $30^{0,}$ $40^{0,}$ $50^{0,}$ and $60^{0}$c. After 3 days of incubation changes in the growth and colour was observed and noted.

**Seed germination test:** Green gram seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min, followed by successive washing with sterile distilled water and then the water was decanted. The seeds were kept for 10 minutes in 48h old cultures. The seeds were kept on sterilized wet blotting paper and incubated at $30^{o}$C for 2-7 days. Seeds were treated with sterilized medium was treated as control. The root and shoot lengths were recorded regularly from 2-7 days (Shende et al.). The vigor index was calculated by using the method given by Ista (1996).

**Morphological characterization of isolates:**The selected bacterial isolates were examined on NA plates for their morphological features. After 3 days of incubation, different colony characteristics such as Gram reaction, shape, motility, elevation, surface, etc. were recorded.

## III. EXPERIMENTAL RESULTS AND DISCUSSION

Over all 180 bacterial strains were isolated from the rhizosphere soils of green gram fields from selected regions by serial dilution method. The Rhizobacteria have been screened for antifungal activity against Rhizoctonia solani, Fusarium oxysporum, Macrophomina phaseolina and Colletotrichum capscici and zone of inhibition was taken as an indicator of antifungal property in the dual culture method. Among the 180 isolates, only 20 were antagonistic to the pathogens. The inhibition percentage was calculated using the formula described by Idris et al. (2007) which is (R - r) / R × 100 **(**r: radial growth of the fungal colony opposite to the bacterial colony, R: the radial growth of the pathogen in control (Table 1).Among antagonistic isolates OUG21, OUG26, OUG38, OUG51, OUG57, OUG61, OUG62 exhibited significant antifungal activity. Isolate OUG38 has shown high level of inhibition against Colletotrichum capscici, Rhizoctonia solani. Isolate OUG61 has shown high level of inhibition against Fusarium oxysporum, Macrophomina phaseolena. All the 20 antagonistic isolates were tested for growth promotion traits.

**Table I:** ***Inhibition percentage of Rhizoctonia solani, Fusarium oxysporum, Colletotrichum capscicii, Macrophomina phaseolena by PGPR isolates in Dual culture method***

| S.No | Isolate | Percentage of inhibition (%) | | | |
|---|---|---|---|---|---|
| | | Fusarium oxysporum | Colletotrichum capscicii | Rhizoctonia solani | Macrophomina phaseolina |
| 1. | OUG2 | 52 | 40 | 48 | 72 |
| 2. | OUG5 | 50 | 60 | 50 | 27 |
| 3. | OUG11 | 54 | 45 | 46 | 22 |
| 4. | OUG13 | 57 | 65 | 56 | 66 |
| **5.** | **OUG21** | **57** | **70** | **68** | **72** |
| **6.** | **OUG26** | **66** | **63** | **54** | **73** |
| **7.** | OUG29 | 57 | 57 | 45 | 24 |
| 8. | OUG35 | 55 | 46 | 49 | 50 |
| **9.** | **OUG38** | **64** | **80** | **76** | 42 |
| 10. | OUG42 | 59 | 70 | 45 | 52 |
| 11. | OUG50 | 64 | 36 | 49 | 22 |
| **12.** | **OUG51** | **72** | **75** | 60 | 57 |
| 13. | OUG53 | 54 | 68 | 65 | 33 |
| **14.** | **OUG57** | 69 | **77** | **70** | 72 |
| 15. | OUG59 | 47 | 45 | 50 | 42 |
| 16. | OUG60 | 57 | 35 | 40 | 48 |
| **17.** | **OUG61** | **78** | 65 | 65 | 78 |
| **18.** | **OUG62** | **73** | 65 | 65 | 53 |
| 19. | OUG63 | 57 | 44 | 53 | 62 |
| 20. | OUG64 | 52 | 45 | 48 | 72 |

*Plant growth promotion was assessed by qualitative and quantitative determination of Indole Acetic Acid (IAA),*

