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Plant Growth Characteristics of Bacteria Isolated from Rhizosphere Region of *Santalum album*

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Plant growth promoting rhizobacteria (PGPR) have gained increasing importance in recent past due to agricultural benefit. On account of that, an attempt was made in the present investigation to study plant growth promoting activities of bacteria isolated from rhizospheric region of *Santalum album* (Sandalwood) plant. A total of ten bacteria were isolated from the soil sample and amongst them two prominent phosphate solubilizers were selected for biochemical characterization and further study on plant growth promoting activities. The most potential among the two was tried with different crop plants to determine effect on germination and plant growth under laboratory and field conditions. Percent germination of *Vigna radiata* (Mung bean) in germination paper is 30%, and 6% in tray with sterilized soil & 5% in Pot culture method increased significantly over respective controls. A significant increase in seedling vigor index. Root length and shoot length at P ≤ 0.05 significant level was also observed. Morphological and biochemical characterization suggests the organism belongs to the genera *Bacillus.* 16S rRNA gene sequence submitted to NCBI gene bank was assigned with the accession number JQ408711.

Key words: PGPR, *Santalum album*, Siderophore, IAA, HCN, 16S rRNA gene sequencing.

Increasing human population has demanded concomitant increase in food productivity which has become a major concern to the scientist. Moreover with the negative environmental impact of artificial fertilizers with their increasing costs, sustainable and approach for organic produce has persuaded search for plant growth promoting rhizobacteria as a part of mainstream agricultural practice which involves use of microorganisms with the aim of improving nutrients availability for plants[16]. Plant Growth Promoting Rhizobacteria (PGPR) is the group of free living bacteria that enhances plant growth via various growth promoting substances[15] by different mechanisms like Phosphate solubilization and nitrogen fixation, making nutrients available for the plant, repression of soil borne pathogens by the production of hydrogen cyanide, Siderophore production and phytohormones such as indole-3-acetic acid[4]. Treatments with PGPR increase germination percentage, seedling vigor, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields etc [17,21]. In view of this an attempt was made to the present investigation to isolate and assess plant growth promoting bacteria from the rhizospheric region of

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Santalum album (sandal wood). It was found that inoculation of mung bean seeds with *Bacillus* sp. significantly increased the number and length of root & shoots and dry weight. To assess this hypothesis, Bacterial isolates were screened for their intrinsic ability to produce plant growth promoting substances and it was tried with mung bean plant and pot experiment was conducted to evaluate the efficacy of isolate to increase the germination (%), survival and other growth related characters in sterile soil and field conditions.

MATERIALS AND METHODS

Sample collection & isolation of bacteria from rhizospheric soils

Soil sample was collected from the rhizosphere region of *Santalum album* (Sandalwood) plant. Intact root system was dug out and the rhizospheric soil sample was carefully taken in plastic bags and stored at 4^0C. Microorganisms were isolated from the rhizospheric sample by serial dilution technique. Total ten different colonies were isolated and named as PGPR-1 to PGPR-10.

Identification, biochemical characterization & enzymatic activities of bacterial isolates

The colonies were then checked for biochemical identification. Bacterial isolates were thus characterized based their staining characteristics and were further investigated for their biochemical properties like Indole, catalase, urease, citrate, ammonia, nitrate producing abilities and enzymatic activities like amylase, cellulase, gelatinase, caesinase and this helped in the bacterial identification up to the genus level[11] by Bergey's manual of determinative bacteriology[12] and PIBWin software[7].

