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Diversity of plant growth promoting rhizobacteria (PGPR) in rice soils of Odisha

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ABSTRACT

Numerous species of soil bacteria which flourish in the plant rhizosphere, but may grow in, on, or around plant tissues, stimulate plant growth by various direct and indirect mechanisms. These bacteria are collectively known as PGPR (plant growth promoting rhizobacteria). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are being made to exploit them commercially as biofertilizers. In this study a total of 27 bacteria having unique colony and cell morphology were isolated from the rice rhizosphere soil. These isolates were screened for plant-growth promoting (PGP) traits, including indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, P-solubilization, and siderophore, cyanide (HCN) and ammonia (NH_3) production. Percentage of isolates having IAA, ACC deaminase, P-solubilization, siderophore, HCN and NH_3 activities was 85.2%, 18.5%, 44.4%, 37%, 25.9% and 48.2% respectively. 11.1% of the total isolates did not have any PGP traits whereas the percentage of isolates having one and six PGP traits was 22.2% and 3.7% respectively. There was a positive correlation (0.630, $p<0.05$) between phosphate solubilizers and NH_3 producers and there was no significant relationship among other PGP traits in rhizobacteria. Further evaluation of the isolates exhibiting multiple PGP traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) offer an environment-friendly means for increasing productivity and sustainability in agriculture. A diverse array of free living bacteria including *Acetobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Klebsiella, Pseudomonas, and Serratia* are reported to enhance plant growth by increasing seed emergence, plant biomass, and crop yield (Glandorf *et al.,* 1994; Rodriguez *et al.,* 2008). These bacteria inhabit plant roots and affect plant growth promotion by mechanisms ranging from a direct influence such as increased solubilization of mineral phosphates and other nutrients, their uptake and/or production of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins etc to indirect effects such as suppression of plant pathogens by producing siderophores, antibiotics (Glick, 1995; Lucy *et al.,* 2004; Raaijmakers *et al.,* 2009), chitinase, b-1,3-glucanase, protease, or lipase (Chet and Inbar, 1994). Many PGPR contains the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves ACC the physiological precursor of ethylene to ammonia and α-ketobutyrate and reduce the level of stress ethylene, conferring resistance and resulting better growth of plants under various abiotic stress (Glick *et al.,* 2007).

In the last 10 years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere and also because mechanisms of action of PGPR having been deeply studied. The beneficial effects of PGPR have been

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demonstrated for many crops. However, inconsistency in their field performance, attributed mainly to poor rhizosphere competence and lack of multiple plant growth-promoting activities, is the major limiting factor in realizing the full potential of these microorganisms (Lottmann *et al.,* 2000; Ahmad *et al.,* 2008). To get maximum benefits from the inoculation, the selection of the most-effective PGPR is a pre-requisite as the use of rhizobacterial isolates directly in the field without screening is a highly laborious procedure. However, there has been no standard approach for the selection of effective PGPR.

Isolation and study of native strains that are adapted to their environment may contribute to the formulation of an inoculant to be used in regional crops. Therefore, this study was designed to isolate rhizospheric bacteria from the soil samples of agricultural fields planted to rice and to screen for their multiple plant growth promoting (PGP) traits like phosphate mobilization, production of siderophore, indole acetic acid (IAA), ammonia, hydrogen cyanide (HCN) and ACC deaminase activity in order to identify potent strain(s) to be used as candidate PGPR with rice crop.

2. Materials and methods

2.1 Sampling and characterization of soils

The root adhering soil (RAS) samples were collected from the experimental rice fields at CRRI, Cuttack during September, 2009 at the tillering stage of the monsoon season (kharif) rice. The rice plants were carefully uprooted along with the soil and brought to the laboratory in polythene bags in portable cool chambers (~4°C). The non-rhizosphere soil was removed by vigorously shaking the uprooted rice hills leaving behind the rhizosphere soil strongly adhering to the roots (Ramakrishna and Sethunathan, 1982). The soil sample was analyzed for physic-chemical characteristics according to Spark *et al.* (1996). The soil was a typic haplaquept having pH 6.16, electrical conductivity 0.5 dS/m, cation exchange capacity 15.0 meq/g soil, organic carbon 0.86 %, total nitrogen 0.09 % and contained 25.9 % clay, 21.6%slit and 52.5%sand. The rhizosphere soil was used for the isolation of bacteria.

