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Microbiology

Exploitation of Rhizobacteria for Functional Traits in Mungbean

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Abstract

The enrichment of plant rhizosphere with beneficial bacteria is a strategy that favours the production of more vigorous seedlings, which is essential for the success of legume cultivation. The aim of this study was to select rhizobacteria that are able to act as plant growth-promoting rhizobacteria (PGPR). A total of 17 rhizobacteria belonging to genera *Bacillus* (8), *Pseudomonas* (5) and *Azotobacter* (4) from mungbean rhizosphere were selected on the basis of their ability to solubilize phosphate. These selected rhizobacteria were further characterized for Plant Growth Promoting (PGP) traits for quantitative phosphate solubilisation, Indole Acetic Acid (IAA), organic acid production and Intrinsic antibiotic spectra (IAR), control of phytopathogen via siderophore and cellulase production and stress tolerant management by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Significantly high phosphate was solubilized by strain of *Bacillus* sp. B2 (9.77 mg ml^{-100}) followed by strain of *Pseudomonas* sp. P10 (9.14 mg ml^{-100}) and strain of *Azotobacter* sp. A3 (8.98 mg ml^{-100}). In the presence of L-trptophan P10, A3 and B2 were also able to produce significant high amount of IAA i.e. 55.6 µg ml^{-1}, 52.6 µg ml^{-1} and 50.3 µg ml^{-1} respectively and they also produced organic acid, siderophore and cellulase. Of 17 rhizobacterial isolates, 11 were found compatible with *Rhizobium* sp. *vigna* (LSMR1) *in-vitro*. IAR pattern revealed 52.9 % of rhizobacteria resistant to ampicillin (10 µg $disc^{-1}$) and chloramphenicol (25 µg $disc^{-1}$). ACC deaminase activity was measured by their growth in DF medium supplemented with ACC (OD 600 ranged from 0.117 to 0.432) as nitrogen (N) source. In the present investigation, rhizobacterial isolates *viz*, B2, P10 and A3 were found most promising for multiple activities (PGP traits, biocontrol and stress tolerant activities) and can be evaluated *in-vivo* for their possible role in mungbean growth and development.

Highlights

17 rhizobacteria , 3 isolates *viz*, B2, P10 and A3 belonging to genera *Bacillus* (8), *Pseudomonas* (5) and *Azotobacter* (4) respectively from mungbean rhizosphere were found most promising for multifunctional traits and compatibiltity with *Rhizobium* (*in vitro*).

Keywords: *Azotobacter*, *Bacillus*, Mungbean, PGP traits, *Pseudomonas*, Rhizobacteria

Mungbean [*Vigna radiata* (L.) Wilczek], is an important short duration grain legume crop with wide adaptability and low input requirements. An important feature of the mung bean crop is its ability to establish a symbiotic partnership with specific bacteria, setting up the biological N_2-fixation in root nodules that supply the plant's needs for N_2, improving soil quality (Mahmood and Athar, 2008; Mandal *et al*., 2009). Mungbean is well suited to a large

number of cropping systems and constitutes an important source of high quality protein in the cereal- based diets of many people in Asia (Sadeghipour, 2009). In last few decades a large array of bacterial genera including species of *Pseudomonas, Azospirillium, Arthrobacteria, Klebsiella, Enterobacter, Alcaligenes, Arthrobacteria, Bacillus and Serratia* colonize the legume rhizosphere have been reported as Plant Growth Promoting Rhizobacteria (Alikhania and Yakhchalia, 2009; Bashan and de-Bashan, 2009). PGPR applied to seeds or roots, colonize plant roots and stimulate plant growth and crop yield by direct and indirect mechanisms and 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 and stresses. Direct plant growth promotion mechanisms include symbiotic and non-symbiotic PGPR which function through production of plant hormones such as auxins, cytokinins, gibberellins, ethylene, *etc.* and by solubilisation of mineral phosphates and other nutrients, enhance resistance to stress, stabilize soil aggregates, and improve soil structure and organic matter content. Production of indole-3-ethanol or indole-3-acetic acid (IAA), the compounds belonging to auxins, have been reported for several bacterial genera (Mishra *et al*., 2010). PGPR retain more soil organic nitrogen (N), and other nutrients in the plant–soil system, thus reducing the need for fertilizer nitrogen (N) and phosphorous (P) and enhancing release of the nutrients (Hayat *et al.*, 2010). Indirect plant growth promotion mechanisms include the prevention of the deleterious effects of phytopathogenic organisms. This can be achieved by the production of siderophores, i.e. small metal-binding molecules (Martinez-Viveros *et al.*, 2010) and synthesis of antibiotics (Castro *et al.*, 2008). Another mechanism by which PGPR can inhibit phytopathogens is the production of hydrogen cyanide (HCN), ammonia and/or fungal cell wall degrading enzymes, e.g., cellulase, chitinase and protease (Pathma *et al.*, 2011). Some PGPR function as a sink for ACC, the immediate precursor of ethylene in higher plants, by hydrolyzing it into α-ketobutyrate and ammonia, and in this way promote root growth by reducing stress due to high indigenous ethylene levels in the micro-rhizo environment (Bangash *et al.*, 2013). PGPR may increase the efficiency of *Rhizobium* inoculation in legumes through these mechanisms and have potential to contribute to in sustainable plant growth promotion. These PGPR also enhance the infection sites for *Rhizobium* by colonizing the root surface (Parmar and Dadarwal, 1999; Contesto *et al.*, 2008). The exploitation of PGPR in combination with *Rhizobium* also constitutes an interesting alternative to improve nitrogen fixation. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield, but only a few isolates have been commercialized (Dey *et al.*, 2004). In the context of increasing international concern for food and environmental quality, the use of PGPR for reducing chemical inputs in agriculture is a potentially important issue (Saharan and Nehra, 2011; Farzana *et al.*, 2009; Kumar *et al.*, 2012). Therefore, screening for the selection of effective PGPR exhibiting multiple PGP traits needs to be very critical. Further exploration and evaluation on soil-plant system is needed to uncover their efficacy as effective PGPR. So keeping in view the above constrains, the present study was designed to screen rhizospheric bacterial isolates for their multiple activities (PGP traits, biocontrol and stress tolerant activities) from rhizospheric soil of mungbean crop.