In vitro screening of isolates for different plant growth promoting activities

The isolates were screened for plant growth promoting activities and phosphate solubilization[11]. On modified Pikovskaya agar with insoluble tricalcium phosphate (TCP), a loop full of each culture was placed on the centre of agar plates and incubated at 30±0.1°C for 5 days. The amount of IAA produced by rhizobacteria is estimated quantitatively by Salkowski method[10]. The cultures were incubated in peptone broth together with tryptophan for 24 and 48 h, After 25min, the absorbance of the colour change was measured spectrophotometrically at 530 nm. Siderophore production by rhizobacterial isolates was described with several modifications[23]. The assay was performed by using CAS agar medium which contains the ternary complex CAS/Fe+3/ hexadecyltrimethyl-ammonium bromide as an indicator. Autoclaved CAS agar medium was poured in each Petri dish. The rhizobacterial inoculum was placed in the center of the medium. The plates were incubated in the dark at 30^0C for 7 days. The CAS agar colour changed from blue to orange surrounding a bacterial colony was scored as positive for Siderophore production. Isolates were further screened for their HCN producing abilities[8]. Bacterial cultures were streaked on nutrient agar medium containing 4.4 g per liter of glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. Plates were sealed with parafilm and incubated at 30±0.1 °C for 4 days.

In vitro antagonism against *Aspergillus* sp., *Fusarium* sp & *Rhizoctonia solani*

Two isolates namely PGPR-2 & PGPR -8 were tested for antibiosis against three common plant pathogen *Aspergillus* sp., *Fusarium* sp., *Rhizoctonia solani* and showed significant halo zone.

Seed germination test

Of the two bacterial isolates, PGPR 8 is more potential and it was tested for Seed germination and plant growth under laboratory conditions**.** Seeds of Moong bean (*Vigna radiata*), collected from Orissa University of Agriculture and Technology were surface sterilized with 0.1% $HgCl_2$ for 2 min and rinsed with sterile distilled water. Bacterial isolates were grown in respective broth on shaking incubator (180 rpm) at 28 ± 2°C for 24 h. Cell densities in the suspension were adjusted to a final density of approximately 10^8 CFU $seed^{-1}$.The surface sterilized seeds of Moong bean were inoculated in broth culture for 30 minutes. Germination tests were carried out by the paper towel method[13] and PGPR-treated seeds and control were seeded onto paper towels. For pot experiment, five Moong bean Seeds were soaked in inoculum for 30 minutes and were sown at 2 cm depth in pot containing 250g soil and for tray experiment 50 seeds were soaked in inoculum for 30 minutes and were sown in tray containing

sterilized soil. A control was also maintained without inoculated seed. Data of germination, growth, shoot, root length, Dry weight & Wet weight were recorded & statistically analyzed by Student '*t*' test. All tests were conducted in triplicate and means were compared between treatments by LSD (Least significant difference) at the 0.05 confidence level. Germination percentage was measured with following formula: Germination percentage = Number of germinated seeds / Number of seeds in sample × 100.Root and shoot length of individual seedling was measured to determine the vigor index with following formula: Vigor index= (mean root length + mean shoot length) × % germination[1].

Molecular identification

After the screening for Plant growth promoting activities, Extraction and amplification of genomic DNA for 16S rRNA gene sequence analysis of PGPR -8 was carried out as it was showing all positive results. The sequencing was done at Xceleris Laboratories Pvt. Ltd., Ahmadabad. The 16S rRNA gene fragment was amplified by using universal primers. Based on 1300 bp long 16S rRNA gene sequences, phylogenetically related bacteria were aligned by using a BLAST search against the GenBank database. Multiple alignments with sequences of related taxa of the genus *Bacillus* were implemented by using CLUSTAL W[3]. The molecular phylogeny has been study by using the computational tools MEGA and BIOEDIT.

RESULTS AND DISCUSSION

Identification and characterization of bacterial isolates

Soil sample was collected from the rhizospheric region of *Santalum album* (Sandalwood) from OUAT premises, India and from ten bacterial isolates only two i.e. PGPR-2 & 8 showed positive results for biochemical and enzymatic activity (Table1).

