In vitro Screening of Native *Bacillus* Isolates for Plant Growth Promoting Attributes

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Abstract

Bacillus spp. are well known rhizosphere residents of many crops and usually show plant growth promoting (PGP) activities that include biocontrol capacity against some phytopathogenic fungi. In this study, a total of thirty *Bacillus* isolates were obtained from the rhizospheric soils of groundnut and redgram crops growing in Rangareddy district, India and identified based on their colony morphology, cell morphology and biochemical characteristics. These thirty isolates were screened for PGP attributes like phosphate solubilization and Indole Acetic Acid (IAA) production. Isolates showing PGP properties were further screened for in vitro antagonism against soil borne phytopathogens viz., *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium solani*. Results revealed that 50% (15 out of 30 isolates) reacted positively for one or more PGP properties. A high prevalence of antagonists was found against the three fungal pathogens. Majority of the bacterial isolates (33%) displayed antagonism through the production of siderophores or HCN and the remaining isolates showed antagonism with negative results for siderophores and HCN indicating that, other mechanisms such as production of mycolytic enzymes and Antibiosis etc. may be involved. It was suggested that the rhizospheric soils are the rich source of *Bacillus* fungal antagonists, which have a potential to be used in the future as PGP inoculants.

1. Introduction

Modern Agriculture is heavily dependent on the application of chemical pesticides for disease control. Due to the concerns regarding both human health and environmental protection, viable alternatives to these chemicals are being sought (Franks et al., 2006). The interest in the use of biological approaches to replace hazardous pesticides in fertilizing soils or improve plant resistance against phytopathogens is steadily gaining worldwide acceptance. In this regard, the use of plant growth promoting rhizobacteria (PGPR) has depicted potential in developing sustainable agricultural systems for crop production and protection (Govindasamy et al., 2011; Erturk et al., 2010). Among the 20 genera of bacteria, *Bacillus* spp., *Pseudomonas* spp. are widely used as biocontrol agents and *Bacillus* spp. has been reported to produce several antibiotics (Ferreira et al., 1991).

Enhancement of plant growth by root colonizing *Bacillus* sp. is well documented (Idris et al., 2007a; Idris et al., 2007b; Kloepper et al., 2004). Studies reveal that *Bacillus* species are among the most prominent bacteria found to colonize plants root and soil populations (Beneduzi et al., 2008). The genus *Bacillus* is characterized by Gram positive, aerobic or facultative anaerobic, rod shaped bacteria that form endospores (Claus and Berkeley 1986).

Quantitative and qualitative relation in these traits allow for these bacteria to inhabit diverse niches in agro ecosystems. Their microscopic size and omnipresence in soil facilitate their colonization of plants.

Bacillus species may protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as, by induction of host resistance. It depends on a wide variety of traits, such as the production of structurally diverse antibiotics (Liu et al., 2006), production of iron chelators, bacterial phytohormones and/or the solubilization of mineral phosphates (Calvo et al., 2010; Viruel et al., 2011) and an ubiquitous presence in soil (Gajbhiye et al., 2010). Keeping in view, the importance of PGPR in sustainable agriculture, the present study aims at (i) Screening of native *Bacillus* isolates for Plant growth promoting attributes such as phosphate solubilization, production of Ammonia, IAA, HCN and siderophores etc., (ii) to identify the organism based on cultural, morphological and biochemical properties and iii) to study the *in vitro* antagonism of these PGPR isolates against *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani*.

2. Materials and Methods

2.1. Soil sampling

Fifteen soil samples were collected from various rhizosphere soils of Groundnut and Red gram crops growing at Rangareddy district of Andhra Pradesh in India during 2012. Crop plants were selected randomly in the field and the intact root system was dug out, carefully taken in plastic bags and stored at 4^0C.

2.2. Isolation of rhizobacterial isolates

Bacterial isolates were obtained from the rhizosphere soils using dilution method (Alexander, 1965) and preserved on the Nutrient agar (NA) plates at 4^oC.

2.3. Screening of bacterial isolates for PGP properties

The isolated *Bacillus* colonies were analyzed for their plant growth promoting characteristics viz. production of IAA, ammonia, siderophores, HCN and their ability to solublize phosphates.