Materials and Methods

Selection of rhizobacteria

A total of 17, out of 48 rhizobacterial isolates belonging to genera *Bacillus* (B1, B2, B3, B6, B7, B8, B10, B11), *Pseudomonas* (P2,P4,P5,P8,P10) and *Azotobacter* (A1, A3, A7, A9) from mungbean rhizosphere were selected on the basis of their ability to solubilize phosphate on NBRIP (National Botanical Research Institute's Phosphate growth) medium. The presence of yellow clear zone around bacterial growth after one week incubation period at 28°C was used as indicator for positive P solubilisation (Nautiyal, 1999). These selected isolates were further characterized for PGP (quantitative phosphate solubilisation, IAA, organic acid production and IAR), biocontrol (Siderophore and cellulase production) and stress tolerant activity (Quantitative assay for ACC deaminase production).

PGP traits

Quantitative measurement of phosphate solubilization

Hundred ml of Pikovaskaya's broth (Himedia, Mumbai) containing grams per liter of distilled water: 10.0 Glucose, 5 $Ca_3(PO_4)_2$, 0.5 $(NH_4)_2SO_4$, 0.2 NaCl, 0.1 $MgSO_4.7H_20$, 0.2 KCl, 0.5 Yeast extract, 0.002 $MnSO_4.7H_20$, 0.002 $FeSO_4.7H_20$, pH 7.0 was dispensed in 250 ml conical flasks. 100 mg P_2O_5 as tri-calcium phosphate (TCP) was added separately to each flask and the contents were sterilized at 121°C for 15 min. The flasks were inoculated with 1 ml suspension of overnight grown culture and incubated at 28±2°C for 15 days. Presence of yellow colour after addition of ammonium molybdate and ammonium vandate in equal

ratio to culture supernatant confirmed phosphate solubilizing activity. The yellow colour intensity of the solution was measured spectroscopically (TechComp UV-VIS spectrophotometer) at 420 nm after 25 minutes incubation for quantitative estimation of phosphate solubilisation (Jackson, 1973).

Measurement of IAA

IAA was quantified by the method of Gordon and Weber (1951). Rhizobacterial isolates were cultured in flasks containing 10 ml of nutrient broth supplemented with tryptophan (L-Trp) 0.2 mM and incubated at room temperature 28±2ºC for 48 h. The cultures were then centrifuged for 15 min at 10 000 rpm. Two ml of the supernatant was mixed with 2 ml of Salkowski's reagent (150 ml H_2SO_4, 250 ml distilled water, 7.5 ml $FeCl_3.6H_2O$ 0.5 M) and incubated at room temperature for 30 min. The presence of IAA was determined by the development of pink color and the IAA concentration was measured spectroscopically (TechComp UV-VIS spectrophotometer) at 520 nm and quantified in an IAA standard curve.

Organic acid production

Qualitative assessment of organic acid production by the rhizobacterial isolates was determined by methyl red test (Sambrook and Russell, 2001). Rhizobacterial isolates were inoculated in MR-VP broth and incubated for 5 days at 28±2ºC. It was then, observed for drop in pH using methyl red as an indicator dye. Isolates having the ability to produce organic acid gave orange to bright red colour while yellow colour indicated a negative reaction and on the basis of intensity of colour developed, the rhizobacterial isolates were categorized into different categories.

