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BIODIVERSITY OF *PSEUDOMONAS* AND *BACILLUS* POSSESSING BOTH BIOANTAGONISTIC AND PLANT GROWTH PROMOTING TRAITS IN CHICKPEA RHIZOSPHERE

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ABSTRACT
A total of 174 rhizobacterial isolates (76 from Kings B medium and 98 from Nutrient Agar) were isolated from 36 rhizospheric soil samples collected from different chickpea growing locations. Of these 174 isolates, 55 were found antagonistic to *Fusarium oxysporum*, 65 to *Rhizoctonia bataticola* and 50 to both. Twenty-nine rhizobacterial isolates (13 from Kings B and 16 from Nutrient agar) with inhibition of >20% were scored as strong antagonists and inhibited mycelial proliferation of both the test fungi. The cultural, morphological and biochemical tests tentatively placed the isolates into two genera *Bacillus* and *Pseudomonas.* Screening of 50 antagonistic bacterial isolates for P-solubilization ability revealed that 19 (10 were representatives of genus *Bacillus* and 9 of *Pseudomonas*) could solubilize tricalcium phosphate. Zn-solubilizing potential was associated with 20 isolates in the range of 1.14-1.77. All the isolates tested positive for gibberellic acid (11.9-36.3 µg/ml) and IAA production, however, presence of tryptophan greatly affected the IAA production pattern of isolates. In the absence of tryptophan, IAA production ranged from 2.4-15.5 µg/ml in contrast to 9.2-48.7 µg/ml in presence of 3mM tryptophan. *Bacillus* isolates were more efficient IAA producers both in presence and absence of tryotophan. Salicylic acid production was exhibited by 47 isolates (22 of *Bacillus* and 25 pseudomonads) while 16 produced siderophore (9 *Pseudomonas* sp. and 7 *Bacillus* sp).

KEYWORDS: Antagonism, P-solubilization, IAA, Gibberellins.

INTRODUCTION

Sustainable agricultural practices which aim at solving multifaceted problems that have resulted due to prolonged and indiscriminate use of chemical based agronomic tools has provoked the search for suitable ecofriendly options to chemical fertilizers and pesticides. Owing to their versatile and unmatchable capacities microbial agents offer a viable and attractive option to develop the biological tools to replace/supplement the chemicals. Exploring the microorganisms that reside in close proximity to the plant is thus a necessary move in the direction to achieve this target (Prashar *et al*., 2013). Plant growth promoting rhizobacteria are defined as ‘‘the soil bacteria that colonize the roots of plants by following inoculation on to seed and that enhance plant growth’’(Kloepper and Schroth, 1978). The range of bacteria being reported to enhance the plant growth and control plant pathogens includes various species of *Pseudomonas, Bacillus, Azospirillum, Azotobacter, Streptomyces, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Flavobacterium, Burkholderia, Bradyrhizobium, Mesorhizobium, Rhodococcus* and *Serratia etc*. (Chen *et al*., 2006; Ahmad *et al*., 2008). However, the predominant bacterial species in the PGPR community which have emerged as the most widely studied and potent candidates for improvement of plant growth and health are *Pseudomonas* and *Bacillus*. Insight into the mechanisms responsible for their favorable activities include solubilization of micro and macro nutrients (Chen *et al*., 2006), production and release of phytohormones like indole acetic acid and gibberellins (Jangu and Sindhu 2011) and biocontrol of soil borne phytopathogens (Cawoy *et al*., 2011). In the last few decades a large body of literature reporting the activities of these two bacterial species, pertaining to plant growth promotion and biocontrol of phytopathogens has been generated that reflects the potential of these PGPR strains to be developed as alternative/ supplementary agrochemicals. The present work was conducted to study the diversity of *Pseudomonas* and *Bacillus* possessing both plant growth promoting and antagonistic traits in chickpea rhizosphere so that they can be exploited as potential bioinoculating agents.

MATERIALS & METHODS

Collection of soil sample

A total of 36 soil samples were collected from the rhizospheric soil of chickpea grown in vicinity of Punjab, UP, Chhattisgarh, Himachal Pradesh, Mumbai and West Bengal (Table 1). The rhizospheric soil samples were collected from 0-15 cm depth by carefully uprooting the plants.

