Journal of Food Legumes 26(3 & 4): 84-89, 2013

Phenotypic characterization of rhizobacteria associated with mungbean rhizosphere

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(Received : May 20, 2013 ; Accepted : October 21, 2013)

ABSTRACT

Plant growth promoting rhizobacteria have been identified in influencing the growth and yield of many plants by direct or indirect mechanisms. In search of efficient PGPR strains with multiple plant growth promoting (PGP) activities, a total of 48 isolates of rhizobacteria were isolated from 35 different samples of mungbean rhizosphere. Out of 48, 34 isolates were characterized and tentatively identified as *Bacillus* spp. (14), *Pseudomonas* spp. (11) and *Azotobacter* spp. (9) on the basis of their morphological and biochemical activities. Thirty five percent of these rhizobacterial isolates were able to solubilize phosphate (P) and showed solubilisation index (SI) from 1.00 to 3.83 being highest with rhizobacterial isolate B2 (3.83). These rhizobacterial isolates were selected and screened *in vitro* for their PGP traits (Indole acetic acid (IAA) production), biocontrol (NH_3 , HCN, chitinase and protease production) and stress tolerant activity (1-amino cyclopropane-1-carboxylic acid (ACC) deaminase). Rhizobacterial isolates (B2, P10 and A3) showed maximum IAA production. Ammonia production was detected in two isolates (B2 and A3) and 5 isolates (B2, B6, P4, P10 and A3) found positive for HCN production. None of the isolates was positive for chitinase production whereas 52.9% of selected isolates were able to produce protease on skimmed milk agar. Seventy percent of the isolates indicated the growth on plates containing Dworkin and Foster (DF) minimal medium with ACC as a sole nitrogen source indicated the presence of ACC deaminase activity. Three rhizobacterial isolates (B2, P10 and A3) were found most promising for multiple activities (PGP traits, biocontrol and stress tolerant activities) and have potential to be used in future as PGP inoculants to improve mungbean crop.

Keywords: *Azotobacter,* ACC deaminase, *Bacillus,* Mungbean, *Pseudomonas,* Rhizobacteria,

Mungbean [*Vigna radiata* (L.) Wilczek], also known as green gram, has gained key importance in intensive crop production systems in India because of its short growing period and better storage ability. Mungbean not only has great dietary value due to its high protein content but also improves soil fertility by fixing atmospheric nitrogen Soil bacteria generally associated with legumes including free living as well as associative and symbiotic rhizobacteria belonging to genera *Acetobacter, Arthrobacter, Azorhizobium, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Pseudomonas, Proteus, Serratia, Rhizobium, Bradyrhizobium, Ensifer, Mesorhizobium* and *Xanthomons,* have reported to enhance plant growth (Bashan and de-Bashan 2010). The application of PGPR in crop production is steadily increasing as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other inputs.

The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include : ability to produce or change the concentration of plant growth regulators like indole acetic acid (IAA) gibberellic acid cytokinins and ethylene (;asymbiotic nitrogen fixation; (3) exhibition of antagonistic activity against phytopathogenic microorganisms by producing siderophores, ß-1,3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide and (4) solubilization of mineral phosphates and other nutrients (Kumar *et al.* 2012, Sahai and Chandra 2010,Mehnaz *et al.* 2010, Mishra *et al.* 2010, Bansal 2009, Joseph *et al.* 2007) In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil The good response obtained *in vitro* cannot always be dependably reproduced under field conditions (Zhender *et al.* 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effect on the plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other micro-organisms (Bent *et al.* 2001).

Therefore, it is necessary to develop efficient strains for field conditions. One possible approach is to explore soil microbial diversity for PGPR having combination of PGP activities and well adapted to particular soil environment. So keeping in view the above constrains, the present study was designed to screen rhizospheric bacterial isolates for their multiple plant growth promoting activities from rhizosphere of mungbean crop.

Isolation and characterization of rhizobacteria

Rhizospheric soil samples (1 kg) were collected from different locations of mungbean growing area of Punjab. Soil samples were stored in refrigerator till further use.

