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RESEARCH ARTICLE

SCREENING OF RHIZOBACTERIA FOR PGPR AND ANTAGONISTIC ACTIVITIES ISOLATED FROM MEDICINAL PLANTS

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ABSTRACT

Rhizosphere soils from nine medicinal plants were used to identify the plant growth promoting ability of rhizobacteria. The bacterial isolates were purified and identified based on their morphological and biochemical characteristics. Altogether twenty nine bacterial species were identified belonging to seven genera (*Staphylococcus, Micrococcus, Bacillus, Pseudomonas, Neisseria, Serratia, Streptococcus*). All the isolates were identified up to genus level except the potential isolates. Results showed that the colonization of rhizobateria vary among the medicinal plants. The rhizobacterial isolates were screened for their plant growth promoting activity such as Ammonia, Indole acitic acid, Phosphate solubilization and Hydrogen cyanide production. Among the isolates *Pseudomonas* sp from *Leucas aspera* and *Bacillus* sp *Cleome viscosa* gave better result for almost all the test. However *Bacillus* sp effectively inhibited the fungal pathogen. Hence it was characterized through 16s r DNA sequencing and identified as *Bacillus cereus* NK2.

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INTRODUCTION

The rhizosphere is a very complex environment in which the effects of plant on soil microorganisms and the effects of microorganisms on plant are interacting and are interdependent (Mukerji *et al*., 2006). Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant root and increase plant growth (Deshwal *et al*., 2011). Tilak *et al*., (2005) demonstrated that a number of bacterial species belonging to genera *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium and Serratia* are associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth. The main mechanisms by which PGPR directly contribute to the plant growth are phytohormone production such as auxins, cytokinins and gibberellins, enhancing plant nutrition by solubilization of minerals such as phosphorus and iron, production of siderophores and enzymes, lowering of ethylene levels and induction of systemic resistance (Bhattacharyya and Jha, 2012). Keeping the above said beneficial properties and importance of medicinal plants the present investigation was carried out with the following objectives. 1. Isolation and identification of PGPR 2. Screening for plant growth promoting activities 3. Testing the ability to control plant pathogen. 4. Molecular characterization of efficient isolate.

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MATERIALS AND METHODS

Study area and sample collection

The rhizosphere soils of medicinal plants were collected from sivaganga district of Tamil Nadu, India. The collected soil samples were transported to the laboratory and kept in refrigerator (4^oC) for further process. Bacteria were isolated using serial dilution technique, 1gm of soil sample was suspended in 100ml autoclaved distilled water. After sedimentation of solid particles, dilution was made upto10^{-7}.0.1ml of each dilution was spread by L-shaped glass rod on nutrient agar. All the isolates were identified at genus level based on morphological and biochemical characteristics.

Indole Acetic Acid (IAA) production

Indole acetic acid production was detected as described by Bric *et al*., (1991). Bacterial isolates were inoculated in nutrient agar

amended with L-Tryptophen and incubated at 37°C for 48hrs. Fully grown cultures were centrifuged at 3000rpm for 30 minutes, the supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowaski reagent (50 ml 35% of perchloric acid, 1ml of 0.5m$FeCl_3$ solution). Development of pink colour was indicative of IAA production.

Phosphates solubilization

Phosphate solublizing ability of the isolate was checked on Pikovskaya (PVK) medium (Pikovskaya1948), incorporated with tricalcium phosphate ($Ca_3 (PO4)_2$). The isolates were spot inoculated on PVK medium. Formation of transparent halo zone around the developing colonies indicated phosphate solublizing ability of the isolates.

Assay for Ammonia production

The rhizobacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated into 10 ml peptone water and incubated at 30°C for 48 hrs. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappucino and Sherman, 1992).

Hydrogen Cyanide production (HCN)

Production of HCN was determined using the modified procedure of Millar and Higgins (1970). All the bacterial strains were grown on Trypticase Soy Agar (TSA) plates. Sterilized Whatman No. 1 filter paper strips were soaked in picric acid solution (2.5 gm of picric acid, 12.5gm of Na_2CO_3, in 1000ml of distilled water). The strips were placed in the lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28°±1°C for 48 hrs. A change in colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN by each strain, respectively.