2.3.1. Phosphate solubilization

The ability of the isolates to solubilize tri-calcium phosphate was observed as per Wahyudi et al., (2011). Pikovskya's Agar was inoculated with the isolates and incubated at 36±2°C for five days. Formation of halo indicated phosphate solubilization.

2.3.2. Production of indole acetic acid (IAA)

The production of Indole Acetic Acid (IAA) was deleted as described by Brick et al. (1991). Bacterial cultures were grown for 72 h in King's B broth at 36±2°C. Fully grown culture was centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of Orthophosphoric acid and 4 ml of the Salkowaski reagent (50 ml 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). The development of pink colour indicates IAA production.

2.3.3. Catalase activity

Catalase test was performed by taking a drop of 3% hydrogen peroxide was added to 48 hr old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick. The effervescence indicated catalase activity.

2.3.4. Ammonia production

All the bacterial isolates were tested for the production of ammonia as described by Joseph et al. (2007). Overnight grown bacterial cultures were inoculated in 10 ml peptone broth and incubated at 30±0.1°C for 48 h in incubator shaker. After incubation 0.5 ml of Nessler's reagent was added. The development of faint yellow to dark brown color indicated the ammonia production.

2.4. Identification of bacterial isolates

All the bacterial isolates were studied for their colony morphology, cell morphology (Gram Reaction), pigmentation and spore production as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

2.4.1. Hydrogen sulphide production

SIM agar medium tubes were stab inoculated by test isolates and incubated for 24-48 h at 37±2°C (Clarke, 1953). Tubes were observed for presence or absence of black coloration along the line of stab inoculation indicating hydrogen sulphide production.

2.4.2. Production of hydrolytic enzymes

The isolates possessing the growth promoting characteristics were further characterized biochemically through tests for the production of oxidase, amylase, gelatinase, urease, lipase and cellulase. The isolates were also tested for their ability to carry out nitrate reduction and carbohydrate fermentation. The biochemical characterization has been carried out as per Cappuccino and Sherman (1992).

2.5. Screening for antagonistic activity

Bacillus isolates showing one or more PGP traits were screened for antagonistic activity against pathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium solani* using Potato Dextrose Agar (PDA) with method described by Skidmore and Dikinson (1976). Bacterial isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at 3 cm of the medium. The barrier between the bacterial isolate and pathogenic fungi indicated antagonist interaction (Inhibition zone) between them.

$$\% \text{ Inhibition} = \frac{\text{Growth of Pathogenin control (mm)- Growth of Pathogen in treatment (mm)}}{\text{Growth of Pathogen in control (mm)}} \times 100$$

2.6. Mechanism involved in antagonism

2.6.1. HCN production

HCN production was tested by the method of Lorck (1948). Nutrient broth was amended with 4.4 g glycine $liter^{-1}$ 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 Para film and incubated at 36 ± 2^0C for 4 days. Development of orange to red color indicates HCN production.

2.6.2. Siderophore production

Siderophores were detected quantitatively by CAS Shuttle Assay (Schwyn and Neilands, 1987). 0.5% of cell free culture supernatant was added to 0.5% CAS (Chrome Azurol Sulphate) assay solution and absorbance was measured at 630 nm against a reference consisting of 0.5 ml uninoculated broth and 0.5 ml CAS reagent. Siderophore content in the aliquot was calculated by using the following formula:

$$\% \text{ siderophore units} = \frac{\text{Ar-As}}{\text{Ar}} \times 100$$

Where, A_r=Absorbance of reference at 630 nm
A_s=Absorbance of sample at 630 nm.

3. Results and Discussion

3.1. Isolation of rhizobacterial isolates

Fifteen soil samples were collected from different places of Rangareddy district for the isolation of rhizobacterial isolates. The soil samples were mainly collected from the rhizosphere of Groundnut and Red gram crop plants. All the soil samples were inoculated on the Nutrient Agar (NA), based on the colony morphology and cultural characteristics of the isolates on the above media, about thirty colonies from above plates were selected and purified. Similar results were observed with Kumar et al., (2010) where they have isolated seven *Bacillus* isolates from the rhizosphere soils of common bean and concluded that they showed PGP and antagonistic activity against ten different phyto-pathogens.

