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Research Article

PLANT GROWTH PROMOTING CHARACTERISTICS OF RHIZOBIAL STRAINS ISOLATED FROM ROOT NODULES OF *Vigna trilobata* CULTIVARS

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Abstract-In the present study, six *rhizobial* strains were isolated from root nodules of *Vigna trilobata* plants raised in soils collected from geographically different areas in A.P., India. All the isolates were biochemically characterized and screened in vitro for their plant growth promoting activities (PGP) *viz.* Indole Acetic Acid production, Chitinase production, Exopolysaccharide production, Siderophore and HCN production. These strains were identified as *Agrobacterium tumefaciens* MRR 108 (KC 415690), *A. tumefaciens* MRR 111 (KC 415692), *Ensifer* sp. MRR 101 (KC 428651), *Ensifer fredii* MRR 110 (KC 415691), *Rhizobium* sp. strain MRR 112(KF 621018) and *Rhizobium* sp. strain MRR 123 (KC 503884), after 16S rDNA sequencing. The results showed that the *rhizobial* isolates differ in the levels of PGP activities. All the six isolates were Indole Acetic Acid and EPS producers while *Ensifer* sp. MRR101 and *Rhizobium* sp. MRR 123 are siderophore producers and *A. tumefaciens* MRR 111 and *Ensifer fredii* MRR 110 are chitinase producers. Except, *Ensifer fredii* MRR 110 and *Rhizobium* sp. strain MRR 112 all are HCN producers. Therefore, these six strains with PGP potential are best biocontrol agents.

Keywords- PGPR, Indole Acid Acetic, Chitinase, Exo polysaccharide, HCN, Siderophore.

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Introduction

Legumes such as beans, clover, soybean and cowpea help to feed the meat producing animals as well as humans. Crop yield is achieved by nodulation by rhizobacteria. In agriculture, 80% of the biologically fixed nitrogen comes from symbiotic association between leguminous plants and bacteria of family *Rhizobiaceae*. The family *Rhizobiaceae* currently includes genera like *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium, Bradyrhizobium* etc., which are collectively referred to as *Rhizobia*.

Rhizobia are usually defined as nitrogen-fixing soil bacteria capable of forming nodules on the roots of leguminous plants in which atmospheric nitrogen is reduced to ammonia for the benefit of the plant.

Plant associated bacteria able to colonize the roots aggressively and facilitating plant growth are in general called as Plant Growth Promoting Rhizobacteria. These bacteria help plants to establish in degraded ecosystems and protect the plants from diseases thereby promoting plant growth.

Plant growth promoting rhizobacteria (PGPR) are known to influence plant growth directly or indirectly. The direct promotion by PGPR entails either providing the plant with bacteria synthesized growth promoting substances or by facilitating the uptake of certain plant nutrients from the soil environment. Indirectly by controlling the pathogenic microorganisms, the PGPR can promote plant growth. Though the exact mechanism involved was not clear, there evidence for the increase of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene [2,14], nitrogen fixation [3], antagonism against pathogenic microorganisms by the production of siderophores [27], antibiotics [29] and cyanide [11] and phosphate solubilization [8,13].

Exo polysaccharide production is another important trait of PGPR bacteria because it helps in preventing the cells against phagocytosis, phage attack and helps in nitrogen fixation by reducing the high oxygen concentrations [34]. Chitinase was produced by microorganisms to utilize chitin as an energy source, whereas fungi and insect produce this enzyme to synthesis their cell wall.

Microorganisms have evolved specialized mechanisms for the assimilation of iron, including the production of siderophores, the low molecular weight iron-chelating compounds, which transport this element into their cells. As one of the major source of iron for plants microbial siderophores play an important role. Siderophores are produced under iron-limiting conditions and change insoluble iron and make it available to plants. They play a vital role in plant growth stimulation and pathogenic control.

A number of rhizobacteria were able to produce volatile compounds such as ammonia and hydrogen cyanide, which play an important role in biocontrol. HCN production in the rhizosphere by selecting rhizobacteria is a potential and environmentally compatible mechanism for biological control of weeds and minimizing the harmful effects of desired plants [21].

Vigna is the major nodulating genera with nearly 150 species in the family *Leguminoseae*. *Vigna trilobata* commonly called as Pillipesara, mainly cultivated as a short term forage crop in India. From the literature on *Vigna-Rhizobium* interactions it was evident that the studies relating to the isolation of *rhizobia* and their cultural and biochemical studies was carried out only on a few species of *Vigna viz. V. mungo, V. unguiculata and V. radiata*. The studies on biochemical and plant growth promoting characteristics of the *Rhizobium* spp. associated with *V. trilobata* was very limited. On *Vigna trilobata* through nodulation was reported much early in 1930's no intensive cultural studies have been carried out so far.