Intrinsic antibiotic spectra

Intrinsic antibiotic spectra (IAR) test was carried out to identify the bacterial sensitivity or resistance to antibiotics. In order to check the sensitivity of the isolate towards different antibiotics, disc diffusion was done where culture was grown on NA in the presence of sterile filter paper discs (HiMedia, Mumbai) impregnated with different concentration of antibiotics viz. tetracycline (30 µg $disc^{-1}$), ampicillin (10 µg $disc^{-1}$), kanamycin (30 µg $disc^{-1}$), erythromycin (15 µg $disc^{-1}$), chloramphenicol (25 µg $disc^{-1}$), amoxycillin (10 µg $disc^{-1}$) and streptomycin (25 µg $disc^{-1}$) After 48 h incubation of plate at 28 °C, either development of zone of inhibition was observed and interpreted as sensitive (S) or resistant (Sarode *et al.,* 2009).

Biocontrol activities

Siderophore production

Rhizobacterial isolates were assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.) described by Schwyn and Neilands (1987). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism and incubated at 28± 2ºC for 48–72 h. Development of yellow–orange halo around the growth was considered as positive for siderophore production.

Cellulase production

Colonies were screened for cellulase activity by plating on Carboxy Methyl Cellulose (CMC) agar (Himedia, Mumbai) according to Ariffin *et al.* (2006). The agar plates were prepared and spot inoculated with test organism and incubated at 28±2ºC for 5 days. Development of halo zone around the colony was considered as positive for cell wall degrading enzyme production. The CMC agar plates were incubated at 28±2ºC for 5 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. The diameter of clear zone was measured in order to select for the highest cellulase activity producer.

Stress tolerant activity

Quantitative estimation of ACC-deaminase production

Selected rhizobacterial isolates were grown individually in liquid DF (Dworkin and Foster, 1958) minimal medium with $(NH_4)_2 SO_4$, with ACC (Sigma, Ltd.) and without ACC. Growth of rhizobacterial isolates in these different media was measured at 600 nm using a TechComp UV-VIS spectrophotometer (Shahzad *et al.,* 2010)

Comptibility test

Compatibility of promising rhizobacteria with Rhizobium sp. vigna (in-vitro)

Rhizobium sp. *vigna* (LSMR1) and promising rhizobacterial isolates were tested for compatibility of growth by disc plate method (Anndaraj *et al.,* 2010). A lawn of *Rhizobium* sp. *vigna* was prepared by spreading culture on NA medium. A sterile disc was dipped in different rhizobacterial culture

and then placed on lawn of *Rhizobium* and observed for compatibility of both cultures. The zone of inhibition indicated the incompatibility of two cultures.

Data analysis

The collected data were analyzed statistically using the CPCS 1 software developed by Department of statistics, Punjab Agricultural University, Ludhiana, Punjab, India. Following the analysis of variance procedure (ANOVA), differences among treatment were determined using comparison method at 5% level of significance.

Results and Discussion

A total of 17, out of 48 rhizobacterial isolates from mungbean rhizosphere were selected on the basis of phosphate solubilisation indicated by presence of yellow zone on NBRIP medium (Figure 1). These 17 rhizobacteria were further exploited for their plant growth promoting, biocontrol and stress tolerant activities .