Antifungal activity

Antifungal activity was estimated from the inhibition of mycelia growth of fungus in the direction of actively growing bacteria. The percentage of inhibition was calculated using the following formula.

% inhibition = (R-r) /R X100

Where 'r' is the radial growth of the fungal colony opposite the bacterial colony and R is the radial growth of the pathogen in control plate (Rabindran and Vidyasekaran, 1996).

Identification of Potential isolate

Molecular characterization of potential isolate was done by PCR amplification of 16s rDNA gene. The 16s rDNA gene of isolate was sequenced using universal primer. The sequence of the isolate was deposited in NCBI and the sequence comparison was done with EMBL.

RESULTS AND DISCUSSION

Plant growth promoting rhizobacteria (PGPR) are usually applied to a wide range of agricultural crops for the purpose of growth enhancement, including increased seed germination, plant weight, and harvest yields. PGPR colonization triggers plant growth by bacterial synthesis of plant hormones including indole-3-acetic acid, cytokinin, and gibberellins as well as by increased mineral and nitrogen availability in the soil. Some of them were also known to protect their host plant from pathogenic microorganisms (Malleswari *et al*., 2014). In our investigation twenty nine rhizobacteria were isolated from nine medicinal plants *viz*., *Phyllanthus amarus, Leucas aspera, Ocinum basillium, Acalypha indica, Adhathoda vesica, Centella asiatica, Cleome viscosa, Cadiospermum helicobabum, Solanium nigrum*. *Leucas aspera* showed more number of bacterial colonization followed by *Cadiospermum helicacabum*.

Table: 1.1 Morphological and biochemical characteristic of isolates from *Phyllanthus amarus*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
PA1	*Staphylococcus* sp	+ cocci	White	Non-motile	-	+	+	-	+
PA2	*Micrococcus* sp	+ cocci	Slight yellow	Non-motile	-	-	+	+	+

Table: 1.2 Morphological and biochemical characteristics of isolates from *Leucas aspera*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
LA1	*Staphylococcus* sp	+ cocci	White	Non-motile	-	+	+	-	+
LA2	*Neisseria* sp	- cocci	Yellow	Non-motile	-	+	+	-	+
LA3	*Bacillus* sp	+ rod	White	Non-motile	-	-	-	+	+
LA4	*Pseudomonas* sp	-rod	Greenish yellow	Motile	-	-	-	+	+
LA5	*Micrococcus* sp	+cocci	Slight yellow	Non-motile	-	+	-	-	-
LA6	*Streptococcus* sp	+cocci	Slight yellow	Non-motile	-	+	-	-	-

Table: 1. 3 Morphological and biochemical characteristics of isolates from *Ocinum basilicum*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
OB1	*Bacillus* sp	+ rod	Pale white	Motile	-	+	-	+	+
OB2	*Bacillus* sp	+ rod	Pale white	Motile	-	+	-	+	+

Table: 1. 4 Morphological and biochemical characteristics of isolates from *Acalypha indica*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
AI1	*Neisseria* sp	- cocci	Yellow	Non-motile	-	+	-	+	+
AI2	*Bacillus* sp	+ rod	Pale white	Non- Motile	-	-	-	+	+

Table: 1. 5 Morphological and biochemical characteristics of isolates from *Adhathoda vesica*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
AV1	*Serratia* sp	-rod	Pink	Motile	-	-	+	+	+
AV2	*Micrococcus* sp	+ cocci	Slight yellow	Non-motile	-	-	+	+	+

Table: 1. 6 Morphological and biochemical characteristics of isolates from *Centella asiatica*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
CA1	*Micrococcus* sp	+ cocci	Slight yellow	Non-motile	-	-	+	+	+
CA2	*Staphylococcus* sp	+ cocci	White	Non-motile	-	+	+	-	+

Table: 1.7 Morphological and biochemical characteristics of isolates from *Cleome viscosa*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/ Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
CV1	*Bacillus* sp	+ rod	Pale white	Non-motile	-	-	-	+	+
CV2	*Bacillus cereus*	+ rod	Pale white	Motile	-	-	-	+	-
CV3	*Bacillus* sp	+ rod	Pale white	Non-motile	-	-	-	-	+
CV4	*Bacillus* sp	+ rod	Pale white	Non-motile	-	-	-	-	+