Hence it was proposed to carry out the studies on plant growth promoting characteristics of *Rhizobial* strains associated with *Vigna trilobata*. There is no much information on these important PGP characteristics of *Rhizobium* strains from *Vigna trilobata*. The present work intended to characterize the effective strains of *Rhizobacteria* with PGPR characteristics like Indole Acetic Acid (IAA) production, Chitinase production, Exopolysaccharide (EPS) production,

Siderophore and HCN production. Among the six *Rhizobial* strains, the potential and better PGP strain can be exploited as bio inoculants.

Materials and Methods

Isolation

Rhizobial strains were isolated from the root nodules of *Vigna trilobata* plants raised in earthen pots filled with soils collected from twenty one districts of Andhra Pradesh and maintained properly in the botanical garden of our university. For the isolation pink coloured healthy root nodules were collected by gently uprooting the plants, twenty one days after sowing, surface sterilized with 0.1% mercuric chloride and washed several times with sterile distilled water. Bacterial suspension was prepared by crushing these nodules with sterile glass rod using sterile distilled water. A loopful of suspension was prepared on media plates containing selective medium yeast extract Mannitol agar medium (YEMA) with 0.1% Congo red and incubated at room temperature for 3 days. After incubation, the white translucent, convex, colonies with produce mucilage were isolated and pure cultures were maintained after subculturing the same medium. Pure cultures were authenticated as *Rhizobium* by performing the appropriate biochemical tests [32], and infection test was conducted to study the nodulation ability on homologous hosts [37]. However, the molecular characterization of the strains through 16S rDNA reveals that of the six only two are *Rhizobium* sp, and remaining four include two species each of *Agrobacterium* and *Ensifer*. Hence, sequences of all the six strains were deposited in the Gene bank and accession numbers are allotted to them.

Plant growth promoting activities

IAA Production:

IAA production was determined by the [16] method. For IAA production, all the six strains were inoculated separately into Erlenmeyer flasks (250ml) containing 100ml of YMB supplemented with L-tryptophan (100mg/ml). The cultures were incubated at 28^0 C on a rotary shaker at 200 rpm for 72hrs. After incubation the culture broth was centrifuged at 10,000 x g for 5 min. and used for IAA extraction [31]. To the 1 ml of the supernatant, 2 ml of Salkowsky's reagent (0.5 M $FeCl_3$ in 35% perchloric acid) was added and incubated for 30 min. under darkness. The absorbance of the colour developed was measured at 530 nm using a spectrophotometer. The amount of IAA produced was calculated by using the standard graph of authentic IAA (Hi-media).

Chitinase assay:

The chitinase was assayed by the method described by [38]. Chitinase activity was determined by incubating 1 ml of crude enzyme with 1 ml of 1% colloidal chitin in 0.05 M phosphate buffer pH 7.0 at 35 °C for 1 h. After centrifugation, 1 ml of reaction mixture was taken and to this 1 ml of distilled water was added, boiled in a glass ball –covered centrifuge tube for 10 minutes and then centrifused. From the supernatant 0.5 ml of aliquot was taken and to this 0.1 ml of Potassium tetraborate was added and boiled for exactly 3 minutes in a water bath. After cooling, 3 ml of P-Dimethyl amino benzaldehyde (P-DMAB) reagent was added, and the absorbance was read at 585 nm against the blank prepared without chitin or enzyme. The amount of N-acetyl D-Glucose amine released in the supernatant was determined using N-Acetyl D-Glucosamine as the standard. One unit of the chitinase activity was defined as the amount of the enzyme products 1µ mole of N- Acetate glucosamine in 1 ml of reaction mixture under the standard assay condition [22].

Stranded graph was prepared with curve for authentic N-Acetyl D-Glucosamine to convert the absorbency values to micro moles of N-Acetyl D-Glucosamine liberated from colloidal chitin.

Exopolysaccharide production

The bacterial isolates were inoculated into Erlenmeyer flasks (250 ml) containing 100 ml YEM broth supplemented with 1% Mannitol. The flasks were incubated at RT on orbital shaker at 200 rpm for 72 h. After incubation the broths was centrifuged at 3000 X g and collect the supernatant was mixed with 2 volumes of chilled acetone. The crude polysaccharide precipitate was collected by centrifugation at 3000 X g for few minutes. The EPS was washed with distilled water and acetone alternately, transferred into a filter paper and final weight was measured after overnight drying at 105^0C [6].