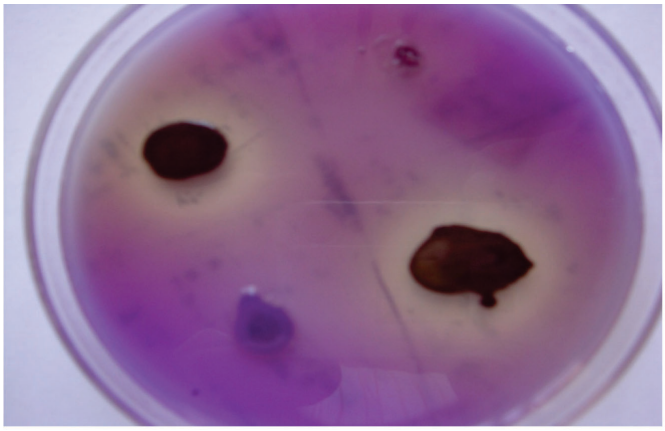

Fig. 1: Yellow zone on NBRIP medium indicated P solubilisation

PGP traits

Quantitative measurement of phosphate solubilization

The relative efficiency of 17 rhizobacterial isolates in solubilizing TCP in liquid medium as a function of time was further investigated at different intervals (3,6,9,12 and 15 days) (Table 1). It was seen that increasing amount of P was released by different isolates with increasing period of incubation upto 12th day. The phosphate solubilizing activity was observed upto 15th days. These isolates showed maximum phosphate solubilization at 12th day ranged between 4.28 to 9.77mg ml^{-100} with decrease in pH of medium (Figure 2). Significantly high phosphate was solubilized by rhizobacterial isolate B 2 (9.77 mg ml^{-100}) followed by P 10 (9.14 mg ml^{-100}) and A3 (8.98 mg ml^{-100}) at 12th day. After 12 days, there was decline in phosphate solubilizing activity, might be due to deficiency of nutrients in the culture medium (Kaur and Sharma, 2013). The ability of several isolates to solubilize tricalcium phosphate *in vitro* shows the possible application of the isolates in crop fields (Ponmurugan *et al.*, 2012). A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere soil (Rodriguez *et al.*, 2006; Suh *et al.*, 1995; Whitelaw *et al.*, 1999; Joseph *et al.*, 2007). *Pseudomonas* and other phosphate solubilizing bacteria (PSB) like *Bacillus* sp. and *Azotobacter* sp. were capable of increasing the availability of phosphorus in soil (Rodriguez and Fraga, 1999). Specifically, all isolates showed their potential to be developed as inoculants for alkaline soil, based on the ability to solubilize phosphate bounded by calcium which mostly exists in alkaline soils, whereas in the acidic soil, phosphate was mostly fixed by Fe or Al (Goldstein, 1995). Our results were similar to earlier result where solubilization of TCP in liquid medium by *Pseudomonas* spp. varied in the range of 24.7- 44.0 mg/ 100 ml (Poonguzhali *et al.*, 2008). Phosphate solubilization by rhizobacterial isolates has been shown to be related to the production of organic acids such as formic, acetic, propionic, lactic, glycolic, fumaric, succinic acids *etc* (Goldstein, 1995; Kucey, 1983). The production of organic acids results in a decrease in soil pH as observed in our study, producing H^+ which replaces Ca^{2+} and release HPO^{-1} to the soil solution. In tropical soil, the low pH influences solubilization of phosphate by rhizobacteria (Yasmin *et al.*, 2009).

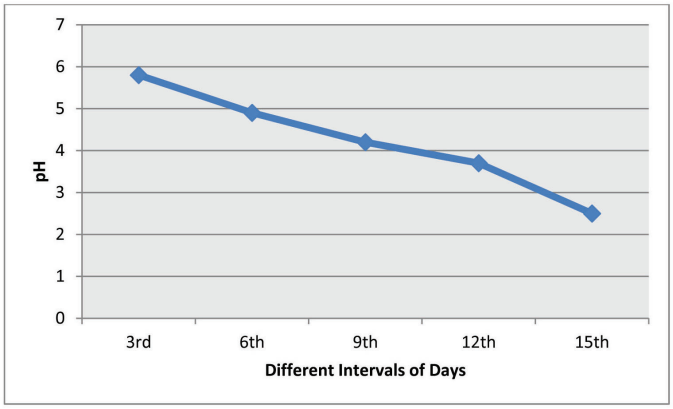

Fig. 2: Decrease in pH at different intervals of time (days)

Table 1: Quantitative measurement of P solubilization by rhizobacteria as a function of time

Rhizobacterial cultures	P-solubilization (mg/100ml)				
	Incubation period (days)				
	3rd	6th	9th	12th	15th
B1	2.80	7.29	8.19	8.27	6.77
B2	3.90	4.56	6.78	9.77	6.45
B3	2.89	4.90	6.97	8.43	7.08
B6	4.65	6.93	6.99	7.07	6.43
B7	5.88	5.21	5.34	6.82	5.67
B8	6.89	7.14	7.89	8.86	7.00
B10	3.14	3.07	3.92	4.28	3.45
B11	1.89	2.99	5.27	7.68	6.20
P2	1.34	2.70	4.23	6.22	6.13
P4	2.33	3.87	6.37	6.99	6.45
P5	1.74	2.67	4.29	5.78	5.22
P8	2.37	3.56	5.44	6.29	5.36
P10	3.76	4.88	7.67	9.14	7.19
A1	2.11	4.25	5.65	6.39	6.31
A3	3.43	4.67	6.32	8.98	7.02
A7	0.86	1.46	2.57	5.66	4.28
A9	1.45	2.90	3.74	6.38	5.40
CD(5%)	0.03	0.03	0.02	0.02	0.03

