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Characterization of potential PGPR and antagonistic activities of *Rhizobium* isolates from different rhizosphere soils

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Abstract
PGPR function in three different ways synthesizing particular compounds for the plants facilitating the uptake of certain nutrients from the soil and lessening or preventing the plants from diseases. Some of Plant growth promoting characteristics such as phosphate solubilisation, Indole acetic acid (IAA) capacity, ability to produce ammonia (NH_3) as sole nitrogen source, siderophore production and production of hydrogen cyanide were evaluated in fifteen *Rhizobacteria* isolated from different rhizosphere soils of Groundnut, Sunflower, Maize, Black gram, Green gram, Rice, Soy bean and Redgram. In this study, 47% *Rhizobial* isolates showed phosphate solubilization.73% of the isolates showed IAA production and 100% for ammonia, 53% for siderophores and 53% isolates showed for HCN production. All the fifteen isolates were examined for the potential to inhibit two fungal pathogens viz., *Rhizoctonia solani* and *Sclerotium rolfsii* under *in vitro* conditions. Out of fifteen isolates, only 3 isolates exhibited inhibition potential against two soil borne plant phytopathogen Among these isolates, RR-1 and GNR-1 were tolerant to all the heavy metals (100 µg ml-1).

Keywords: *Rhizobium*, PGPR, Rhizosphere, IAA, Siderophore, HCN

1. Introduction
Soil bacteria of the family Rhizobiaceae are called Rhizobia (Werner, 1992) [15], which wraps an array of bacterial genera;containing *Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium, Sinorhizobium and Azorhizobium.* From more than a century it has been known that growth of legumes can be promoted by rhizobia via formation of nitrogen-fixing nodules but the rhizobial interaction with non-legumes erstwhile ignored as an investigational system. Efforts on the interaction of rhizobia with non-legumes have increased during the previous couple of decades, and it has been established that roots of nonlegumes could also be associated with rhizobia, devoid of effective nodule formation. Also, several mechanisms e.g., phytohormones production (Zahir *et al*., 2010) [18], siderophore production (Meyer, 2000) [10], enzymes generation (Yang & Hoffman, 1984) [16] and increased availability of insoluble phosphorus (Fatima *et al*., 2006; Pandey & Maheshwari, 2007) [6, 11] have been proposed by which Rhizobia can stimulate the growth of non-legumes directly and indirectly via suppressing or eliminating deleterious microbes by producing antibiotics (Antoun & Prevost, 2000) [1], HCN (Antoun *et al*., 1978), and by producing exopolysaccharides (EPS). Several environmental factors adversely affect the plant growth and development and final yield performance of a crop. Drought, salinity, nutrient imbalances and extremes of temperature are among the major environmental constraints to crop productivity worldwide. Soil pollution, is a very important environmental problem and it has been attracting considerable attention in recent years (Garbisu and Marques 2001) [7]. Human activities, such as mining operations and the discharge of industrial wastes, have resulted in the accumulation of metals in the environment. It has been reported that microorganisms become adapted to these environments by the acquisition of specific resistance systems [Yilmaz, 2003] [17]. Rhizobacteria have been classified into beneficial, deleterious and neutral according to their effect on host [Benzri, 2001]. Development of crop plants with stress tolerance is a very important research. Recently, the scientists try to improve plant tolerance to extreme environmental conditions through the biofertilizers treatments (symbiotic nitrogen fixing bacteria, asymbiotic nitrogen fixing bacteria and mycorrhiza). *Rhizobium* population tolerance to major environmental factors than their host legumes. *Rhizobium* symbiosis with leguminous plants and fix atmospheric N2. *Rhizobium* spp. are gram-negative soil bacteria that have a profound scientific and agronomic significance due to their ability to establish nitrogen-fixing symbiosis with leguminous plants, which is of major importance to the maintenance of soil fertility [Somasegan, 1994].

For this reason and taking into consideration the importance of legumes in animal and human consumption, some attention has been given to the effects that heavy metals exert on *Rhizobium* isolates as free-living organisms or symbiotically associated with legumes [Ibekwe, 2000].

2. Materials and Methods

2.1. Collection of Sample

Eight different rhizospheric soil samples were collected from Groundnut, Sunflower, Maize, Black gram, Green gram, Rice, Soy bean and Redgram. field grown in PJTSAU Rajendranagar, Hyderabad. The sample was collected in 1cm depth and it was packed in a sterile polythene bag and labelled properly.