Siderophore production

Siderophore production was detected on chrom azurol S (CAS) medium following the method of [28]. Rhizobial strains were spotted on CAS medium and all plates were incubated at 28 ^{0}C for 72 h. Development of orange hallo around the colonies confirmed the Siderophore production.

HCN production

Actively growing bacterial cultures were streaked on YEMA plates amended with Glysine at 4.4 g/l. A Whatman filter paper No.1 (9 cm in diameter) soaked in 2% Picric acid solution was placed in the upper lid of the Petri dish. Plates were sealed with parafilm. The plates were incubated for 7 days at room temperature. Change in colour from yellow to light brown, moderate (brown) or strong (reddish brown) indicated hydrogen cyanide production. Control plates did not receive inoculum.

Results and Discussion:

Characterization of PGPR:

All the six isolates were screened for their plant growth promoting activities include Indole Acetic Acid production, EPS production, Chitinase, Siderophore and HCN production. The results showed that all the isolates do not possess all the PGP activities. The positive isolates for each of PGP activities varied greatly presented in [Table-1]. All the isolates were IAA and EPS producers while siderophore production was observed in only two isolates *Ensifer* sp. MRR 101 and *Rhizobium* sp. MRR 123 and chitinase production was exhibited by *Agrobacterium tumefaciens* MRR 111 and *Ensifer* sp. MRR 110. Except *Ensifer* sp. MRR 110 and *Rhizobium* sp. MRR 112, all isolates exhibited the HCN production character.

Table-1 *Plant Growth promoting characteristics of Rhizobium strains from Vigna trilobata*

Rhizobium strains	IAA	EPS	Siderophore	Chitinase	HCN
***Agrobacterium tumefaciens* MRR 108**	+	+	-	-	+
***Agrobacterium tumefaciens* MRR 111**	+	+	-	+	+
***Ensifer* sp. MRR 101**	+	+	+	-	+
***Ensifer* sp. MRR 110**	+	+	-	+	-
***Rhizobium* sp. MRR 112**	+	+	-	-	-
***Rhizobium* sp. MRR 123**	+	+	+	-	+

IAA Production: The amount of IAA produced varied from strain to strain and relatively more amount was observed in *Agrobacterium tumefaciens* MRR 111 with 46.5µg/ml followed by and *Ensifer* sp. MRR 110 with 42.5 µg/ml of production in YEM medium supplemented with 0.1 mg/ml L-tryptophan after 72h of incubation [Table-2]. *Rhizobium* sp. MRR 123 and *Rhizobium* sp. MRR 112 produced 33µg/ml and 32.5µg/ml respectively.

Rhizobium strain BH 14 from root nodules of Barseem (*Trifolium alexandrium* L.) was reported to produce a maximum of 176 µg/ml of IAA production in YEM

medium supplemented with 0.1 mg/ml L-tryptophan after 72h of incubation [12] while the *Rhizobium* strains in the present study produced only the minimum quantity of IAA. *Ensifer* sp. isolated from soybean was reported to produce a maximum of 30.90 µg/ml of IAA in the presence of L-tryptophan (0.01%) [19]. *Rhizobium* species reported to produce more amount of IAA than *Ensifer* and *Agrobacterium*, however, no much variation was observed among the isolates in the present study. Production of IAA varied greatly among different species and also influenced by culture conditions, growth stage and availability of substrates [36]. IAA production in the presence of L- tryptophan was reported by many of the previous workers. *Rhizobium* sp. isolated from root nodules of *Desmodium gangetium* and *Clitoria ternata* L. [26], *Sinorhizobium* sp. KCC5 from *Cajanus cajan* [7,9]. However, IAA synthesis was also reported to occur in the presence of tryptamine in *Agrobacterium tumefaciens* and indole 3-acetonitreile in *Alcaligenes faecalis* and *A. tumefaciens* [5].