*Ammonia ($NH_3$), Hydrocyanic acid (HCN), Seed germination (Table 2, Fig. 1). Among 20 antagonistic isolates all are ammonia and IAA producers while only 4 (OUG5, OUG11, OUG13, OUG26) isolates shown the ability to produce HCN. OUG42, OUG50, OUG58, OUG61, OUG62, OUG63, were the best producers of ammonia, High production of IAA was associated with OUG21, OUG26 and OUG63 isolates, while the isolates OUG60 and OUG13 were low producers of IAA. On the other hand, a negative response to HCN production was evident in all the antagonistic isolates except OUG5, OUG11, OUG13, and OUG26.And all are capable to germinate seeds, OUG63 shows high root length and OUG35 shows high shoot length (Table 2).*

**Table II*: Production of $NH_3$, IAA, HCN and Seed germination by PGPR isolates from rhizosphere of Green gram***

| S.No | Isolate Name | PGP Traits | | | Seed germination | |
|---|---|---|---|---|---|---|
| | | $NH_3$ Production | IAA Production µg/ml | HCN Production | RL(Avg) | SL(Avg) |
| 1. | OUG2 | + | 42 | - | 7.5 | 12.5 |
| 2. | OUG5 | + | 70 | +++ | 7.0 | 14.3 |
| 3. | OUG11 | +++ | 40 | +++ | 8.5 | 13.9 |
| 4. | OUG13 | ++ | 18 | +++ | 9.2 | 13.2 |
| 5. | OUG21 | + | **85** | - | 9.4 | 14.7 |
| 6. | OUG26 | ++ | **87** | +++ | 12 | 14.0 |
| 7. | OUG29 | +++ | 46 | - | 10.5 | 16.0 |
| 8. | OUG35 | + | 50 | - | 9.0 | **18.5** |
| 9. | OUG38 | + | 49 | - | 8.9 | 15.7 |
| 10. | OUG42 | +++ | 8.5 | - | 8.0 | 12.9 |
| 11. | OUG50 | +++ | 8.5 | - | 5.7 | 11.5 |
| 12. | OUG51 | ++ | 46 | - | 8.0 | 12.1 |
| 13. | OUG53 | +++ | 46 | - | 5.6 | 8.6 |
| 14. | OUG57 | ++ | 59.5 | - | 9.1 | 14.7 |
| 15. | OUG58 | +++ | 36.5 | - | 9.0 | 13.6 |
| 16. | OUG60 | ++ | 10 | - | 10.0 | 13.5 |
| 17. | OUG61 | +++ | 27 | - | 12.0 | 15.5 |
| 18. | OUG62 | +++ | 33 | - | 9.5 | 14.3 |
| 19. | OUG63 | ++ | **86** | - | **14.0** | 14.0 |
| 20. | OUG75 | +++ | 42 | - | 12 | 14.9 |

*- = No production; + = Weak production; ++ = medium production; +++ = high production; RL=Root length; SL=Shoot length.*

Production of extracellular enzymes by microorganisms plays an important role in the management of plant pathogens as well as holds enormous economic potential. In view of the significance of extracellular enzymes all the 20 PGPR isolates selected from the antifungal and growth promotion screening were tested for their extracellular enzyme production like phosphatases, catalase, lipase, amylase, chitinase, pectinase, cellulase and gelatinase activity.The bacterial isolates OUG13, OUG26, OUG29, OUG42, OUG50, OUG57, OUG58 showed all pgp activities, and OUG11 could not produce catalase but are capable of solubilizing phosphates and exhibited lipase, protease, amylase, chitinase, pectinase, cellulase and gelatinase enzyme activities (Table 3).