Table: 1. 8 Morphological and biochemical characteristics of isolates from *Cadiospermum helicacabum*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
CH1	*Micrococcus* sp	+ cocci	Pale white	Non-motile	-	-	-	+	+
CH2	*Bacillus* sp	+ rod	White	Non-motile	-	-	-	+	+
CH3	*Staphylococcus* sp	+ cocci	White	Motile	-	-	+	+	+
CH4	*Streptolococcus* sp	+ cocci	Yellow	Motile	-	-	+	+	+
CH5	*Neisseria* sp	- cocci	Yellow	Non-motile	-	-	-	+	+

Table: 1. 9 Morphological and biochemical characteristics of isolates from *Solanium nigrum*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	Gramstaining/Shape	Colony colour	Motility	Indole	MR	VP	Citrate	Catalase
SN1	*Bacillus* sp	+ rod	White	Non-motile	-	-	-	-	+
SN2	*Bacillus* sp	+ rod	White	Non-motile	-	-	-	+	+
SN3	*Neisseria* sp	- cocci	Yellow	Non-motile	-	-	-	+	+
SN4	*Pseudomonas* sp	-rod	Greenish yellow	Motile	-	-	-	-	+

Table: 2.1. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Phyllanthus amarus*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN production	Phosphate solubilization	Zone of inhibition (%)
PA1	*Staphylococcus* sp	_	+	-	-	-
PA2	*Micrococcus* sp	+++	+++	+	-	20.00

Table: 2.2. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Leucas aspera*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN production	Phosphate solubilization	Zone of inhibition (%)
LA1	*Staphylococcus* sp	++	+	-	-	-
LA2	*Neisseria* sp	+++	+++	+	-	-
LA3	*Bacillus* sp	-	+	-	+	-
LA4	*Pseudomonas* sp	+++	+++	+	-	30.00
LA5	*Micrococcus* sp	-	++	-	-	-
LA6	*Streptococcus* sp	-	++	-	-	-

Table: 2.3. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Ocinum basilicum*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN production	Phosphate solubilization	Zone of inhibition (%)
OB1	*Bacillus* sp	_	+	-	-	10%
OB2	*Bacillus* sp	++	+	-	-	-

Table: 2.4. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Acalypha indica*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN production	Phosphate solubilization	Zone of inhibition (%)
AI1	*Neisseria* sp	_	+	-	-	-
AI2	*Bacillus* sp	++	+	-	-	-

Table: 2.5. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Adhathoda vesica*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN production	Phosphate solubilization	Zone of inhibition (%)
AV1	*Serratia* sp	++	+	-	-	-
AV2	*Micrococcus* sp	++	+	-	-	-

Table: 2.6. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Centella asiatica*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN Production	Phosphate solubilization	Zone of inhibition (%)
CA1	*Micrococcus* sp	_	+	-	-	-
CA2	*Staphylococcus* sp	++	+	-	-	-

Table: 2.7. Growth promoting and antagonistic activity of Rhizobacterial isolates from *Cleome viscosa*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN Production	Phosphate solubilization	Zone of inhibition (%)
CV1	*Bacillus* sp	+++	+++	++	-	20.00
CV2	*Bacillus cereus*	+++	+++	++	+	60.00
CV3	*Bacillus* sp	+++	+++	++	-	-
CV4	*Bacillus* sp	+++	+++	++	-	-

Table: 2.8. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Cadiospermum helicacabum*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN Production	Phosphate solubilization	Zone of inhibition (%)
CH1	*Micrococcus* sp	_	+++	-	-	-
CH2	*Bacillus* sp	++	+++	++	-	-
CH3	*Staphylococcus* sp	++	+++	++	-	50.00
CH4	*Streptolococcus* sp	_	++	-	-	-
CH5	*Neisseria* sp	_	+	-	-	-

Table: 2.9. Growth promoting and antagonistic activity of Rhizobacterial isolates of *Solanium nigrum*

Rhizobacterial Isolates	Name of the Rhizobacterial Isolates	NH_3 Production	IAA Production	HCN Production	Phosphate solubilization	Zone of inhibition (%)
SN1	Bacillus sp	+++	+++	++	-	-
SN2	Bacillus sp	+++	+++	-	-	-
SN3	Neisseria sp	+++	+++	-	-	-
SN4	Pseudomonas sp	+++	+++	-	-	-