2.1.2. Isolation of *Rhizobium* Isolates

The isolation of *Rhizobium spp*. from soil samples, 1g of soil sample was serially diluted in sterile distilled water, 0.1 ml of soil suspension from 10-1 to 10-6 was spreaded on yeast extract mannitol agar [Callivino, 2010]. (Vlassak *et al.* (1992)

2.1.3. Identification of *Rhizobium spp.* The bacterial isolates were identified by using cultural, morphological and biochemical characteristics features described in Bergey's manual of determinative bacteriology and stored at 4 °C on slants and maintained through sub-culturing. The isolates were characterized by Gram staining, motility test, Methyl Red, Voges Proskauer, Citrate, oxidase test, catalase test, H_2S production and starch hydrolysis as per the standard methods.

2.1.4. *In vitro* Screening of Multiple Plant Growth Promoting Activities of *Rhizobium spp*.

2.1.5. Production of Indole acetic acid Bacterial cultures were grown for 3 *Rhizobium* on their respective media at 36±2 °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl3 solution). Development of pink colour indicates IAA production.

2.1.6. Production of HCN

All the isolates were screened for the production of hydrogen cyanide by adapting the method. 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 at the top of the plate. Plates were sealed with parafilm and incubated at 36±2 °C for 4 days. Development of orange to red colour indicated HCN production. Bacterial cultures were grown in a nutrient agar medium for 18-24 h at 36±2 °C. The cultures were mixed with appropriate amount of H2O2 on a glass slide to observe the evolution of oxygen

2.1.7. Ammonia production

The isolates were tested for ammonia production by inoculating the isolates in to 10 ml of pre-sterilized peptone water in the test tubes. The tubes were incubated for 48-72h at 36±2 ^{0}C. Nessler's reagent (0.5 ml) was added in each tube. Change in colour of the medium from brown to yellow colour was taken as positive test for ammonia production.

2.1.8. Phosphate Solubilization

Phosphate Solubilization Bacterial isolates were evaluated from the ability to solubilize inorganic phosphate. Pikovskaya's agar medium (HiMedia, Mumbai) containing calcium phosphate as the inorganic form of phosphate was used in this assay. A loopful of bacterial culture were placed on the plates and kept for incubation at 28 °C for 7 days. The presence of clear zone around indicate phosphate solubilization.

2.1.9. Siderophore Production

Siderophore production was estimated qualitatively. Chrome Azurol S (CAS) Agar medium (Schwyn and Neilands, 1987) [12]: For the detection of siderophores, each *Pseudomonas* isolate was grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 h on a rotary shaker at room temperature. Chrome Azurol S (CAS) assay is used to detect the siderophores. The CAS plates were used to check the culture supernatant for the presence of siderophores. Culture supernatant was added to the wells made on the CAS agar plates and incubated at room temperature for 24 h. Formation of yellow to orange coloured zone around the well indicates siderophore production.

2.2 Antagonistic Activity

Pure isolates of common disease causing soil phytopathogens viz., *Rhizoctonia solani*, *Sclerotium rolfsii* were obtained from the Dept. of Plant Pathology, College of Agriculture, Rajendranagar.

Antagonistic activity was verified by following dual culture technique (Skidmore and Dickinson, 1976). First, the bacterial isolates were streaked on respective media plates and incubated at respective temperature and time. Loop ful of each bacterial isolate was streaked on the potato dextrose agar plate at one end, which was pre-inoculated with 5 days old, 5mm mycelial disc of test pathogen at the other end. Control plate was maintained by placing only pathogen mycelial disc in the centre without bacteria.

The assay plates were incubated at 28$\pm$1^{0}C for 5 days and observations were made on inhibition of mycelial growth of the test pathogens. For each bacterial isolate three replications were maintained with suitable controls.

The per cent growth inhibition over control was calculated by using the formula:

Percent Inhibition

Growth of pathogen in control (mm)-growth of pathogen in treatment (mm) x100 Growth of pathogen in control (mm)

Note: In this the percent inhibition in control is taken as zero percent.

3. Results and Discussion

3. 1 Screening of pure isolates for PGPR properties

Plant root colonizing bacteria can function as harmful, deleterious rhizobacteria (DRB) or beneficial, plant growth promoting rhizobacteria (PGPR). PGPR colonize roots of monocots and dicots, and enhance plant growth by direct and indirect mechanisms. Modification of root system architecture by PGPR implicates the production of phytohormones and other signals that lead, mostly to enhanced lateral root branching and development of root hairs. PGPR also modify root functioning, improve plant nutrition and influence the physiology of the whole plant.