Plant growth promoting activities of the bacterial isolates

In search of efficient PGPR strains with multiple activities, total of ten bacterial isolates were screened for phosphate solubilization on modified PVK agar, of which two isolates showed the development of sharp phosphate solubilization zones i.e. PGPR-2(13mm) & PGPR-8(20mm). Similar findings were also observed[22] while solubilize precipitated phosphates and enhance phosphate availability to chickpea that represent a possible mechanism of plant growth promotion under field condition. The two bacterial isolates also able to produce Siderophore whereas iron is an essential growth element for all living organisms including plant pathogens and the scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition[18]. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere[4]. The ability of bacteria to produce phytohormone like Auxin i.e. IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. The bacterial isolates also produced plant growth promoting hormone i.e. IAA. The two bacterial isolates also exhibited strong production of ammonia, which is taken up by plants as a source of nitrogen for their growth[2].

Antibiosis

Two isolates namely PGPR-2 &-8 were tested for antibiosis against three common plant pathogen *Aspergillus* sp., *Fusarium* sp. and *Rhizoctonia solani* and showed significant halo zone(Table-2).

Seed germination test

In this study, an increase in the plant growth by seed bacterization has been demonstrated. It is a well-established fact that improved phosphorous nutrition influences overall plant growth and root development[14]. Among the two bacterial isolates PGPR 8 is more potential than PGPR-2 & positively affected the germination of *Vigna radiata* seeds. Highest root elongation was recorded when seeds were pre-treated with PGPR-8 isolate. PGPR-8 showed significant increase in germination, root length, shoot length in germination paper, tray and pot culture method (Fig.1).

Molecular identification

PGPR-8 was initially identified by biochemical characterization, and 16S rRNA homology of 1300-bp partial sequence confirmed that PGPR-8 belongs to *Bacillus* genus and it was assigned with the accession number JQ408711. *Bacillus* sp. result corroborated with the fact of their abundance in soil and high adaptability to

Table 1. Biochemical characterization & Enzymatic activities of bacterial isolates

Isolate code	Gram Staining	I	MR	VP	C	Cat	U	Lac	Glu	Mn	Fru	Su	Nitrate	Amylase	Cellulase	caesinase	Gelatinase	Ammonia	Organism
PGPR-2	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+	+	*Bacillus* sp.
PGPR-8	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	*Bacillus* sp.

I- indole, MR- methyl red , VP-vogues Lac – lactose, Glu-glucose, Mn- Mannitol, Fru- fructose Su-sucrose

Table 2. Bacterial isolates showing different plant growth promotion activities and Antibiosis

Isolate code	IAA	Siderophore	Phosphate	HCN	*Aspergillus* sp.	*Fusarium* sp.	*Rhizoctonia solani*	Organism
PGPR-2	+	-	+	-	+(20mm)	+(18mm)	+(15mm)	*Bacillus* sp.
PGPR-8	+	+	+	-	+(15mm)	+(22mm)	+(20mm)	*Bacillus* sp.

- : Negative, +: Positive

the rapidly changing environmental conditions, In this case, bacterial population inhabiting in the rhizospheric region of the plants overcome the limiting nitrogen and phosphorus content[19]. In the phylogenetic tree, PGPR-8 and other *Bacillus* species were grouped together (Fig-4)

Table 3. Standard deviation (S.D),'t' value & least significant difference (LSD) at 0.05 level

Moong bean	Standard deviation	Control	*Bacillus* sp.JQ408711
Root length , Germination paper	S.D	± 3.47	±3.59
t value		3.52	
Shoot length, Germination paper	S.D	±3.68	± 4.04
t value		3.86	
Root length, Tray	S.D	± 0.74	±2.54
t value		0.71	
Shoot length,Tray	S.D	± 0.2	±0.40
t value		0.15	
Root length, Pot	S.D	± 3.67	±4.02
t value,		7.85	
Shoot length, Pot	S.D	± 1.61	± 3.92
t value		2.99	