3.2. Screening of bacterial isolates for PGP properties

Majority of the isolates showed one or more of the PGP properties and were presented in the Table 1.

3.2.1. Phosphate solubilization

Of the thirty isolates, eight isolates showed zone of phosphate solubilization activity on Pikovskya's medium. Out of eight, SFRB and MGB showed highest zone with 60% Solubilization Efficiency (SE), followed by AGB and CFRB showed 40% S.E. Several microorganisms are able to make insoluble soil phosphorous available to plants by solubilizing mineral phosphates through the production of organic acids or phosphatases. *Pseudomonas* and *Azotobacter* are two of the most reported phosphate solubilization genera; however *Bacillus* strains also have this capacity (Chatli et al., 2008; Nautiyal, 1999; Vessey, 2003). The phosphate solubilization abilities of our isolates were in conformity with those reported for other *Bacillus* tested under similar assay conditions (Chatli et al., 2008; Nautiyal, 1999).

3.2.2. Indole acetic acid (IAA) production

Nine of the *Bacillus* isolates were identified as IAA producers, of which SFRB and MGB was found to be strong. IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. It has been reported that IAA production by PGPR can vary among different species and strains, and also influenced by culture conditions, growth stage and substrate availability. The role of bacterial IAA in different plant-microbe interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms (Ahmad et al., 2008; Samuel and Muthukkaruppan, 2011).

3.2.3. Catalase activity

All the bacterial isolates in the present study were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield, and contribute to the protection of plants against certain pathogens and pests (Dey et al., 2004; Herman et al., 2008; Kloepper et al., 2004; Minorsky, 2008).

3.2.4. Ammonia production

Another important trait of PGPR is the production of ammonia that indirectly influences the plant growth. All the isolates were able to produce ammonia. It has been reported that ammonia production indirectly influences the plant growth. *Bacillus subtilis* strain MA-2 and *Pseudomonas fluorescens* strain MA-4 was efficient in ammonia production and significantly increased biomass of medicinal and aromatic plant such as *Geranium* (Mishra et al., 2010).

3.3. Identification of bacterial isolates

Cultures on NA showed irregular, flat, dull white colored colonies. The bacterial isolates were found to be gram-positive rods with endospore, when observed under microscope (Table 2). The isolates were positive for some of the biochemical tests viz. Citrate Utilization, Gelatin Liquefaction, Starch Hydrolysis (Table 3). They also utilize carbon sources viz., mannitol, glucose and lactose. They were negative for Voges-Proskauer test, production of indole, hydrogen sulphide and utilization of lipids and cellulose. Finally, these bacterial isolates were identified and grouped as *Bacillus* sp. based on the colony morphology, Gram reaction and Biochemical properties as

Table 1: Plantgrowth promoting characteristics of *Bacillus* isolates

Isolate	Plant growth promoting characteristics			
	IAA	Phosphate Solubilization Efficiency (SE) in %	Ammonia production	Catalase activity
SFGB	+	20	+	+
SFRB	+++	60	+	+
AGB	+	20	+	+
ARB	++	-	+	+
DGB	-	30	+	+
DRB	+	-	+	+
KGB	-	-	+	+
KRB	++	40	+	+
SBGB	-	-	+	+
SBRB	++	20	+	+
CFGB	-	-	+	+
CFRB	-	-	+	+
YGB	++	40	+	+
YRB	-	-	+	+
MGB	+++	60	+	+

per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

3.4. Screening for antagonistic activity

Out of the thirty isolates, fifteen isolates showing PGP properties were observed for biocontrol activity and the results revealed that *Bacillus* isolates were able to reduce the growth of all the three phytopathogens viz. *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani* with inhibition ranging from 19.2% to 38.8%, 44.4% to 52.9% and 14.3% to 45.6% respectively (Table 4).

Results of this research evidenced a high prevalence of antagonistic *Bacillus* towards *R. solani*, *S. rolfsii* and *F. solani* in the rhizospheres of both the legume crops. In other studies where a collection of *Bacillus* isolates has been challenged against *R. solani*, only 9.5 to 36% were antagonistic to this pathogen (Cho et al., 2007; Mojica-Marin et al., 2008). *In vitro* antagonism against these three pathogens have been found in *Bacillus* sp. isolated from several sources (Calvo et al., 2010; Nihorimbere et al., 2010), including the legume rhizospheres (Maheswar and Sathiyavani, 2012).