TABLE 1: Sources of rhizospheric soil samples

State	Location	No. of samples
Chhattisgarh	Raipur	2
	Dhamtari	4
Himanchal Pradesh	Solan	1
	Mandi	1
Mumbai	Mumbai	1
Punjab	PAU	7
	Bhatinda	1
	Mavi Kalan	1
	Hosiarpur	1
	Gurdaspur	1
	Kartarpur	1
	PAU entomology field	1
	Ropar	1
	Faridkot	2
	Dhanera	1
	Balbira	2
	Moga	1
	PAU agronomy field	1
	Talwandi field	1
Uttar Pradesh	Pantnagar	4
West Bengal	Aasansol	1
Total		36

Isolation of rhizobacteria

The isolation of rhizobacteria was done using two different media *viz.* Nutrient agar (NA) for *Bacillus* and Kings B (King *et al.*, 1954) for *Pseudomonas.*

Screening for antagonistic rhizobacteria

Antagonistic activity of the bacterial isolates against *Fusarium oxysporum* and *Rhizoctonia bataticola* was evaluated based on dual plate technique. 5mm mycelia plug of the test fungus was inoculated at the centre of plate containing Potato dextrose agar. Rhizobacterial isolates were streaked 3 cm apart from the fungal inoculum and inhibition zone was observed.

Cultural and morphological Characterization of bacteria

Initial characterization of all the isolates was done on the basis of colony morphology and gram's staining. The cultural characterization of isolates was done on solid agar medium.

Biochemical characterization of rhizobacteria

Biochemical characterization of bacterial isolates was done on the basis of catalase production, nitrate reduction, starch hydrolysis and methyl red test. These were conducted as per the standard methods (Cappuccino and Sherman, 1992).

Assessment of biofertilizing traits of antagonistic bacteria

P-solubilization

The P-solubilization ability of rhizobacterial isolates was tested using Pikovskaya agar medium (Glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.1g; yeast extract 0.5 g; $MnSO_4$. and $FeSO_4$ trace; D/W, 1 l; agar, 15 g; pH 7). Overnight grown bacterial cultures were spot inoculated and plates were incubated at 28^0C for 5 days. The isolates forming halo zone around the colony were considered as phosphate solubilizers.

Zn-solubilization

The Zn-solubilization ability of rhizobacterial isolates was tested using modified Pikovskaya medium (Glucose, 10 g; $Ca_3(PO_4)_2$, 5.0 g; $(NH_4)_2SO_4$, 0.5g; NaCl, 0.2g; $MgSO_4.7H_2O$, 0.1 g; KCl, 0.2 g; Yeast extract, 0.5 g; $MnSO_4$, Trace; $FeSO_4.7H_2O$, Trace; Agar, 15 g; Water, 1000 ml; pH, 7.0±0.2) containing 1% insoluble zinc compound (ZnO). All the plates were incubated for 48 h at 28°C. The isolates forming halo zone around the colony were considered as zinc solubilizing bacteria.

Gibberellic acid production

The gibberellic acid production by rhizobacteria was determined as per the method by Borrow *et al.* (1995). Cultures inoculated in their respective broth were incubated at 37°C for seven days and then centrifuged at 8000 rpm for 10 min. Fifteen ml of the supernatant was pipetted out separately into the test tubes and two ml of zinc acetate solution (21.9 g of zinc acetate in 80 ml of distilled water and one ml of glacial acetic acid was added and volume made to 100 ml with distilled water) was added. After 2 min, two ml of potassium ferrocyanide solution (10.6 g of potassium ferrocyanide in 100 ml of distilled water) was added and centrifuged at 8000 rpm for 10 min. Five ml of supernatant was added to five ml of 30 per cent hydrochloric acid and the mixture was incubated at 27°C for 75 min. The blank was prepared with five % hydrochloric acid. Absorbance was measured at 254 nm in a UV-VIS spectrophotometer. From the standard graph prepared by using gibberellic acid solutions of known quantities, the amount of GA_3 produced by the culture was calculated and expressed as μg 25 ml^{-1} broth.

IAA production

IAA production was detected by the modified method as described by Gorden and Weber (1951). Bacterial cultures were inoculated in Luria broth and incubated for 72 hours at 30^0C. Fully grown cultures were centrifuged and 2 ml supernatant was mixed with two drops of orthophosphoric acid followed by 4 ml of the Salkowski reagent and incubated for 25 minute at room temperature for development of pink color. Absorbance was recorded at 530 nm.

