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In vitro screening and characterization of bacteria isolated from the rhizospheric soil of *Ocimum sanctum* for multiple plant growth promotion activities

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Abstract : *Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, in the rhizosphere, which, when applied to seeds or crops, influences the plants physiology to a great extent. In our study, soil samples from rhizosphere of medicinal plant Ocimum sanctum were collected from different locations in Phulwarisharif, Patna. Total of sixteen bacterial strains were isolated out of which six strains were selected and screened for in vitro plant growth promoting activities like ammonia production, Indole Acetic Acid (IAA) production, Phosphate solubilization, Hydrogen Cyanide (HCN) production and antifungal activities. All the six bacterial strains that showed plant growth promoting activities were subjected to cell wall degrading enzyme production and intrinsic antibiotic resistance. The selected six strains were further identified as Klebsiella sp., Bacillus sp., Rhizobium sp., Pseudomonas sp., and Azotobacter sp. Our results showed that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers and these PGPR isolates may be used as biofertilizers to enhance the growth and productivity of commercially grown medicinal and aromatic plants under local agro-climatic conditions of Bihar.*

Key words: *PGPR, Biofertilizer, Ocimum sanctum, Rhizosphere.*

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Introduction :

India has one of the richest medicinal plant cultures in the world. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers all plants as potential sources of medicinal substances (Malleswari and Bagyanarayan, 2013).

There are thousands of herbal plants in the world but Tulsi (*Ocimum sanctum*) is considered to be the "queen of herbs" due to its greater medicinal values (Asha et al., 2011). Medicinal plants support a great diversity of microflora surrounding

the root. The beneficial microorganisms surrounding the roots provide nutrients for the plants. They not only promote plant growth but also help in sustainable agricultural development and protecting the environment .All this activity makes the rhizosphere the most dynamic environment in the soil (Madhuri and Sahasrabudhe, 2011).

Numerous microorganisms such as algae, bacteria, protozoa and fungi coexist in the rhizospheric region, but bacteria are the super abundant among them. The bacteria colonizing this habitat are called "Rhizobacteria". A large number of plant growth promoting rhizobacteria(PGPR) including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Alkaligenes, Arthrobacter, Bacillus, Rhizobium* and*Serratia* are reported to enhance plant growth (Malleswari and Bagyanarayan, 2013).

The PGPR inhabiting plant roots exert a positive effect ranging from direct influence mechanisms to an indirect effect**.** Direct mechanisms include fixation of atmospheric nitrogen, solublization of minerals such as phosphorus and synthesis of plant growth hormones i.e. indole-3-acetic acid (IAA), cytokines, and ethylene. PGPR also help in enhance other beneficial bacteria or fungi, control fungal and bacterial diseases and help in controlling insect pests (Saharan and Nehra, 2011).

Indirect mechanisms involves the biological control of plant pathogens and deleterious microbes through the production of antibiotics, lytic enzymes, hydrogen cyanide, and catalase or through competition for nutrients and space can improve significantly plant health and promote growth as evidenced by increase in seedling emergence, vigor and yield (Malleswari and Bagyanarayan, 2013).

PGPR are environmental friendly and offer sustainable approach to increase production of crops and health. Direct use of these microorganisms to promote plant growth and to control plant pests continues to be an area of rapidly expanding research as the capacity of specific root-colonizing bacteria, or rhizobacteria, to increase growth and yield of crop plants currently is attracting considerable attention (Suslow et al.1979). Therefore, these isolates can also be explored for bioprotectants (suppression of plant disease), biostimulants (phytohormone production) or biofertilizer formultion (improved nutrient acquisition), under local agro-climatic conditions of Patna, Bihar (Saharan and Nehra, 2011 and Malleswari and Bagyanarayan, 2013).

Materials and Methods :

Collection of soil samples : Soil samples were collected from the rhizosphere of medicinal plant, Tulsi (*Ocimum sanctum)* growing locally in Phulwarisharif, Patna, Bihar. Intact root system was dug out and the soil samples from the rhizosphere were carefully collected in plastic bags and stored at 4°C.

Isolation and Preliminary screening of the bacterial isolates : The plant growth promoting rhizobacteria (PGPR) were isolated from the rhizospheric soil samples by serial dilution plate technique in which dilution series upto10^{-7} was prepared. 100µL of each sample dilution of $10^{-7}$was spread on Nutrient and King's B agar plates respectively as described by Malleswari and Bagyanarayan (2013). The plates were incubated at 30°C for 24-48 h. Colonies were picked from these plates and maintained as pure cultures in respective medias with periodic transfer to fresh media and stocked for further use. All the isolates were maintained at 4°C.