For identification of efficient PGPR strains with multiple activities, microbial isolates (*Rhizobium and Pseudomonas fluorescence*) were subjected to further studies to understand their Plant Growth Promoting Properties (PGPR) under *in vitro* conditions. Plant growth promoting attributes of *Rhizobial* isolates presented in Table. 4.9

3.2. IAA production

Out of fifteen *Rhizobial* isolates 11 were able to produce IAA. Further, out of 15 isolates GNR-1(24.12 µg ml^{-1}) showed maximum IAA, followed by SFR-1(15.20 µg ml^{-1}), GGR-2(14.24 µg ml^{-1}), MR-1 (13.24 µg ml^{-1}), GGR-1(12.24 µg ml^{-1}), RGR-1 (12.22 µg ml^{-1}), RR-1(12.14 µg ml^{-1}), GNR-2 (11.41 µg ml^{-1}), SFR-2(11.34 µg ml^{-1}), MR-2 (11.25 µg ml^{-1}),RR-2 (9.14 µg ml^{-1}). [Plate 3.2(a)].

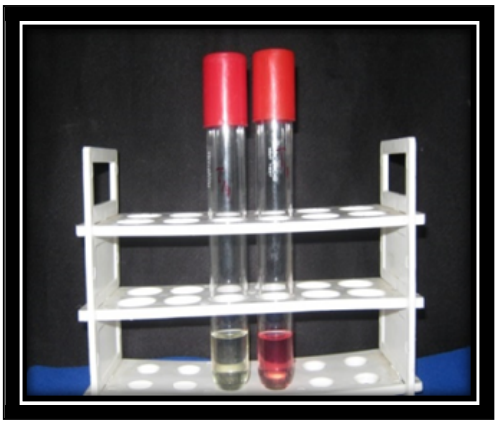


Plate 1 (a). IAA production

3.3. Ammonia production

Out of 15 *Rhizobium* isolates 15 were able to produce ammonia. Further, out of 15 isolates RR-1 exhibited strong (+++) Ammonia production and MR-1,MR-2,MR-3,BGR-1,GNR-1,GNR-2,GGR-1,SFR-1,SFR-2, RGR-1 and SYR-1 produced moderately (++). Whereas the remaining 3 isolates viz., RR-2, BGR-1, MR-4 and GGR-2 were scored as weak (+) for Ammonia production. Plate 3.3(a)].

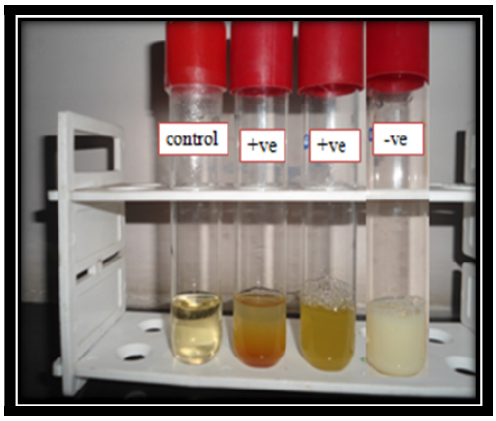


Plate 2 (a). Ammonia production

3.4. Phosphate solubilization

Collavino (2010) [5] reported that phosphate-solubilizing bacteria native to acid soil had ability to promote *Phaseolus vulgaris* growth. The study is conducted to characterize three bacterial strains in solubilising rock phosphates as well as their impact in promoting soybean growth under pot grown conditions

Among 15 *Rhizobial* isolates 7 isolates were able to solubilize phosphate on pikovskaya's media containing Tri calcium phosphate in the range of 10mm to 25mm. Among 7 *Rhizobial* isolates SFR-2 recorded the highest solubilization zone (22.00mm) followed by RR-1 & MR-1 (19 mm), GNR-2 (18.00 mm), RR-2 (14.00mm) and less solubilization by GNR-1 & SYR-1(10.00mm). Plate 3.4(a)].