Measurement of IAA

A total of 13 rhizobacterial isolates were selected based on qualitative screening of IAA production (data not shown) and assayed for their ability to produce indole acetic acid in pure culture in the presence and absence of precursor L-tryptophan. In the absence of L-tryptophan, all the selected PGPR isolates produced very low amount of IAA ranged from 0.8 to7.4 µg ml^{-1} (Figure 3). In the presence of L-tryptophan the concentration of IAA produced by the rhizobacterial isolates ranged from 19.7 µg ml^{-1} to 55.6 µg ml^{-1}. Out of the 13 rhizobacterial isolates P10, A3 and B2 were also able to produce significantly high amount of IAA i.e. 55.6 µg ml^{-1}, 52.6 µg ml^{-1} and 50.3 µg ml^{-1} respectively, in the presence of L-trptophan.on the basis of above observations, these isolates were characterized into high, medium and low IAA producing genera (Figure 4). Based on earlier reports 80% of microorganisms isolated (*Azospirillum*, *Pseudomonas*, *Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus*, *A. chroococcum* and *Bradyrhizobium japonicum*) from the rhizosphere of various crops have the ability to produce IAA which help in stimulating plant growth (Saharan and Nehra, 2011; Bhattacharayya *et al.*, 2012). It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Kaur and Sharma , 2013). Similarly, our results are corroborated with earlier finding of Ahmad *et al*., (2008) where all tested *Bacillus* sp. showed IAA production in culture supplemented with tryptophan (Trp), in the range of 0.81 to 86.82 mg/L. Furthermore, there was an increase in the level of IAA with the increasing concentration of tryptophan (10-100µg/ml) as evident by (Vetrivelkalai *et al*., 2010). Bacterial IAA stimulates the development of the host plant root system (Joshi and Bhatt, 2011).

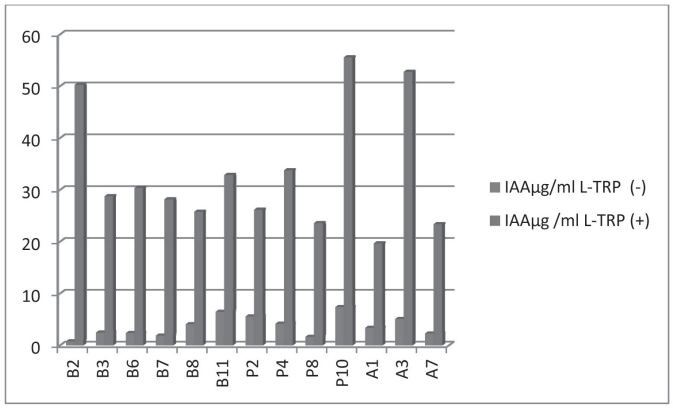

Fig. 3: Indole acetic acid (IAA) production by rhizobacteria in the absence and presence of L-Tryptophan (TRP). Each column represents a mean of triplicate data and following the analysis of variance procedure (ANOVA), differences among treatment were determined using comparison method at 5% level of significance. All the values were significantly greater than the lowest value

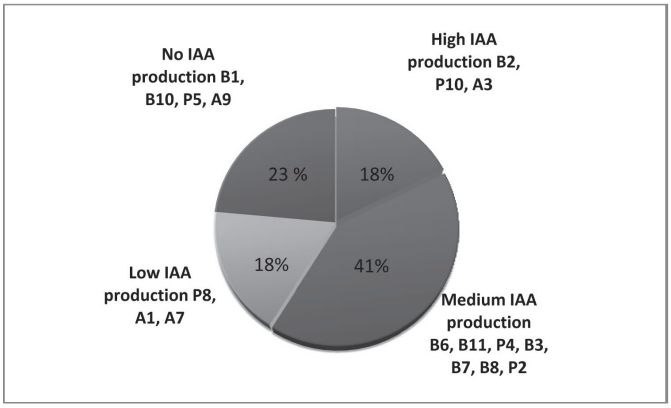

Fig. 4: Percentage of IAA producing genera

Organic acid production

Organic acid plays an important role in chelation and mineralization of minerals in the soil was produced by 70.8

% of rhizobacterial isolates capable of solubilizing the phosphate in medium (Table 3). On the basis of intensity of colour developed, the rhizobacterial isolates were categorized into different categories. Two rhizobacterial isolates, B2 and P10 showed highest organic acid production followed by A3 whereas 9 rhizobacterial isolates showed only low level of organic acid production. Organic acid production is directly related to phosphate solubilisation as indicated by our results. Similarly, other workers reported the production of organic acids by endophytic rhizobacteria from different crops (Vetrivelkalai *et al*., 2011; Hung *et al*., 2007).

Intrinsic Antibiotic Spectra

Out of 17 rhizobacteria from mungbean rhizosphere tested for their reactivity to antibiotics (Figure 5) revealed that 52.9 % of isolates were resistant to ampicillin and chloramphenicol whereas 47% showed resistance to erythromycin and 29.4% rhizobacterial isolates were resistant to tetracycline, amoxicillin, and streptomycin (Table 2). Resistance to erythromycin was shown by 23.5% of isolates. Resistance of PGPR to several antibiotics might have an ecological advantage of survival in the rhizosphere when they introduced as inoculum. Similarly, several workers revealed that rhizobacterial isolates were intrinsically resistant to antibiotics tested (Yasmin *et al*., 2004; Siddiqui *et al*., 2006; Kundu *et al*., 2009).