Salicylic acid (SA) production

Salicylic acid (SA) production was determined as per the method described by Meyer and Abdallagh (1978). The

rhizobacteria were grown in the standard succinate medium at 28°C for 48 hrs and cells centrifuged at 8000 rpm for 5 min and resuspended in 1ml of 0.1 M phosphate buffer. A 4ml cell free culture filtrate was acidified with 1 N HCl to pH 2.0 and SA was extracted in $CHCl_3$. Four ml of water and 5 µl of 2M $FeCl_2$ were added to the pooled $CHCl_3$ phases. The absorbance of the purple iron- SA complex, which developed in the aqueous phase, was read at 527 nm. A standard curve was prepared with SA prepared in succinate.

Siderophore production

Siderophore production was assayed using chromeazurol S agar (CAS) (Schwyn and Neilands, 1987). Cultures positive for siderophore production produced an orange halo around the colony.

RESULTS & DISCUSSION

Isolation of rhizobacteria

The rhizosphere supports the development and activity of a huge and diversified microbial community including microorganisms capable of promoting plant growth. In an attempt to tap this microbial diversity, a total of 174 rhizobacterial isolates were isolated from 36 rhizospheric soil samples collected from different chickpea growing locations. Out of these, 76 isolates were selected from Kings B medium and 98 from Nutrient Agar. Most of the isolates from Kings B medium showed the characteristic fluorescent green pigmentation. Kings B media has been proposed to general medium for the non-selective isolation and pigment production of *Pseudomonas* species. The production of pigments such as fluorescein, pyorubin and non-fluorescent blue pigment, pyocyanin is readily demonstrated by culturing on Kings Medium B (King 1951). However, production of fluorescence by pseudomonads is not constant during incubation and not common to all species of this group. The isolates from nutrient agar medium differed in their colony morphology although most of them appeared off-white to creamish in colour. The predominance of *Pseudomonas* and *Bacillus* sp. in legume rhizosphere has been reported by many workers. Joseph *et al* (2007) reported that populations of *Pseudomonas*, *Bacillus* and *Azotobacter* predominantly colonized the rhizosphere and rhizoplane of healthy chickpea plants. They found that bacterial population ranged from 0.5- 2.1×10^6 of *Bacillus* sp., 1.1-2.1×10^6 of *Pseudomonas* sp. and 0.3-1.7×10^6 of *Azotobacter* sp. Hynes *et al.* (2008) reported that plant growth promoting bacteria isolated from the roots of pea, lentil and chickpea were representatives of the families pseudomonadaceae and 36%-42% of the enterobacteriaceae.

In vitro screening for antagonistic rhizobacteria

In plant disease management programmes, the use of a rapid method for screening efficient biocontrol agents is a prerequisite (Anith *et al.*, 2003). Microorganisms that grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens (Nautiyal, 1997). Antagonistic potential of 174 rhizobacterial isolates collected from chickpea rhizosphere was evaluated against *Fusarium oxysporum* sp. *ciceris* and *Rhizoctonia bataticola* using dual plate assay under *in vitro* conditions. Out of total 174 isolates, 55 isolates were found antagonistic to *Fusarium oxysporum*, 65 to *Rhizoctonia bataticola* and 50 to both. On the basis of fungal growth inhibition under dual plate assay, isolates were characterized into weak (1-10%), medium (>10-20%) and strong antagonists (>20%) (Table 2). Twenty-nine rhizobacterial isolates (13 from Kings B and 16 from Nutrient agar) were scored as strong antagonists and inhibited mycelial proliferation of both the test fungi. Kaur *et al.* (2007) reported antagonistic effect of *Pseudomonas* isolates from chickpea rhizosphere against *Fusarium oxysporum* sp. *ciceris*, they observed that 14 out of 96 isolates were highly antagonistic to the phytopathogen and showed inhibition zone ranging from 5-7 mm under *in vitro* growth conditions.