2.2 Isolation and characterization of rhizospheric bacteria

For the isolation of rhizospheric bacteria, 1 g of closely associated rhizospheric soil was added to 9 ml of sterile water and shaken for 30 min on a mechanical rotary shaker. Six fold dilutions were made and plated on to seven media, Jensen's N free medium, Luria Bertani (LB) agar medium, nutrient agar (NA), tryptose soy yeast extract agar (TSY), 1/1000 dilution of TSY media (TSY/1000), casein–peptone–starch–glycerol agar (CPSG) and soil extract agar (SEA) and incubated for 72 hours at 30^0C (Reichardt *et al.,* 1997). Rhizobial colonies were chosen based on their colony morphology and purified by streaking on nutrient agar plates (Holt *et al.,* 1994). Bacterial cultures were maintained on the respective slants at 4 ^{0}C and in 65% glycerol at -80 ^{0}C till further use.

Morpho-physiological and biochemical characters of the bacterial isolates were examined according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Individual cultures grown on NA medium at 30^0C were examined for the colony morphological features. Motility and morphology were studied by phase contrast microscopy (Olympus BX-51, Olympus America Inc., USA). Gram staining was performed as per standard procedures with exponentially growing cultures. The bacterial isolates were tested for biochemical characteristics using standard methods (Cappuccino and Sherman, 1992).

2.3 Screening of bacterial isolates for their plant growth promoting traits

IAA production by the isolates in the presence of 0.2% L-Tryptophan (L-Trp) was detected by the method of Salkowski (Glickmann and Dessaux, 1995). Uninoculated control was kept for comparison. The quantity of IAA produced was determined by UV-VIS spectrophotometry (Specord 200, Analytic Jena, Germany) against a standard curve of IAA ranging from 0.01 to 0.1 mM (Leveau and Lindow, 2005). All measurements were made in five replicate samples and averaged.

Screening of bacterial isolates for ACC deaminase activity was based on their ability to use ACC as a sole nitrogen source in the minimal medium. The cultures were spot inoculated on DF salts minimal medium supplemented with 3 mM ACC as a nitrogen source. The plates were incubated for 3-4 days at 28 ± 2^0C. Isolates growing on DF minimal medium with ACC were purified and assayed for enzymatic activity by monitoring the amount of α-ketobutyric acid generated from the cleavage of ACC (Penrose and Glick, 2003). The ACC deaminase activity was expressed as the amount of α-ketobutyrate produced per mg of protein per hour.

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 and incubated for 48-72 h at 28 ± 2^0C. Development of brown to yellow colour following nesslerization was a positive test for ammonia production (Cappuccino and Sherman, 1992).

All the isolates were screened for the production of

hydrogen cyanide by adopting the method of Lorck (1948). 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 ± 2^0C for 4 days. Development of orange to red colour indicated HCN production.

Bacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.) described by (Louden *et al.,* 2011). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (10 ml of 10^6 CFU/ml) and incubated at 28 ± 2°C for 48-72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production.

For studying phosphate solubilization, 5 il of overnight grown culture was spotted onto Pikovskaya's agar plates containing 2% tricalcium phosphate. The plates were incubated at 28°C for 24-48 h and observed for the appearance of the solubilization zone around the bacterial colonies (Mehta and Nautiyal, 2001).

3. Result and discussion

3.1 Isolation and characterization of bacteria

A total of 46 bacteria were isolated using seven different culture media. Highest numbers of the bacteria (9) were isolated using LB agar medium which was followed by Jensen's agar medium (8) and equal numbers of bacteria (6) were isolated by using NA, TSY, TSY/1000 and CPSG agar medium and lowest number of bacteria (5) were isolated using SEA media. After growing all the isolates on NA medium to study colony morphology and cell morphology under phase contrast microscope, 27 numbers of bacteria having unique colony morphology and cell morphology were selected for further characterization Table 1. Morphological and biochemical characteristics of the 24 isolates having PGP activities were studied (Table 2). Except CR6, 7 and 46 all the strains were gram positive in reaction and almost all the isolates were rod shaped except CR29 and 33.