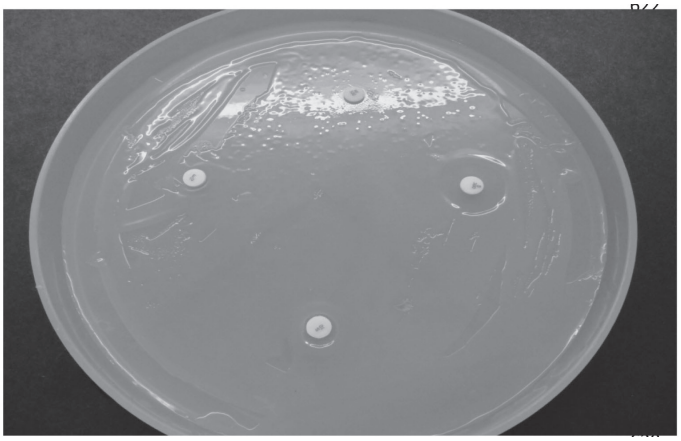

Fig. 5: Intrinsic antibiotic spectra of rhizobacteria

Biocontrol activities

Siderophore production

Siderophore production was further tested quantitatively by streaking the rhizobacterial isolates on Chrome Azurole S (CAS) agar medium. Formation of orange halo zone around the inoculated bacterial colonies on CAS agar indicated siderophore production (Figure 6). Of 17 selected rhizobacteria, 8 were able to form the orange zone after incubation for 48 hrs (Table 3). The diameter of orange zone for rhizobacteria ranged between 0.9 cm to 1.5 cm, being highest for B2 (1.5 cm) followed by P10 (1.4 cm) and A3 (1.4 cm). Several workers reported the production of siderophores by rhizospheric genera belonging to

Table 2: Intrinsic antibiotic spectra of rhizobacteria

Rhizobacterialcultures	Tetracycline	Amphicillin	Kanamycin	Erythromycin	Chloramphenicol	Amoxycillin	Streptomycin
B1	R	S	R	S	S	S	S
B2	S	R	S	R	S	S	S
B3	S	S	S	R	S	S	R
B6	R	S	R	S	S	S	R
B7	S	S	R	S	R	S	S
B8	S	R	S	S	S	R	R
B10	S	R	R	R	S	S	S
B11	S	R	S	R	S	R	S
P2	S	R	S	R	S	S	S
P4	R	R	S	R	R	R	R
P5	S	R	S	S	R	S	R
P8	R	S	S	S	R	S	S
P10	R	S	S	R	R	R	S
A1	S	R	S	S	R	S	S
A3	S	S	S	S	R	R	S
A7	S	R	S	S	R	S	S
A9	S	S	S	R	R	S	S

R=Resistance, S= Sensitive

Table 3: Multiple PGP activities of selected rhizobacteria from mungbean rhizosphere

Rhizobacterialcultures	Organic acid production	Siderophoreproduction	Cellulaseproduction	Compatibilitywith *Rhizobium* sp. *vigna*
B1	+	-	+ (0.8 cm)	-
B2	+++	+++ (1.5 cm)	+++ (2.1 cm)	+
B3	-	+ (1.1 cm)	+ (1.0 cm)	+
B6	+	-	++ (1.6 cm)	-
B7	+	-	+ (2.0 cm)	+
B8	-	++ (1.3 cm)	-	-
B10	+	-	++ (1.8 cm)	-
B11	-	+(0.9 cm)	-	+
P2	+	-	++ (1.9 cm)	+
P4	+	++ (1.2 cm)	++ (1.4 cm)	+
P5	-	+ (0.9 cm)	-	-
P8	+	-	-	+
P10	+++	+++ (1.4 cm)	++ (1.9cm)	+
A1	+	-	-	-
A3	++	+++ (1.4 cm)	++ (1.7 cm)	++++
A7	-	-	+++ (2.0 cm)	++++
A9	+	-	-	++++

(+ = low, ++ = medium, +++ = high & - = absent), (++++ = excellent growth, - = no growth)