TABLE 2: Antagonistic potential of rhizobacterial isolates

Bacterial genera	Characterization	Range of inhibition (%)	Total no. of isolates	No. of antagonists		
				F. oxysporum	*R. bataticola*	Both
Pseudomonas	Weak antagonists	1.0-10	76	7	8	6
	Medium antagonists	>10-20.0		10	11	8
	Strong antagonists	>20.0		15	17	13
Bacillus	Weak antagonists	1.0-10	98	3	4	3
	Medium antagonists	>10-20.0		5	4	4
	Strong antagonists	>20.0		18	21	16
Total			174	58	65	50

Characterization of rhizobacterial antagonists

The bacterial antagonists from previous experiment were subjected to cultural, morphological and biochemical characterization as given in Bergey's manual of systematic bacteriology. The isolates were assessed for gram reaction and differences in colony morphology. Of the isolates which showed rapid growth on Kings B medium at 28°C, 15 produced characteristic fluorescent yellow to green pigmentation. All the fluorescence producing isolates were gram negative rods. The isolates were indole, methyl red, Voges-Proskauer negative, citrate and catalase positive. They were also able to hydrolyze starch and reduce nitrate. Isolates from nutrient agar were found to be gram-positive, rod-shaped bacteria. These isolates from chickpea rhizosphere were found to show rapid growth on NA media at 28°C. These were methyl red negative, Voges-Proskauer, citrate and catalase positive. The cultural, morphological and biochemical tests tentatively placed the isolates into two genera *Bacillus* and *Pseudomonas* (Table 3). Bacteria representing genera *Bacillus*, *Pseudomonas* and *Rhizobium* which are associated with plant rhizosphere have been reported as potential biocontrol agents. Joseph *et al.* (2007) in their studies for characterization of plant growth promoting rhizobacteria associated with chickpea reported that out of 150 isolates, 40 belonged to genus

Bacillus, 35 to *Pseudomonas*, 40 to *Azotobacter* and 35 to *Rhizobium*. Hynes *et al.* (2008) found that 36-42% isolates from pea, lentil and chickpea rhizosphere that were positive for growth promotion traits were members of the *Pseudomonadaceae*. Sivaramaiah *et al.* (2007) found that out of 124 rhizobacterial isolates obtained from the rhizosphere of field-grown healthy chickpea plants, 45 were gram-positive, sporulating rods.

TABLE 3: Cultural and biochemical characteristics of rhizobacterial isolates

Test character	Rhizobacteria isolated from Kings B media	Rhizobacteria isolated from Nutrient agar media
Gram's reaction	-ve	+ve
Shape	Rods	Rods
Pigment	+/-	- /+
Pigment colour	Fluorescent green/yellow/-	Black/brown/-
Starch hydrolysis	+	+
Catalase production	+	+
Methyl red test	-	-
VP test	-	+
Citrate	+	+
Nitrate production	+	+
Tentatively assigned genera	*Pseudomonas*	*Bacillus*

Plant growth promoting traits of antagonistic rhizobacteria

Phosphate solubilizing microorganisms play an important role in utilization of unavailable native phosphates as well as added phosphates. Screening of 50 antagonistic bacterial isolates for P-solubilization ability revealed that 19 of these could solubilize tricalcium phosphate, however, the P-solubilizing potential varied amongst these isolates (Table 4) as evidenced by the size of halo on Pikovskaya's agar plates. Of these 19 isolates, 10 were representatives of genus *Bacillus* and 9 of *Pseudomonas*. Phosphate solubilizing microorganisms largely include bacteria and fungi *viz.* species of *Bacillus*, *Pseudomonas*, *Penicillium* and *Aspergillus* (Tilak *et al.*, 2005). Chen *et al.* (2006) reported that bacterial genera *Bacillus*, *Rhodococcus*, *Arthrobacter* and *Serratia* were powerful P-solubilizers. In a similar experiment conducted for selecting Zn-solubilizing bacteria showed that 20 isolates were able to solubilize zinc oxide (Table 4) in the range of 1.14-1.77. In soil, both macro and micronutrients undergo a complex dynamic equilibrium of solubilization and insolubilization that is greatly influenced by the soil pH and microflora and that ultimately affects their accessibility to plant roots for absorption as noted by Altomare *et al.* (1999). Exploring microorganisms which can solubilize both micro and macro nutrients could efficiently improve plant growth by reducing the load of chemical fertilizers. Application of phosphate solubilizing bacteria to improve plant growth by solubilizing sparingly soluble inorganic phosphates in soil is well documented (Rodriguez *et al.*, 2006).