**Table III:** ***Extracellular enzyme activity of selected rhizobacterial isolates from Green gram rhizosphere***

| *S.No* | *Isolate Name* | *Psolubilization* | *Catalase activity* | *Protease activity* | *Lipase activity* | *Amylase activity* | *Gelatinase activity* | *Chitinase activity* | *Pectinaseactivity* | *Cellulose activity* |
|---|---|---|---|---|---|---|---|---|---|---|
| *1.* | *OUG2* | - | - | ++ | - | - | +++ | - | +++ | - |
| *2.* | *OUG5* | - | - | ++ | - | - | ++ | - | - | + |
| *3.* | *OUG11* | +++ | - | +++ | +++ | + | +++ | + | ++ | +++ |
| *4.* | *OUG13* | +++ | ++ | +++ | +++ | - | +++ | + | ++ | +++ |
| *5.* | *OUG21* | - | ++ | ++ | ++ | - | ++ | ++ | - | + |
| *6.* | *OUG26* | ++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| ***7.*** | *OUG29* | ++ | ++ | ++ | + | ++ | +++ | +++ | ++ | + |
| *8.* | *OUG35* | - | - | ++ | - | ++ | +++ | - | +++ | + |
| *9.* | *OUG38* | - | ++ | ++ | - | ++ | ++ | - | ++ | +++ |
| *10.* | *OUG42* | +++ | +++ | +++ | +++ | +++ | ++ | +++ | ++ | +++ |
| *11.* | *OUG50* | +++ | +++ | +++ | ++ | +++ | ++ | +++ | +++ | +++ |
| *12.* | *OUG51* | - | - | ++ | +++ | - | ++ | - | ++ | + |
| *13.* | *OUG53* | - | - | ++ | ++ | ++ | ++ | - | ++ | - |
| *14.* | *OUG57* | +++ | +++ | +++ | + | +++ | ++ | +++ | + | +++ |
| *15.* | *OUG58* | ++ | +++ | +++ | - | +++ | ++ | +++ | ++ | - |
| *16.* | *OUG60* | - | +++ | +++ | +++ | +++ | ++ | - | ++ | - |
| *17.* | *OUG61* | - | ++ | ++ | - | - | ++ | - | +++ | - |
| *18.* | *OUG62* | - | - | ++ | - | - | ++ | - | - | - |
| *19.* | *OUG63* | - | - | ++ | ++ | - | ++ | - | ++ | - |
| *20.* | *OUG75* | - | - | ++ | - | - | ++ | - | ++ | - |

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

Based on antagonism, pgp and enzyme activities of six isolates viz., OUG26, OUG29, OUG42, OUG50, OUG57, OUG58 were selected for further studies. The colony morphology, growth at different concentrations of NaCl and at different temperatures was studied for all the six selected isolates. These isolates are gram +ve bacilli, showing motility. They are salt tolerant and mesophilic i.e. all the selected isolates were able to grow at 20 - 50°C and at 10°C, 60 °C the growth was not observed (Table 4).

**Table IV:** ***Gram staining, shape, motility, salt and temperature tolerance of the selected PGPR isolates***

| Isolates Code | Gram staining | Shape of the Bacteria | Motility | Growth in NaCl (%) | | | Growth at temperatures ($^0$C) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 5 | 7.5 | 10 | 10 | 20 | 30 | 40 | 50 | 60 |
| OUG26 | +ve | Bacilli | Motile | ++ | + | - | - | ++ | ++ | + | + | + |
| OUG29 | +ve | Bacilli | Motile | ++ | + | _ | _ | ++ | ++ | + | + | + |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OUG42 | +ve | Bacilli | Motile | ++ | + | _ | _ | ++ | ++ | + | + | + |
| OUG50 | +ve | Bacilli | Motile | ++ | + | _ | _ | ++ | ++ | + | + | + |
| OUG57 | +ve | Bacilli | Motile | ++ | + | _ | _ | ++ | ++ | + | + | + |
| OUG58 | +ve | Bacilli | Motile | ++ | + | _ | _ | ++ | ++ | + | + | + |