Table-2 *IAA production by Rhizobium strains from Vigna trilobata*

S.No.	Strain Name	IAA Production (µg/ml)
1	*Agrobacterium tumefaciens* MRR 108	30
2	*Agrobacterium tumefaciens* MRR 111	46.5
3	*Ensifer* sp. MRR 101	24.5
4	*Ensifer* sp. MRR 110	42.5
5	*Rhizobium* sp. MRR 112	32.5
6	*Rhizobium* sp. MRR 123	33

EPS production: Highest EPS production was observed in *Rhizobium* sp. MRR 123 (816 mg/ml) and *Ensifer* sp. MRR 110 (692 mg/ml) isolates followed by *Ensifer sp.* MRR 101 (585 mg/ml), *Agrobacterium tueifaciens* MRR 111 (534 mg/ml) and *Rhizobium* sp. MRR 112 (471 mg/ml) [Table-3]. *Rhizobium* sp. MRR112 and 123 produced 471 and 861 mg/ml of EPS respectively. This clearly indicates that these strains are considered as copious producers of EPS as the previous reports shows that *Rhizobium* sp. can produce only small amount of EPS. *Rhizobium* sp. from *Crotalaria saltiana* produced 16 µg /ml [23] while that from *Vigna mungo* produced a maximum EPS of 1680 µg/ml [24] and *Rhizobium tropici* produced 2-4 g/L of EPS production [4].

Table-3 *EPS production by Rhizobium strains from Vigna trilobata*

S.No.	Strain Name	EPS Production(mg/ml)
1	*Agrobacterium tumefaciens* MRR 108	556
2	*Agrobacterium tumefaciens* MRR 111	534
3	*Ensifer* sp. MRR 101	585
4	*Ensifer* sp. MRR 110	692
5	*Rhizobium* sp. MRR 112	471
6	*Rhizobium* sp. MRR 123	816

Similarly, the *Agrobcaterium* and *Ensifer* strains in the present study also produced copious amounts of EPS than those of the previous reports - *Agrobacterium sp. CFR 24* produced 11 g/l of EPS [30] *Rhizobium miluonense* CC-B-L1 and *Ensifer adherence* CC-GSB4, produced 212 and 198 mg/ l of EPS, respectively, in yeast extract mannitol (YEM) medium [10].

The polysaccharides produced by Rhizobium help to promote infection and enhance nodulation of legumes [15]. Plant growth promoting rhizobacterial exo polysaccharides are highly important in promoting plant growth as they act as an active signal molecule during beneficial interactions, and provide a defense response during infection process [25]. Rhizobacterial exo polysaccharides can also bind to cations, including Na^+ suggesting a role in the mitigation of salinity stress by reducing the content of Na^+ available for plant uptake [1].

Chitinase production:

Out of six, two strains were able to produce Chitinase activity on chitin agar medium [Table-4]. Maximum chitinase production was observed in *Ensifer* sp. MRR 110 (0.30 U/ml) and the *Agrobacterium tumefaciens* MRR 111 (0.20 U/ml) produced the minimum chitinase production [Table-4]. There were no previous reports on *Ensifer* and *Agrobacterium* sp isolated from legume root nodules on chitinase production. Chitinase producing ability and its role in disease suppression has been visualized by several workers [20].

Table-4 *Chitinase Production by Rhizobium strains from Vigna trilobata*

S .No.	Strain Name	Chitinase Production(U/ml)
1	*Agrobacterium tumefaciens* MRR 108	-
2	*Agrobacterium tumefaciens* MRR 111	0.20
3	*Ensifer* sp. MRR 101	-
4	*Ensifer* sp. MRR 110	0.30
5	*Rhizobium* sp. MRR 112	-
6	*Rhizobium* sp. MRR 123	-

Siderophore and HCN production

Among the rhizobacterial strains studied two strains *viz.*, *Ensifer* sp. MRR 101 and *Rhizobium* sp. MRR 123 showed the Siderophore production. Similarly, using CAS screening method different *Bradyrhizobium* and *Rhizobium* strains, *viz. R. meliloti* [28]; *Rhizobium* spp. infecting *Cicer*, *Vigna*, *Medicago* and cluster bean [33] and *B. japonicum* [17] were reported to be siderophore-positive. [9] observed that the *Ensifer* sp. KCC5 and *Ensifer* sp. KCC2 from *Cajanus cajan*. showed siderophore activity on CAS agar medium, which was revealed by orange halo around their colonies after 12 h of incubation at 28 ^{0}C.

The four strains *viz., Agrobacterium tumefaciens* MRR 108, *A. tumefaciens* MRR 111, *Ensifer* sp. MRR 101, and *Rhizobium* sp. MRR 123 showed the HCN production. [35] reported that most of the rhizosphere isolates produced HCN and helped in the plant growth. Similarly, the rhizosphere isolates of chickpea also exhibits more than two or three PGPR traits including HCN production, which promotes plant growth directly or indirectly was reported by [18].

Conclusion

Present investigation was the first report of Agobacterium, Ensifer species in addition to Rhizobium from the root nodules of *Vigna trilobata*. All the five isolates exhibited the plant growth promoting characters like IAA, EPS, Chitinase production.

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