***In vitro* screening of isolates for multiple plant growth promoting activities**

Screening for Indole acetic acid production : Fifty milliliter of Luria Bertani (LB) broth containing 0.1% DL tryptophan was inoculated with 24 h old bacterial cultures and incubated in refrigerated incubator shaker at 30±0.1°C at 180 rpm for 48 h in dark. The bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4°C. Estimation of indole-3-acetic acid (IAA) in the supernatants was done using colorimetric assay.

Colorimetric estimation: Two millilitre of supernatant was mixed with 4 ml Salkowski reagent (1ml of 0.5M $FeCl_3$in 50ml of 35% $HClO_4$) and absorbance of the resultant pink color was read after 30 min at 535 nm in calorimeter. Appearance of pink color in test tubes indicated IAA production. The IAA production was calculated from the regression equation of standard curve and the result was expressed as mg/ml over control (Malleswari and Bagyanarayan, 2011). Standard graph of IAA was prepared as mentioned by Madhuri and Sahasrabudhe (2011).

Screening for ammonia producion : All the bacterial isolates were tested for the production of ammonia as described by Malleswari and Bagyanarayan (2013). Isolates were grown in peptone water at 30°C for 4 days. At the end of incubation period, 1 ml of Nessler's reagent was added to each tube. The development of faint yellow to dark brown color indicated the production of ammonia.

Screening for HCN production : All the isolates were screened for the production of Hydrogen cyanide by adapting the method of Ahmad et al. (2006). Briefly, Nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 28° C for 4 days. Development of orange to red colour indicated HCN production.

Screening for Phoshphate solubilization : All isolates were screened on Pikovskaya's agar plates for phosphate solubilization as described by Malleswari and Bagyanarayan (2013). Bacterial cultures were inoculated on centre of agar plate. Inoculated plates were incubated for 3 days at 30°C. Appearance of clear halo zone on Pikovskaya's agar plates indicates positive phosphate solubilization ability.

Antifungal activity : The bacterial isolates were screened for the ability to inhibit the pathogenic fungi *Alternaria sp.* by employing dual culture method on Potato Dextrose Agar (PDA) plates. Individually a loopful of 2 day old bacterial cultures grown in nutrient agar media was streaked on one side leaving 1 cm from the margin, and then loopful of fungal pathogen culture was placed at the other side. Plates without antagonist served as control. The plates were inoculated at 26±2°C for 4-5 days.

Screening of bacterial isolates for hydrolytic enzyme production

Production of Protease : The qualitative assay for protease production was performed on sterile Skimmed milk agar plates. Isolates were spot inoculated followed by incubation at 30°C for 24 to 48 hours and zone of clearance around the colony indicated the enzymatic degradation of protease (Duza and Mastan 2013).

Production of Amylase (Starch hydrolysis): Isolates was streak inoculated on starch agar plates. The plates were incubated for 24-48 hours at 37°C. After incubation, the plates were flooded with iodine solution with dropper for 30 second .The excess of iodine solution was poured off and

examined for starch hydrolysis around the line of growth of organism (Duza and Mastan 2013).

Production of Cellulase : Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 minutes at room temperature. One molar NaCl was thoroughly used for counter staining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis (Duza and Mastan 2013).

Catalase activity: The selected PGPR isolates were streaked on nutrient agar medium and incubated for 48 h at 30°C, while holding the inoculated plate, 3-4 drops of hydrogen peroxide (H_2O_2) was allowed to flow over the growth of each plate. Observations were made for the production of gas bubbles within a minute indicating the production of catalase (Duza and Mastan 2013).

Intrinsic Antibiotic Resistance (IAR) test : IAR test was carried out to identify the bacterial sensitivity or resistance to antibiotics A plate of suitable culture medium was incubated by spreading an aliquot of bacterial culture evenly across the agar surface. Filter paper discs containing different concentration of antibiotics, Streptomycin 10 µg mL^{-1}, Ampicillin 30 µg $mL^{-1}$and Tetracycline 30 µg mL^{-1} were then placed on the plate. Plates were incubated at 28 ± 2°C for 3 days. The presence of inhibition zones around the discs of the different antibiotics were noted (Yasmin et al., 2009).

Identification : Identification of the isolates was done by morphological characterization which included Gram staining, study of colony colour, texture, margin, shape and elevation. Biochemical characterization of isolates was done by performing following test which included Carbohydrates fermentation (Lactose and Dextrose) test, Urease test, Hydrogen sulphide production test, Nitrate Reduction test, Gelatin hydrolysis test and IMViC test (Aneja, 2007).