*= No Growth; + = Medium growth; ++ = moderate growth*

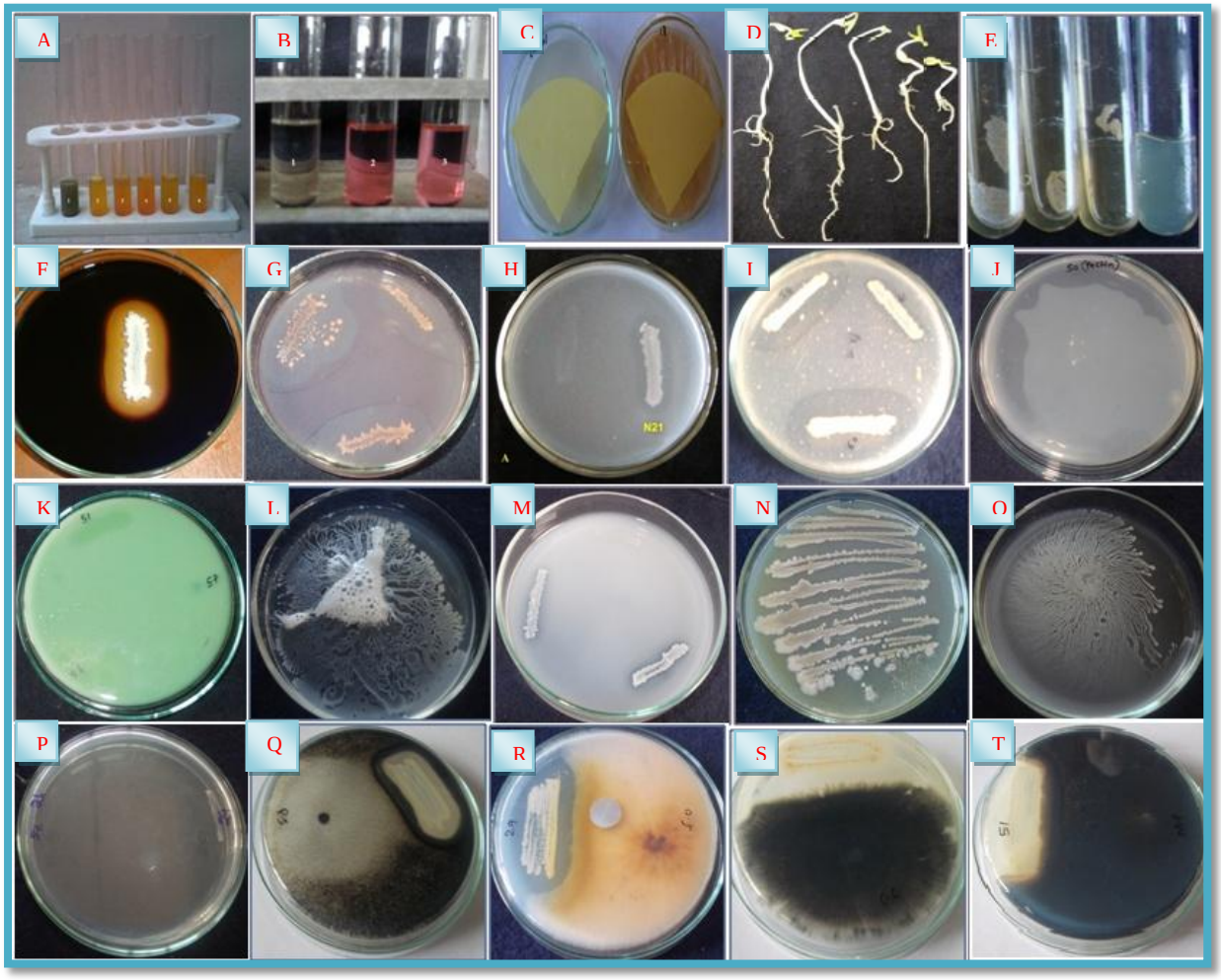


**Figure:1 A**. Ammonia Production 1- Control, 2- Low Production, 3 & 4 -High Production, 5 & 6-Moderate Production; **B.** IAA Production 1- Control, 2- High Production, 3- Low Production; **C**. HCN production; **D.** Seed germination; **E.** Gelatinase activity; **F.** Amylase activity; **G.** Cellulase activity; **H.** Chitinase activity; **I.** Protease activity; **J.** Pectinase activity; **K.** Lipase activity; **L.** Catalase activity; **M.** Phosphate solubilization; **N.** Growth at $60^0$c temperature; **O.** Morphology of OUG26 **P.** Salt tolerance of OUG26 & 29 **Q.** Antifungal activity of Rhizoctonia solani / G-2, **R.** Fusarium oxysporum / G-29 **S.** Colletotrichum Capscici / G-11 **T.** Macrophomina Phaseolena / G-51