Table 4.'t'value of Fresh weight & Dry weight of shoot and LSD at 0.05 level

Fresh wt. (gm) Germination paper	t value	1.44
Dry wt. (gm) Germination paper	t value, P≤0.05	* 0.30
Fresh wt. (gm) Tray	t value, P≤0.05	1.21
Dry wt. (gm) Tray	t value, P≤0.05	1.80
Fresh wt. (gm) Pot	t value, P≤0.05	3.79
Dry wt. (gm) Pot	t value, P≤0.05	1.95

Fig. 1. Effect On Growth Of Moong Bean In Germination Paper and Tray

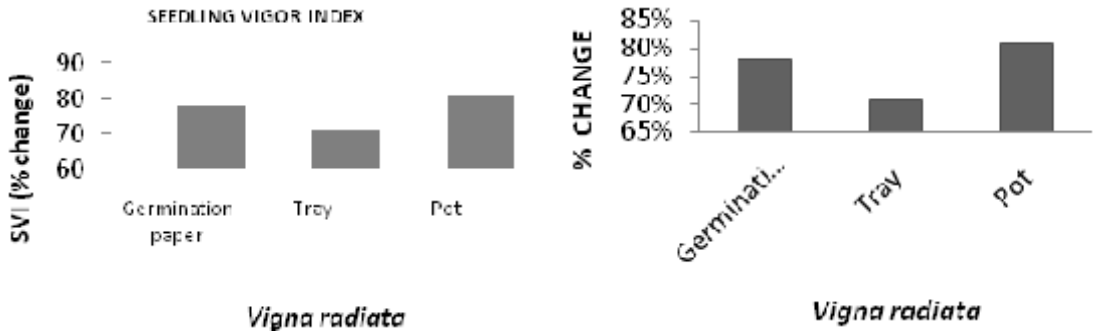

Fig. 2. Seedling vigor index & Germination percentage change of *Vigna radiata* in Germination paper, Tray and Pot

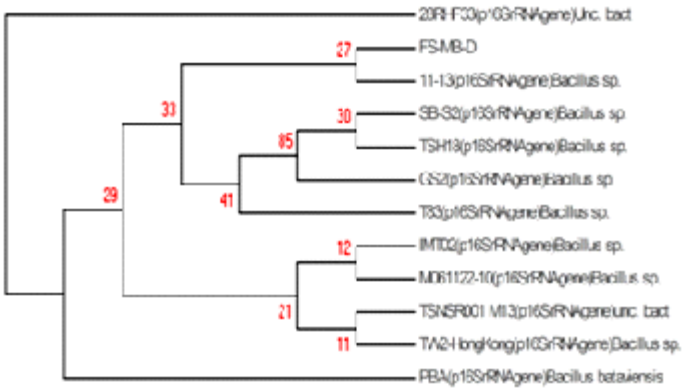

Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA sequences showing relationships between *Bacillus* sp. JQ408711 and related *Bacillus* sp

CONCLUSION

From the present study it is concluded that two Gram positive isolates were obtained from the rhizosphere region of *Santalum album* of the genus *Bacillus* showed certain PGPR characters like Phosphate solubilization, Ammonia production, Nitrate production, Siderophore production. They also showed antibiosis against various plant pathogens like *Aspergillus* sp. & *Fusarium* sp. PGPR-8 under study shows plant growth promoting characters. It is promoting growth of Mung bean plant and also significant increase in Seedling Vigor index, significant increase in germination index, significant increase in root length and shoot length. Of the two bacterial isolates viz. PGPR-2 & PGPR-8, PGPR 8 is more potential than PGPR-2. Molecular identification 16S rRNA sequencing and genomic DNA isolation of PGPR-8 was assigned with the accession no. JQ408711 by NCBI, USA GenBank. PGPR can affect plant growth directly and indirectly. Studies on the use of PGPR inoculants have been conducted under lab condition. Thus, the potential bacteria *Bacillus* sp (JQ408711) further investigated to increase productivity under field condition and application of PGPR strains can provide plant growth as well as effective, economical and practical way of plant protection via disease suppression.

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