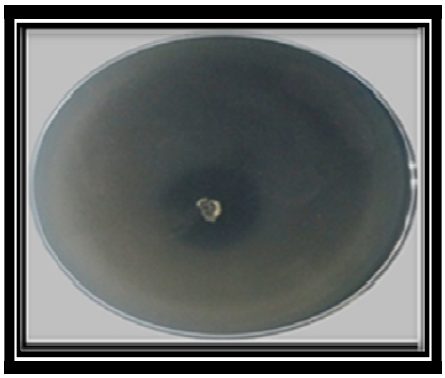


Plate 3 (a). Phosphate solubilization

3.5 Siderophore production

Out of 15 *Rhizobium* isolates 8 were able to produce siderophores. Further, out of 8 isolates RR-1 exhibited strong (+++) Siderophore production and GNR-1 and SYR-1 produced moderately (++). Whereas the remaining 5 isolates viz., RR-2, BGR-1, GNR-2, GGR-1 and SFR-2 were scored as weak (+) for Siderophore production [Plate 3.5(a)].

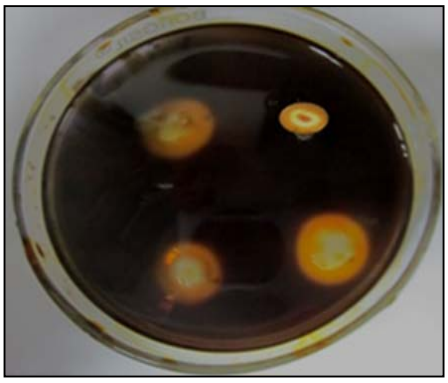


Plate 4(a). siderophore production

Arora *et al*. (2001) [3] stated that siderophore production by *Rhizobial* strains has been considered as a potential way to improve nodulation and N_2 fixation in iron deficiency conditions. The beneficial effect of using siderophore producing strains of *Bradyrhizobium sp*. and *Rhizobium meliloti* might favour the persistence of *Rhizobia* in iron deficient soils.

3.6 HCN Production

Out of 15 *Rhizobium* isolates, 8 produced HCN. Further, out of 8 isolates RR-1 exhibited strong (+++) HCN production and GNR-1 scored as moderate (++) for HCN production. Whereas the remaining 6 isolates viz., MR-2, BGR-1, GNR-2, GGR-2, SFR-2, SYR-1, found to be weak (+) in HCN production Plate 3.6(a)].

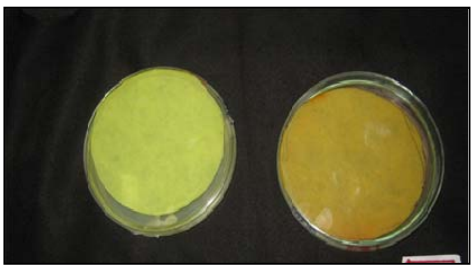


Plate 5 (a). HCN Production

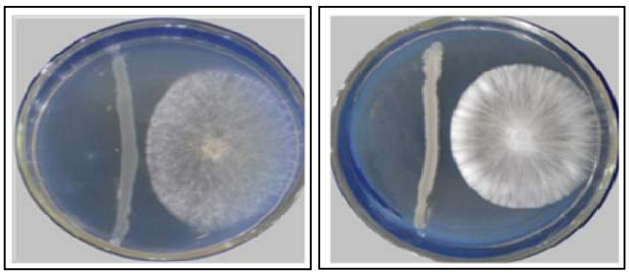
3.7 Antagonistic Activity of *Rhizobium* isolates

Out of 15 *Rhizobium* isolates 4 isolates showed inhibition potential against *Rhizoctonia solani*, viz. RR-1 (36.60%), GNR-1 (36.05%), SFR-2 (36.60%) and SYR-1 (36.60%).The maximum per cent inhibition against *Rhizoctonia solani* was showed by SYR-1 (36.66%) with inhibition zone 4 mm Plate 3.7(a)].

Four out of 15 isolates were inhibitory to *Sclerotium rolfsii*, viz. RR-1 (36. 05%), GNR-1 (37.50%), GNR-2 (36.60%) and SFR-2 (31.10%) The maximum per cent inhibition against *Sclerotium rolfsii*, was showed by GNR -1 (37.50%) with inhibition zone 3 mm [Plate 3.7 (b)].

Out of 15 *Rhizobium* isolates 3 isolates viz., RR-1, GNR-1and SFR-2 showed inhibition potential against both *Rhizoctonia solani* and *Sclerotium rolfsii*. The isolate that showed maximum inhibition potential against *Rhizoctonia solani* was also inhibitory to *Sclerotium rolfsii* to a lesser extent based on per cent inhibition and vice versa. Hence it can be inferred that the *Rhizobium* isolates RR-1, GNR-1and SFR-2 could be considered for their bio control activity.

Rhizobium* with *Rhizoctonia solani



Rhizobium with *Rhizoctonia solani (b)Rhizobium* with *Sclerotium rolfsii*

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