## IV. DISCUSSION:

A wide array of beneficial rhizosphere bacteria have been categorized as PGPR including mainly diazotrophs, bacilli, pseudomonads and rhizobia (Antoun and Prevost, 2005). PGPR may induce plant growth promotion through different direct or indirect modes of action (Glick et al., 1999; Antoun and Prevost, 2005).In the present study, Isolation of bacterial cultures from rhizosphere soil samples of green gram from different regions of Khammam district. The rhizosphere soil supported a total of 180 PGPR isolates with varied

characteristics. Among 180 isolates 20 Bacterial strains were selected based on antagonism . All the isolates were screened for their plant growth promoting activities viz., Indole acetic acid production (IAA), Ammonia production, Phosphate solubilization, HCN production, other lytic enzymes like Catalase, Protease, Lipase, Amylase, Cellulose, Pectinase, Chitinase, Gelatinase, Antagonistic activies and Seed germination parameters. Characterization of selected rhizobacterial isolates by using conventional methods like morphological characters, cultural characteristics on agar plate, growth on broth media, growth on NaCl, and growth at different temperature was done as described in Bergy's Manual of Systematic Bacteriology (Tein et al., 1979). All isolates are gram positive, motile and rod shaped.

Most of the PGPR's are known to produce IAA (Gaudin et al., 1994; Asghar et al., 2002; Vessey, 2003). Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia. All the selected isolates were positive for ammonia production and produced significant amount of IAA. A secondary metabolite produced commonly by rhizosphere pseudomonads is Hydrogen Cyanide (HCN), a gas known to negatively affect root metabolism and root growth (Schippers et al., 1990) and is a potential and environmentally compatible mechanism for biological control of weeds (Heydari et al., 2008).Phosphorous is a major essential macronutrient for biological growth and development. In our study, from 20 bacterial isolates 5 (OUG11, OUG-13, OUG-42, OUG-50, OUG-57) were able to solubilize phosphate in the plate-based assay, by showing a clear halo zone around the colony. Among which OUG-57, OUG-42 showed more production then the other isolates.Fungal plant diseases are one of the major concerns to agricultural production all worldwide. Antagonistic microorganisms is a potential non-chemical mean of plant disease control. Many strains of Bacillus have been shown to be potential biocontrol agent against fungal pathogens. In the present study, 20 isolates were selected for green gram plant growth promoting and bio protecting activity which was originally isolated from green gram rhizosphere. Among antagonistic isolates OUG21, OUG26, OUG38, OUG51, OUG57, OUG61, OUG62 exhibited significant antifungal activity. Isolate OUG38 has shown high level of inhibition against Colletotrichum capscici, Rhizoctonia solani. Isolate OUG61 has shown high level of inhibition against Fusarium oxysporum, Macrophomina phaseolena.

Most of the rhizobacteria showed multiple traits for plant growth promotion and disease suppression. The predominant trait observed was IAA production, Ammonia production, phosphate solubilization followed by Antagonism. Bacterial inoculants are able to increase plant growth and germination rate, improve seedling emergence, responses to external stress factors and protect plants from disease (Lugtenberg et al., 2002). In the present study it was observed that the length of root and shoot were enhanced by 70% compared to the control due to seed treatment with PGPR. This might be due to the production of growth hormones like IAA, gibberellins, auxins by bacteria (Mordukhova et al., 1991; Glick 1995)**.** More than 80% of isolates showed positive response towards germination. The highest response for seed germination was observed with isolate OUG63 showing 93.3% followed by OUG35. OUG26,OUG29,OUG42, OUG50,OUG57 and OUG58 were positive for chitinase activity. OUG5 was negative for pectinase activity. Gelatinase and protease activity was detected in all isolates. OUG2, OUG53, and OUG60 not showed cellulase activity, remaining isolates are positive. Among 20 isolates only 5 isolates not shown catalase and amylase activity. 7 isolates not shown lipase activity.

Salinity is an ever-increasing problem in many regions of the world, particularly in arid and semi-arid regions (Flowers, 2004; Munns, 2005; Ashraf and Foolad, 2007). Salinity stress decreases crop growth and productivity due to salt-induced reduction in the photosynthetic activity (Ashraf, 2004). In this study, the ability of the isolates to withstand high salt concentration was a unique feature which may facilitate competitiveness in soil ecosystem (rhizosphere), where these isolates could be used. Selected six isolates were tested for salt tolerance capabilities and observed that all the 6 isolates were observed to grow at high salt concentration 5% 7.5%. While none of the isolates were failed to show growth at 10 % and at 20%, 30%, 40% NaCl concentrations. All the selected isolates were able to grow at $20^0$c - 50°c, 60 °c and at 10°c the growth was not observed.

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