3.2 Plant growth promoting traits of selected isolates

Extensive research has demonstrated the potential of PGPR in plant growth improvement (Govindarajan *et al.*, 2006; Cakmakci *et al.*, 2007). The abilities of PGPR bacterial isolates on production of plant growth promoting substance (Khalid *et al.*, 2004; Bal *et al.*, 2012) have been well documented and were focused in this study. Screening results of PGP traits have been depicted in Table 1 and 3. The results showed that 11.1% of the total isolates did not have any PGP traits and 3.7% isolates showed all the six PGP traits studied. Highest number of the rhizobacteria (29.6%) showed three PGP traits. Percentage of rhizobacteria displaying single PGP traits was 22.2 % which was followed by 14.8% bacteria having five PGP traits, 11.1% bacteria having two PGP traits and 7.4% bacteria having four PGP traits.

IAA production was reported in 85.2% of the isolates (Fig.1) highlighting the enormous potential these organisms have to contribute to a plant's endogenous pool of IAA. Generally, microorganisms isolated from the rhizosphere and rhizoplane of various crops have revealed higher potential of IAA production than those from the root free soil (Sarwar and Kremer, 1995a, b; Arshad and Frankenberger, 1998).

Table 1
Isolation of bacteria from rice rhizosphere and screening for PGP traits

Media	No. of bacteria isolated	IAA	ACCd	P-Solubilization	Siderophore	HCN	NH_3
TSY (%)	4/27(14.8%)	3/23(13.0%)	1/5(20%)	2/12(16.7%)	1/10(10%)	1/7(14.3%)	2/13(15.4%)
TSY/1000(%)	6/27(22.2%)	6/23(26.1%)	0	3/12(25.0%)	2/10(20%)	2/7(28.6%)	3/13(23.1%)
NA(%)	3/27(11.1%)	3/23(13.0%)	0	1/12(8.3%)	1/10(10%)	0	2/13(15.4%)
LB(%)	2/27(7.4%)	1/23(4.3%)	0	0	1/10(10%)	0	1/13(7.7%)
Jensen's(%)	5/27(18.5%)	4/23(17.4%)	1/5(20%)	3/12(25.0%)	2/10(20%)	2/7(28.6%)	2/13(15.4%)
CSGP(%)	4/27(14.8%)	3/23(13.0%)	2/5(40%)	2/12(16.7%)	1/10(10%)	1/7(14.3%)	2/13(15.4%)
SEM(%)	3/27(11.1%)	3/23(13.0%)	1/5(20%)	1/12(8.3%)	2/10(20%)	1/7(14.3%)	1/13(7.7 %)
SUM (%)	27	23/27(85.2%)	5/27(18.5%)	12/27(44.4%)	10/27(37.0%)	7/27(25.9%)	13/27(48.1%)

Table 2
Biochemical characterization of the isolates

Biochemical test	Isolates											
	CR1	CR5	CR6	CR7	CR8	CR9	CR10	CR11	CR12	CR14	CR15	CR16
Gram reaction	+	+	-	-	+	+	+	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Thin, rod	Rod	Rod	Rod	Thick, Rod	Rod	Rod
Colony Color*	W	Y	LO	Y	P	T	T	P	W	Y	W	Y
Motility	+	+	+	-	-	+	+	+	+	+	-	+
MR	+	+	+	-	+	-	+	+	+	-	+	-
MRVP	+	-	-	+	+	-	-	-	+	+	-	+
Citrate	-	+	+	-	+	+	+	-	+	-	-	+
Nitrate	-	-	+	+	+	+	-	-	-	+	+	-
Oxidase	+	+	+	-	+	-	+	+	+	-	+	-
Catalase	-	-	-	-	+	+	-	-	+	+	-	+
Starch	-	-	+	+	-	-	+	-	-	+	-	+
Tributyrin	+	-	-	+	-	+	-	+	-	+	-	-
Tween 80	-	+	-	+	-	-	-	+	-	-	+	+
Gelatin	-	-	+	-	-	+	-	-	+	-	-	+
Casein	-	+	-	-	+	-	+	+	-	-	+	-
Urease	+	-	-	-	+	+	+	-	+	+	+	+