The production of phytohormones such as auxins and gibbrellins by PGPR is one of the most important mechanisms by which many rhizobacteria promote plant growth. In most cases these phytohormones are believed to change assimilate partitioning patterns in plants and affect growth patterns in roots to result in bigger, more branched roots, and/or roots with greater surface area. Gibberellic acid (GA) is a plant growth regulator of economic importance (Gelmi *et al.*, 2002). Various gibberellins are available and are associated with several plant growth and development processes, such as seed germination, stem elongation, flowering, and fruit development (Boğa *et al.*, 2009). All the isolates tested positive for gibberellic acid production and it ranged from 11.9-36.3 µg/ml. However, pseudomonads were observed to be more diverse in production of gibberellins as compared to bacillus isolates. The production of IAA was also observed by all the rhizobacteria, however, presence of tryptophan greatly affected the IAA production pattern of isolates; although it is also thought to be strain dependent (Ahmad *et al.*, 2008). In the absence of tryptophan, IAA production ranged from 2.4-15.5 µg/ml in contrast to 9.2-48.7 µg/ml in presence of 3mM tryptophan after 5 days of incubation. *Bacillus* isolates were found to be more efficient producers of IAA both in presence and absence of tryotophan. Joseph *et al.* (2007) isolated 150 rhizobacterial isolates from chickpea rhizosphere and screened for *in vitro* plant growth promoting activities. They recorded IAA production in all the isolates of *Pseudomonas*, *Bacillus* and *Azotobacter* (100%) followed by *Rhizobium* (85.7%). Ahmad *et al.* (2005) found that *Pseudomonas* isolates were able to produce IAA without tryptophan in the range of 5.34-22.4 µg/ml. A further increase in IAA production was observed in the presence of tryptophan.

Role of salicylic acid and sideophores by rhizobacteria has been implicated as both antagonistic and plant growth promoting trait. Salicylic acid (SA) is a plant phenolic and hormone-like endogenous regulator and its role in the defense mechanisms against biotic and abiotic stress has been well documented (Szalai *et al.*, 2000). Salicylic acid production was exhibited by 47 isolates, 22 of which belonged to *Bacillus* genus (Table 4) while rest 25 included all the pseudomonads. Change in blue colour of CAS medium to orange-yellow after 24 hours of incubation with rhizobacteria confirmed production of siderophores, reaching a maximum after 72 h. Out of 50 isolates, 16 produced distinct orange halo on CAS plates indicating siderophore production. Out of these 9 belonged to *Pseudomonas* sp. (Table 4.16) and 7 to *Bacillus* sp. (Table 4). Siderophore production was found to start after 24 h of incubation, reaching a maximum after 72 hrs, when organism had entered into stationary phase. In a similar study conducted by Akhtar and Siddiqui (2009), siderophore production by *Pseudomonas* sp. isolated from chickpea rhizosphere were reported and the halos formed

on CAS medium ranged from 1.6-1.7 cm. Joseph *et al* (2007) reported that the isolates of *Pseudomonas* from chickpea rhizosphere were strong siderophore producers (74.2%) while few isolates of *Bacillus* were able to produce siderophores (12.5%).

TABLE 4: Plant growth promoting traits of antagonistic rhizobacteria

Test character	*Pseudomonas* sp.		*Bacillus* sp.	
	No. of isolates	Range	No. of isolates	Range
P-solubilization index	10	1.14-2.33	9	1.14-2.31
Zn-solubilization index	11	1.14-1.77	9	1.24-1.63
Gibbrellins production (µg/ml)	27	11.9-36.2	23	14.5-30.7
IAA production (L-TRP -) (µg/ml)	27	2.4-15.5	23	4.8-26.1
IAA production (L-TRP +) (µg/ml)	27	9.9-28.7	23	9.2-48.7
Salicylic acid production (µg/ml)	25	5.75-42.5	22	1.3-65.5
Siderophore index	9	1.24-2.94	7	1.2-2.11

CONCLUSION

The present study concludes that rhizosphere supports a rich diversity of *Pseudomonas* and *Bacillus* isolates which possess both antagonistic and plant growth promoting attributes. Exploring and exploiting these rhizobacteria could be a thriving step towards developing eco-friendly and safe replacement for chemical based fertilizers and pesticides.

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