Result and Discussion :

Plant rhizosphere is known to be preferred ecological niche for soil microorganisms due to rich nutrient availability. In the present study, isolation of bacterial strains from the rhizosphere soil samples of *Ocimum sanctum* was made. The rhizosphere soils supported a total of 16 PGPR isolates with diversified characteristics suggesting the importance and richness of the niche as a source of plant microbe interactions. These bacterial isolates were picked up on the basis of differences in their colony morphology. Among 16 isolates, 6 strains were selected on basis of difference in physical characteristics. All the isolates were screened for their plant growth promoting activities *viz*., ammonia production, indole acetic acid (IAA) production, phosphate solubilization, HCN production, and antifungal activity. In the rhizosphere of *Ocimum sanctum*, microbial population was high and these bacterial strains possessed multiple PGP activities. The range of percentage of positive isolates for each of PGP activities varied greatly. The results showed that not all the 6 isolates possessed all PGP activities. These results will lead to developing a biofertilizer consortium for these commercially grown medicinal plants (Malleswari and Bagyanarayan, 2013)

In our study, all the strains were able to produce significant amount of IAA. IAA production ranges from 4.0ìg/ml to 28.5 ìg/ml. The results were in accordance to Azeem et al., 2007 who reported that the differences differences in the performance of auxin-producing PGPR may be attributed to the inherent properties of the individual bacteria (as shown in Figure 1 and 2).

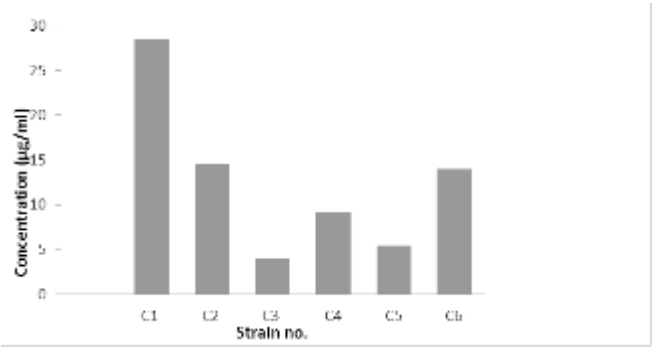

Figure 1. Graph showing IAA Production by the isolates

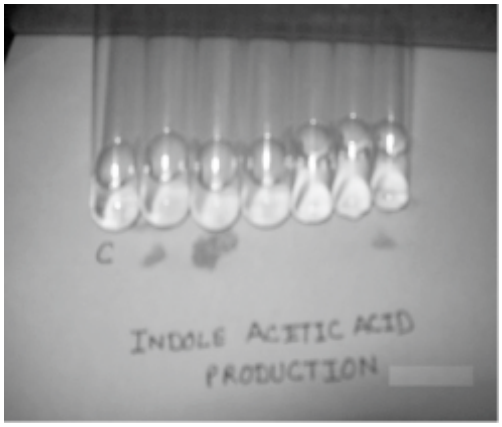

Figure 2. IAA production test

Another important trait of PGPR is the production of ammonia that indirectly influences the plant growth. All the selected isolates (C1, C2, C3, C4, C5 and C6) were positive for ammonia production (as shown in Figure3). According to Kumar et al (2012) production of ammonia is another important trait of PGPR that indirectly influences the plant growth.

Figure 3. Ammonia production test

HCN known to be both beneficial and harmful property for plants. The production of HCN in excess may play a critical role in the control of fungal diseases. Among the six isolates, only one strain (C1) was recorded positive for HCN production as indicated by the colour of filter paper from orange to brown colour (Figure 4). The results obtained were similar to the work done by Kumar et al (2012).

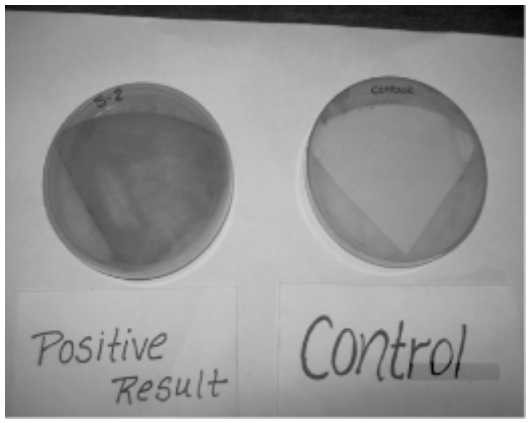

Figure 4. HCN production

Out of the six bacterial isolates, two strain (C1 and C5) were able to solubilize Phosphate in the plate-based assay, as evidenced by the formation of a clear halo around the colony as shown in Figure 5. The work was relevant in accordance to Dhanya et al (2013) and Kumar et al (2012) that higher concentrations of phosphate- solubilizing bacteria are commonly found in the rhizosphere soil as compared to non rhizospheric soil.