3.5. Mechanism of antagonism

Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, including *Bacillus* sp. under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi (Whipps, 2001). In the present study, seven (46%) of the isolates showed biocontrol activity by the production of siderophore and in contrast, some of (66.6%) the isolates were able to inhibit fungal growth through the production of HCN (Table 4). In addition to siderophore, there are other mechanisms of biocontrol including antibiotic compounds, elicitation of induced systemic resistance (ISR) of plant, and lytic enzyme secretion (Haas and Defago, 2005).

A relatively wide range of antagonistic performances among *Bacillus* isolates was observed here. Given that more than one mechanism may be involved, a complex response with a range of antagonistic effects among *Bacillus* isolates and distinct responses by different pathogens could be expected as was observed here. Some of the isolates showing antagonism with negative results for siderophores and HCN indicate that, some other mechanisms such as production of mycolytic enzymes and antibiosis etc. may be involved in inhibiting the fungal pathogen. Similar results were reported by Kumar et al. (2012) where, of the 28 *Bacillus* sp. isolates tested, 20 isolates produced HCN whereas, 13 isolates exhibited Siderophore production.

Table 2: Morphological characterization of Bacterial isolates

No. of isolates	Colony Morphology					Cell morphology			
	Size	Shape	colour	Elevation	Pigmentation	Shape	Arrangement	Gram Reaction	Spore forming
Fifteen	Medium	Irregular	White or Dull white	Flat	No	Rod	Single, short	Gram+ve	Yes

Table 3: Biochemical properties of bacterial isolates

Property	Number of isolates														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Indole production	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl Red	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Voges Proskaur	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4^0C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 41^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+indicates Positive for the test; -indicates Negative for the test

Table 4: Antagonistic activity of *Bacillus* isolates against *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani*

Isolates	*Inhibition (%)			Mechanism of Antagonism	
	Rhizoctonia solani	*Sclerotium rolfsii*	*Fusarium solani*	Siderophores	HCN
SFGB	32.5	46.2	37.4	45	+++
SFRB	32.2	34.8	35.3	00	++
AGB	35.1	50.3	45.6	39	-
ARB	37.0	52.5	45.6	47	++
DGB	33.7	44.4	36.9	00	++
DRB	27.4	38.1	45.1	32	-
KGB	35.1	49.2	44.0	40	+
KRB	34.7	20.3	43.0	00	++
SBGB	27.4	48.8	42.5	45	+++
SBRB	28.1	39.6	37.9	00	+
CFGB	28.5	27.7	29.2	00	-
CFRB	38.8	49.2	34.3	43	++
YGB	19.2	21.4	14.3	00	-
YRB	27.0	24.0	24.0	00	-
MGB	36.6	36.6	35.3	00	++
Control	00	00	00	00	00
SEm±	0.4	0.2	1.1	-	-
CD (p=0.05)	1.4	0.8	3.4	-	-

*Mean of three replications; HCN- Hydrogen cyanide; + indicates Weak production; ++ indicates Moderate production; +++ indicates Strong production

4. Conclusion

Results obtained indicate that the rhizosphere of these two legume crops (Groundnut and Red gram) are the rich source of potential PGPR isolates of *Bacillus*. All the isolates reacted positively for more or at least one of the PGP properties. Some of the isolates produced IAA, while some of them showed phosphate solubilization. The isolate SFRB showed best PGP attributes compared to SBRB, MGB. The isolate ARB was the best in terms of antagonistic activity against three fungal pathogens (*Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani*) tested followed by MGB and SFRB. From the present investigation, it is concluded that MGB could be the choice as it showed good in vitro antagonism, in addition to the moderate activity in terms of PGP attributes.

5. Further Research

The isolates showing PGP attributes and antagonistic activity are studying for Molecular characterization to identify the genetic relatedness among the bacterial isolates. Further, the best isolates are going to be studied for their performance under greenhouse condition as well as field conditions.

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