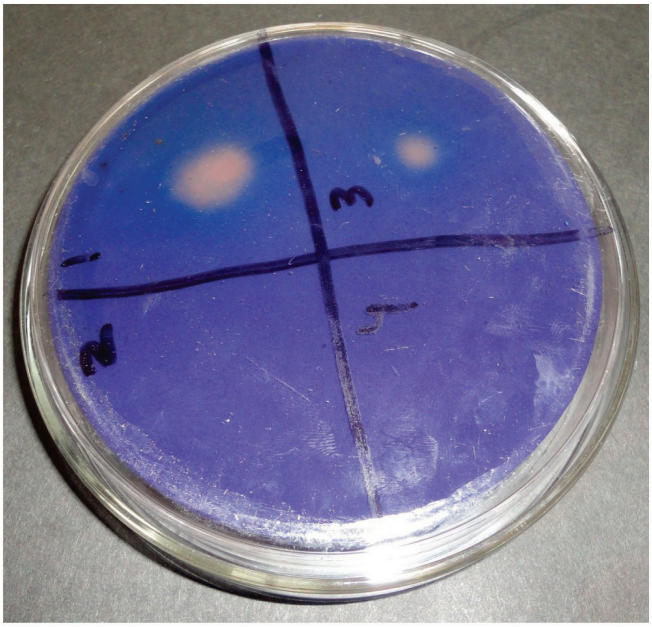

Fig. 6: Yellow-halo zone on Chrome azurol S agar indicated siderophore production

Pseudomonas, *Bacillus*, *Azotobacter*, *Azospirillum* and *Serrtia* (Saharan and Nehra, 2011; Nautiyal, 1999; Yasmin *et al*.,2009). Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, including *Bacillus* sp. under iron limiting condition and facilitate the uptake of iron from environment (Wahyudi *et al*.,2011). 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). Siderophores chelates iron and other metals contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Loper and Henkels, 1997). Siderophore may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria. Similarly, maximum siderophore production was reported for *Pseudomonas fluorescence* (diameter ranged from 0.72-2.6 cm) (Chaiharn, *et al*., 2008). There isolates of *Pseudomonas* spp. (74.2%) and *Bacillus* (12.5%) from chickpea rhizosphere were also reported as strong siderophore produdcers by Joseph *et al*.(2007).

Cellulase production

Production of fungal cell wall degrading enzymes was analysed because this is an important mechanism of fungal inhibition. Screening of bacteria was conducted by using the Congo red test as a preliminary study for identifying cellulase producers. After 5 days of incubation, 64.7 % of rhizobacterial isolates showed signs of growth on CMC agar and demonstrated positive results in the Congo red

test (Table 3). Since the sole carbon source in CMC agar was CMC (cellulose), therefore the result of the test was a strong evident that cellulase was produced in order to degrade cellulose. The diameter of the halo zone was measured and ranged between 0.8 cm to 2.1 cm. Maximum diameter of halo zone was observed for B2 (2.1cm) followed by P10 (1.9 cm) and A3 (1.7 cm) as compared to other rhizobacteria and indicated the highest cellulase production. Similarly, other workers reported 6% to 75% of rhizobacterial isolates positive for cellulase, a fungal cell wall degrading enzyme and control phytopathogens (Chaiharn *et al*., 2008;Asilah *et al*., 2009). It has been hypothesized by Asilah *et al*. (2009) that cellulases also involved in the degradation of cell wall polymers and facilitate invasion of N_2-fixing bacteria in leguminous plants.

Stress tolerant activity

ACC deaminase production

Based on preliminary qualitative test (data not shown) for screening of rhizobacteria positive for ACC deaminase, a total of 12, among these isolates were selected for their ability to utilize ACC as a sole source of N with different degrees of efficacy was assessed on the basis of bacterial growth in liquid medium in terms of optical density (OD ë 600). Improved growth of these rhizobacterial isolates was observed in DF medium supplemented with $(NH_4)_2$ SO_4 (ranged between 0.234 to 0.541) as compared to DF medium supplemented with ACC (ranged between 0.117 to 0.432). This indicated their ability to use ACC as N source due to the presence of ACC deaminase activity (Table 4). Growth in DF medium without ACC was negligible due to the absence of N source. All rhizobacterial isolates, originated from mungbean rhizosphere soil were capable of utilizing ACC and showed variable growth rates in DF medium containing ACC as sole N source. B2 rhizobacterial isolate showed significantly highest growth in DF medium with ACC (0.432) followed by P10 (0.384) and A3 (0.345) (Table 3). This may implied that ACC-deaminase enzyme in different rhizobacteria have variable potential to hydrolyze ACC and, thus could have differential affect on growth of inoculated plants. Several bacterial species belonging to different genera such as *Azospirillum, Agrobacterium, Achromobacter, Burkholderia, Enterobacter, Pseudomonas* and *Ralstonia* have been reported to exhibit variable ACC-deaminase activity which reduces the level of stress ethylene conferring resistance and stimulating growth of plants (Saravanakumar, 2012; Arshad *et al*., 2008; Nukui *et al*., 2000). Our results were similar to earlier study of Govindasamy *et al*., (2008), where out of 236 bacterial isolates screened from the wheat rhizosphere, 40 isolates showed growth on DF minimal medium containing ACC. Similarly, three groups of lentil rhizobacteria, were identified for ACC substrate as compared to ammonium sulphate depending upon their OD value at 550 nm (strains with higher (OD > 0.7), medium (OD 0.5-0.69) and lower (OD < 0.5) growth) (Zafar-ul-Hye *et al*., 2007). A direct correlation has been reported in bacterial ACC-deaminase activity and root growth (Mayak *et al*., 2004; Shaharoona *et al*., 2006). Presence of ACC-deaminase enzyme activity could be a useful tool for screening effective inoculants (i.e. rhizobacteria) to promote seedlings growth.