Figure 5. Phosphate solubilization

Antifungal activity was estimated from the inhibition of mycelial growth of fungus in the

direction of actively growing bacteria observed in strains C2, C4 and C5.

Antagonistic microorganisms are a potential non-chemical means of plant disease control. Many strains of *Bacillus* have been shown to be potential biocontrol agents against fungal pathogen. The results obtained were similar to work of Malleswari and Bagyanarayan (2013) who also reported antifungal activity in *Bacillus subtilis* and *Pseudomanas* sp. isolated from medicinal plant.

Bacterial strains which showed PGP activities are shown in Table1

Table 1. Bacterial Isolates with different plant growth promoting activities

PGPR Isolates	Ammonia Production	HCN Production	IAA Production µg/ml	Phosphate Solubili-zation	Antifungal Activity
C 1	+	+	28.5	+	-
C 2	+	-	13	-	+
C 3	+	-	4.0	-	-
C 4	+	-	10	-	+
C 5	++	-	5.0	+	+
C 6	+	-	12	-	-

++ =Maximum, + =Positive, - = Negative

The bacterial strains that produce different hydrolytic enzymes (Table 2) affect antagonistically the soil fungi. Strain C2 and C5 showed positive result for Protease production. Strain C1, C2, and C3 were positive for amylase production and form zone around bacterial growth. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. All the selected strains were negative for catalase and cellulase enzyme production.

Table 2. Bacterial isolates with Hydrolytic enzyme production

PGPR Isolates	Catalase Production	Cellulase Production	Protease Production	Amylase Production
C 1	-	-	-	+
C 2	-	-	+	+
C 3	-	-	-	++
C 4	-	-	-	-
C 5	-	-	+	-
C 6	-	-	-	-

++ =Maximum, + =Positive, - = Negative

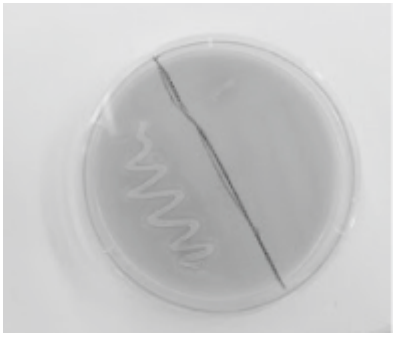

Figure 6. Protease production

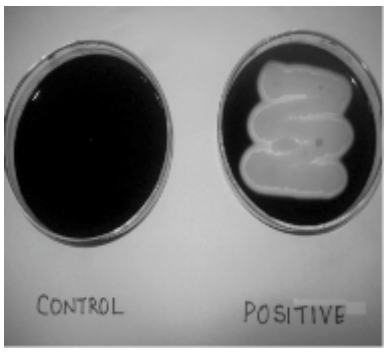

Figure 7. Amylase production

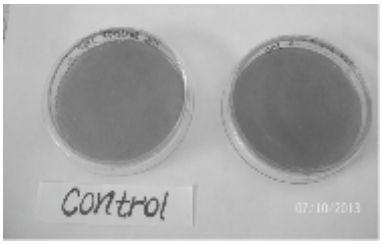

Figure 8. Cellulase test

Intrinsic antibiotic resistence test was also performed (Table 3a, 3b). Strain C1 was resistance to streptomycin and strain C2 was resistant to tetracyclin while the strain C3 was sensitive to all antibiotics. Strain C4 and C5 were resistance to ampicilin while the strain C6 was resistant to all antibiotics (Figure 9). Productions of antibiotics which are deleterious to the growth or other metabolic activities of other microorganisms (Yasmin et al., 2009). The antibiotic production is an important mechanism. 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 (Malleswari and Bagyanarayan, 2013).