Biochemical test	Isolates											
	CR21	CR28	CR29	CR33	CR34	CR36	CR37	CR39	CR41	CR43	CR44	CR46
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	-
Cell shape	Rod	Rod	Coccus	Coccus	Rod	Rod	Thick rod	Rod	Thick rod	Rod	Rod	Rod
Colony Color*	T	W	W	W	W	W	BT	W	BT	W	ST	O
Motility	-	+	+	-	-	+	-	+	+	+	-	-
MR	+	-	+	+	+	-	+	-	+	+	+	-
MRVP	+	+	-	-	-	+	+	-	+	-	-	+
Citrate	-	+	-	+	+	+	-	+	-	-	+	-
Nitrate	+	-	+	+	-	+	+	-	-	+	+	-
Oxidase	-	+	+	-	-	-	-	+	+	+	-	+
Catalase	+	+	-	-	+	-	+	-	-	+	+	-
Starch	-	-	+	-	-	+	-	+	-	-	-	+
Tributyrin	+	-	-	+	-	-	+	+	-	+	+	-
Tween 80	+	+	-	-	+	-	-	-	+	+	-	+
Gelatin	-	-	+	+	-	-	+	+	-	+	+	-
Casein	+	-	+	+	-	+	-	-	-	+	-	+
Urease	-	-	+	+	+	-	-	-	+	+	-	-

*Colony color; T-Translucent; W-White; BT-Blue and translucent; ST-Semi-translucent; O-Orange; Y-Yellow; LO-Light orange; P-Pink

Table 3
PGP activities of the selected isolates from rice rhizosphere

Isolates	IAA	ACCd	P-Solubilization	Siderophore	HCN	NH_3
CR1	+	+	+	+	+	+
CR3	-	-	-	-	-	-
CR5	+	-	+	-	-	+
CR6	+	-	-	-	-	-
CR7	+	-	+	+	+	+
CR8	+	-	-	+	-	+
CR9	+	-	+	-	-	-
CR10	+	-	+	-	+	+
CR11	+	-	-	-	-	-
CR12	+	-	-	-	-	-
CR14	+	-	-	+	-	+
CR15	+	-	+	-	-	+
CR16	+	-	-	-	-	-
CR20	-	-	-	-	-	-
CR21	+	-	-	+	-	+
CR28	+	-	+	+	+	+
CR29	+	+	+	+	+	-
CR30	-	-	-	-	-	-
CR33	+	-	+	-	-	+
CR34	+	-	-	-	-	-
CR36	+	+	+	-	+	+
CR37	-	-	+	+	-	+
CR39	+	+	-	-	-	-
CR41	+	-	-	-	-	-
CR43	+	-	+	+	-	+
CR44	+	-	-	+	+	-
CR46	+	+	-	-	-	-

Microorganisms having ACC deaminase activity enhances plant growth indirectly by reducing the stress ethylene level in plant in presence of different abiotic stress. In this study only 18.5% of the rhizobacterial strains developed colony on DF salt minimal medium containing ACC as the sole nitrogen source implying presence of ACC deaminase activity in these isolates. However, presence of ACC deaminase activity was much less common than any other PGP traits. This is in agreement with earlier reports that only a minority of soil microorganisms possess ACC deaminase (Honma and Shimomura, 1978; Glick *et al.,* 1999).