Table 4: Relative efficacy of ACC-utilization by selected rhizobacteria

Rhizobacterial cultures	ACC deaminaseactivity (quantitative)at 600 nm		
	DF+$(NH_4)_2SO_4$	DF+ACC	DF-ACC
B1	0.266	0.199	0.023
B2	0.541	0.432	0.211
B3	0.452	0.219	0.286
B6	0.478	0.267	0.182
B7	0.390	0.117	0.03
B8	0.490	0.301	0.197
B11	0.234	0.187	0.108
P4	0.310	0.256	0.126
P10	0.478	0.384	0.124
A1	0.334	0.289	0.192
A3	0.486	0.345	0.189
A7	0.319	0.250	0.027
A9	0.263	0.151	0.057
CD(5%)	0.022	0.022	0.024

Compatibility test

Compatibility of rhizobacteria with Rhizobium sp. vigna (in-vitro)

The compatibility of rhizobacteria was tested *in-vitro* with *Rhizobium* sp. *vigna* (LSMR1) by keeping sterile disc dipped in rhizobacterial culture on *Rhizobium* lawn and observed for their growth. Out of 17 rhizobacterial isolates, 11 rhizobacterial isolates were found to be compatible with each other and were able to grow simultaneously without any inhibition in growth (Table 4). The remaining six incompatible rhizobacterial isolates resulted into the formation of zone of inhibition to *Rhizobium* growth (Figure 7). Our results were similar to earlier results of Ostwal and Bhide (1972) and Saxena and Tilak (1994) who

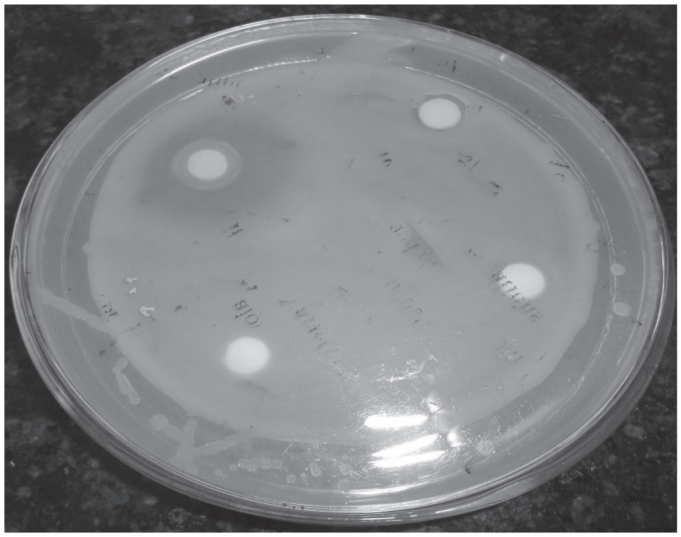

Fig. 7: Compatibility and incompatibility of rhizobacteria with *Rhizobium* sp. *vigna* (LSMR1) indicated by growth

reported favourable effect of *P. fluorescens* on *Rhizobium* growth *in vitro* condition and may be attributed to enhanced activity of microorganisms in association due to synergistic interaction. Interaction between plants and microorganisms in the rhizosphere (rhizobacteria) can affect crop yields. When these microbial strains are made into an inoculum consortium, synergistic interactions result an increase in growth promotional activities *in vitro*. Earlier, microbial study performed without plants indicated that some combinations allow the bacteria to interact with each other synergistically, provide nutrients, remove inhibitory products, and stimulate each other through physical and biochemical activities that may enhance some beneficial aspects of their physiology (Bashan, 1998). Co-inoculation and co-culture of microbes have been observed to perform the tasks better than the individual microbes (Seneviratne, 2003). Successful establishments of these types of combinations *in-vivo* have also been reported by other workers (Chanway *et al.*, 1989; Dashti *et al.*, 1997; Dashti *et al.*, 1998; Grims and Mount, 1984; Li and Alexander, 1988).

Conclusion

This study showed that three rhizobacterial isolates (B2, P10 and A3) were most promising for PGP traits, biocontrol and stress tolerant activities and have potential to be used in future as PGP inoculants to improve growth and yield in mungbean crop. Further investigations, including efficiency test under greenhouse and field conditions are needed to clarify the role of rhizobacteria exhibiting multiple activities as biofertilizers that exerts beneficial effects on Mungbean productivity.

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