Table 3(a). Bacterial Isolates with average value of zone of inhibition

PGPR Isolates	Zone of inhibition in cm		
	Ampicilin 30 µg mL^{-1}	Tetracyclin 30 µg mL^{-1}	Streptomycin 30 µg mL^{-1}
C1	0.9	0.5	0
C2	1.3	0	1.1
C3	0.8	1	1.4
C4	0	1.4	1.3
C5	0	0.5	1.8
C6	0	0	0

Table 3(b). Bacterial Isolates showing Intrinsic Resistance Test

PGPR Isolates	Zone of inhibition		
	Ampicilin 30 µg mL^{-1}	Tetracyclin 30 µg mL^{-1}	Streptomycin 30 µg mL^{-1}
C1	+	+	-
C2	++	-	+
C3	+	+	++
C4	-	+	+
C5	-	+	++
C6	-	-	-

(where,++ =Highly Sensitive, + =Less Sensitive , - Resistant)

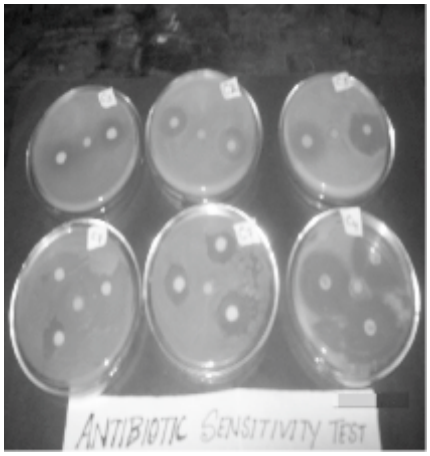

Figure 9. Antibiotic Assay

The results of morphological and biochemical characteristics (Table 4a, 4b) of all the selected six strains were referred to Bergey's Manual of Determinative Bacteriology for further identification and the strains were identified as,C1 –*Klebsiella* sp. ,C2 –*Bacillus* sp., C3 –*Rhizobium* sp.,C5 - *Pseudomonas* sp., and C6 –*Azotobacter* sp. The strain C4 was unidentified (Table 4c).

The results were in accordance to work of Raval and Desai (2012) and Malleswari and Bagyanarayan (2013) who reported that *Bacillus* and *Pseudomonas* sp are the dominant genera in rhizosphere of medicinal plants.

Table 4(a). Bacterial colonies with morphological characteristics

PGPR Isolates	Colour	Texture	Margin	Shape	Elevation
Colony 1	Creamy	Slimy	Regular	Circular	Flat
Colony 2	Creamy	Slimy	Irregular	Circular	Opaque
Colony 3	White	Slimy	Regular	Circular	Flat
Colony 4	White	Rough	Regular	Circular	Opaque
Colony 5	Creamy	Slimy	Entire	Circular	Flat
Colony 6	White	Rough	Irregular	Circular	Flat

Table 4(b). Bacterial isolates with Gram's Staining and Identification

PGPR isolates	Gram's Staining	Shape Observed
Colony 1	Negative	Coccobacillus
Colony 2	Positive	Rod
Colony 3	Negative	Rod
Colony 4	Positive	Bacilli
Colony 5	Negative	Coccobacilli
Colony 6	Positive	Bacilli

Table 4(c). Biochemical characterization of bacterial isolates

PGPR Isolates	MR test	VP test	Urease test	H2S prod.	Nitrate red. Test	Citrate Utiliza-tion	Gelatin hydro-lysis	Indole prod. Test	Fermentation test			
									Lac.		Dex.	
									A	G	A	G
C1	++	-	+	-	+	+	-	+	-	-	+	-
C2	-	+	+	-	+	+	-	-	-	-	+	-
C3	-	-	+	-	+	-	-	+	-	-	-	-
C4	++	+	+	-	-	+	+	+	-	-	+	-
C5	+	+	+	-	+	-	-	+	-	-	+	-
C6	+	-	+	-	+	+	-	+	-	-	+	-

(*where, ++ =Maximum, + =Positive, - = Negative)

Conclusion :

From the present study, it can be concluded that PGPR are able to produce IAA, solubilize the phosphate and show antagonism towards pathogens thereby may improve the growth of plants. In addition, production of enzymes and resistance of PGPR to several antibiotics also have an ecological advantage of survival in the rhizosphere when they are introduced as inoculum. The selected effective PGPR isolates *Klebsiella* sp. (C1), *Bacillus* sp. (C2), *Pseudomonas* sp. (C5)

which as a result of their multiple PGPR traits, could prove effective in improving the seed germination rate, plant growth and vigor index of plants apart from *Rhizobium* (C3) and *Azotobacter* (C6) which are already being used in plant growth promotion. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers which is an efficient approach to replace chemical fertilizers and these PGPR isolates may be used as biofertilizers to enhance the growth and productivity of commercially grown medicinal and aromatic plants under local agro-climatic conditions of Bihar.

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