Phosphorus is one of the major nutrients, second only to nitrogen in requirement for plant growth. The capacity to solubilize precipitated phosphates and enhance phosphate availability to rice is a promising attribute for the selection of bacteria capable of increasing available P in rhizosphere under field conditions (Verma *et al.*, 2001). In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere. In this study 44.4% of the total isolates were having P-solubilizing activity (Fig.1). Another important trait of PGPR, that may indirectly influence the plant growth, is the production of siderophores. They bind to the available form of iron Fe^{3+} in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plant health. In the present investigation 37% of the total isolates were siderophore producing rhizobacteria (Fig.1).

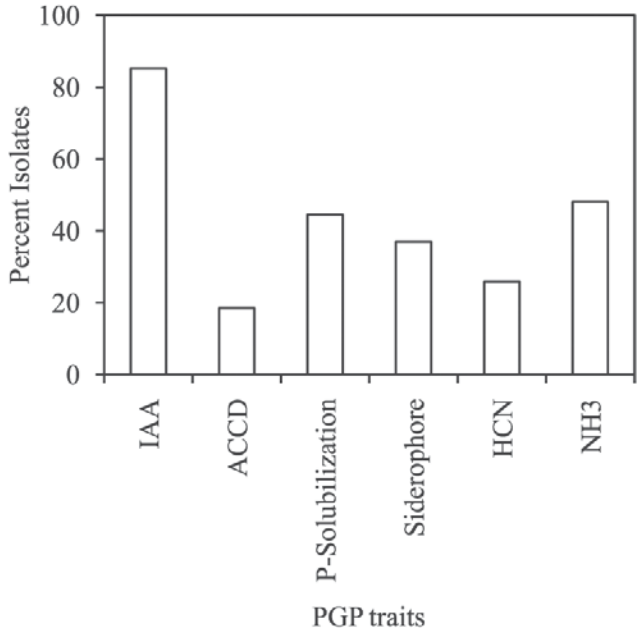

Fig.1. Percent of isolates having different PGP activities

solubilization and NH_3 production PGP traits of the isolates. Nevertheless, there was no significant relationship among other PGP traits possessing rhizobacteria.

Some of the above-tested isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. Multiple PGP activities among PGPR have been reported by other workers while such findings on indigenous isolates of India are less commonly explored (Gupta *et al.*, 1998). Further studies on the performance of such native isolates and their mutants on the growth of plant will uncover the mechanism and potential of these PGPR exhibiting multiple PGP traits.

4. Conclusion

The search for a diverse group of rhizobacteria with useful PGP traits from various crop sources paves the way for the reduction of costs associated with the use of fertilizer nutrients as well as minimizes the risk of pollution from continuous application of chemical fertilizers. Use of strains with multiple PGP properties in particular would help to increase crop productivity on a sustainable basis. The effects of PGPRs on the growth of plants are well known for many crops. Indigenous wild isolates exhibiting multiple PGP traits *in vitro* are expected to influence plant growth and yield of the crops alone or in combination with other PGPR.

Table 4
Correlation coefficients of analysis of relationship among all the six PGP traits of the isolated rhizobacteria

	IAA	ACCd	P Solubilization	Siderophore	HCN	NH_3
IAA	1					
ACCd	0.199	1				
P- Solubilization	0.163	0.149	1			
Siderophore	0.104	0.029	0.240	1		
HCN	0.247	0.371	0.491	0.421	1	
NH_3	0.193	-0.078	0.630*	0.489	0.276	1

*Significant at $p \leq 0.05$

In the present investigation 25.9% and 48.1% of the total isolates were positive for HCN and NH_3 production (Fig.1). These two traits are very important when considering field applications, as plant resistance in a non-sterile environment will be potentially increased if the associated bacteria having HCN production potential. The capacity of some bacterial species to produce NH_3 also enhances plant growth. These traits can influence plant growth in various ways, although it is probably the combination of the diverse PGP traits of the used bacterial strain that is responsible for increase in growth of the cultivated crop.

Table 4 shows the results of the correlation analysis among the PGP traits possessing rhizobacteria. There was a negative correlation (-0.078, $p \leq 0.05$) between ACC deaminase and NH_3 production traits of the isolates. There was a positive correlation (0.630, $p \leq 0.05$) between phosphate

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