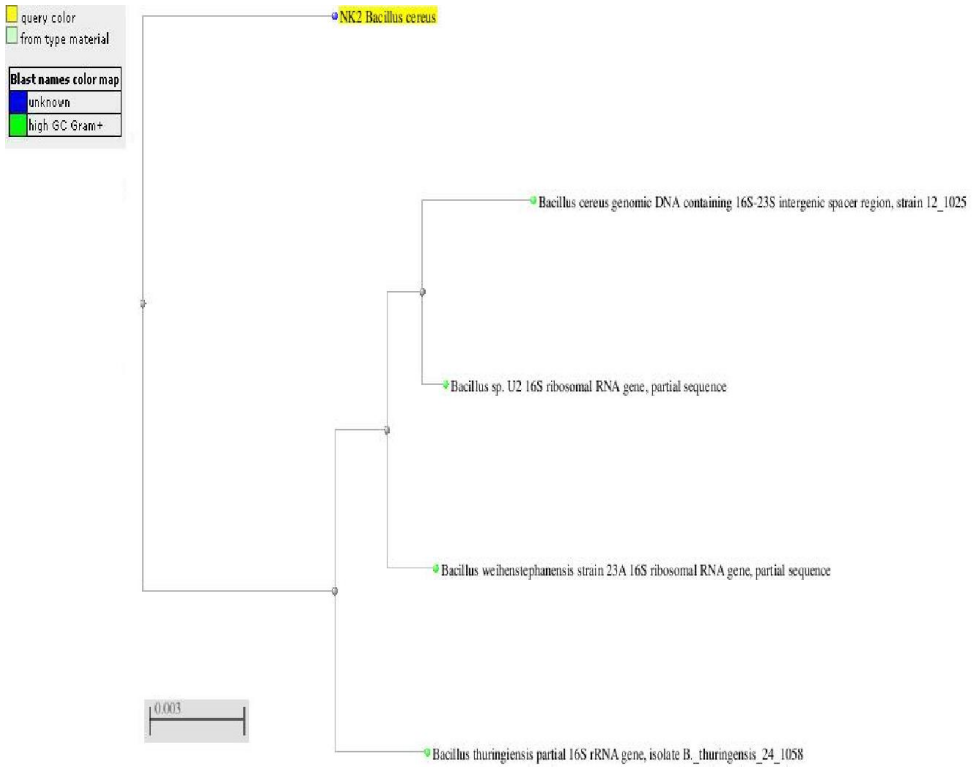

Fig 1. Phylogenitic analysis of Potential isolates

Similarly Ahmed *et al*., (2014) isolated hundred and twelve bacterial culture from eleven medicinal plants. The isolates were identified on the basis of morphological and biochemical characteristic (Table 1.1 to 1.9). They were tested for their plant growth promoting activities and antifungal activities (Table 2.1 to 2.9). Among the twenty nine isolates only few isolates showed better results (Kavith *et al*., 2014). *Pseudomonas* sp from *Leucas aspera* and *Bacillus* sp *Cleome viscosa* gave very good result for almost all the test. *Bacillus* sp *Cleome viscosa* effectively inhibited the fungal pathogen *Fusarium sambucinum* (60%) than *Pseudomonas* sp from *Leucas aspera* (30%). Based on the above result *Bacillus* sp was selected for molecular characterization through 16s rDNA sequencing. The sequences were deposited in Gen Bank and we got the accession number KR909310.

The sequence comparisons with the sequence in the EMBL revealed that the sequence of the isolates is similar (98%) to the existing rhizobacterial clone of *Bacillus cereus*. The phylogenetic relationship of this potential isolates shown in the Fig.1. This findings were supported by previous report of Malleswari, (2014) who identified the potential antagonistic isolate *Bacillus subtilis* from medicinal plant Rhizosphere.

Conclusion

The results of this present research strongly suggest that the PGPR isolated from the rhizosphere of medicinal plants possess the very good plant growth promoting and antagonistic activities. Since India has vast number of medicinal plant, the efficient isolate *Bacillus cereus* NK2 can be used to protect the endangering medicinal plants.

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