Ten gm of soil samples from mungbean rhizosphere were shaken in 90 ml sterilized distilled water for 10 min (Saxena and

Matta 2005). The bacterial strains were isolated by a serial dilution plate technique using Crystal Violet (CV) and Methyl Red (MR) agar for the isolation of Gram negative and Gram positive bacteria, respectively. Plates were incubated at 28±2°C. The colonies were then further transferred on Nutrient Agar (NA) plates. Bacterial colonies were carefully isolated and streaked over the surface their specific media viz. NA for *Bacillus* species, King's B (King *et al.* 1954) for *Pseudomonas* species and Jensen's (Jensen 1942) for *Azotobacter* species. The bacterial isolates were maintained on Trypticase soybean agar (TSA) slants (Narula *et al.* 2006) at 4°C temperature.

The thick bacterial smear of all the isolates was gram stained and morphological characterized on the basis of colony morphology including shape, elevation, texture, margin, color, odour, size and pigmentation. Biochemical characterization of PGPR was done on the basis of oxidase , catalase, citrate utilization, Methyl red (MR), Voges-Proskauer(VP), nitrate reduction and indole production test as per standard procedure (Cappuccino and Sherman 1992).

Plant Growth Promoting (PGP) activities

Assay for P solubilisation

P solubilization ability of plant associated bacteria was determined qualitatively by streaking strains 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). Solubilization index (SI) was calculated by using following formula:

SI Index= A/B

A= total diameter (colony + halo zone), B= diameter of colony.

Promising P solubilizers were further tested for growth promotional, biocontrol and stress tolerant activities.

Qualitative Analysis of Indole Acetic Acid (IAA)

Selected bacterial isolates were cultured on Nutrient Agar (NA) medium amended with L-tryptophan (Trp) and overlaid with cellulose membrane (Whatman filter paper) and incubated for 48h at 28°C (Shobha and Kumudini 2012). Salkowski's reagent was added on the cellulose membrane after 48h of incubation. Pink colouration indicated production of IAA. The results were also analyzed visually on a three point scale (+ - low; ++ - medium and +++ - high).

Biocontrol activities

NH_3 production

Selected rhizobacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated into 10 ml peptone water in each tube and incubated for 48 h at 28°C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman 1992).

HCN production

Exponentially grown different selected rhizobacterial isolates were separately streaked on NA medium supplemented with 4.4 g of glycine per litre with simultaneous supplementation of a filter paper soaked in 0.5% picric acid in 5% Na_2CO_3 in the upper lid of Petri dish. The plates were incubated at 28±1ºC for 2to 3 days. Change in colour from yellow to light brown for moderate (brown) or strong (reddish-brown) indicated HCN production (Bakker and Schippers 1987).

Chitinase and Protease production

Chitinase and protease activity (casein degradation) was determined from clear zone on chitin and skimmed milk agar respectively. The agar plates were prepared and spot inoculated with test organism and incubated at 30°C for 5 days. Development of halo zone around the colony was considered as positive for chitinase and protease production (Chaiharn *et al.* 2008).

Stress tolerant activity

ACC Deaminase activity

The qualitative estimation was done by the method prescribed by Govindasamy *et al.* (2008). Rhizobacterial isolates were streaked on plates containing Dworkin Foster (DF) (Dworkin and Foster 1958) minimal medium with ACC as a sole nitrogen source. The plates were incubated for 3-4 days at 28±1°C and observed for growth.

Morphological characterization of rhizobacteria

A total of 48 isolates of rhizobacteria from 18 soil samples collected from different rhizospheric locations of Punjab were isolated on crystal violet (CV) (28 isolates) and methyl red (MR) (20 isolates) agar media and further streaked on NA. Eleven isolates obtained at CV agar plates produced round shaped and raised colonies having smooth, shiny surface with smooth margin and light yellow to off white in color, but all were odourless while these isolates produced a fluorescent green pigment on King's B medium. These were tentatively assigned to genera *Pseudomonas* on the basis of cultural and morphological appearance Nine isolates from CV agar produced transparent, glistening and shiny colonies when streaked on nitrogen-free Jensen's medium and were assigned as *Azotobacter* species On MR agar, 14 out of 20 isolates produced large spreading, irregular shaped, off-white and rough colonies and were tentatively assigned to genera *Bacillus* Microscopic examination also revealed some characteristics of rhizobacterial isolates on the basis of their shape, gram's reaction and motility (Table 1). *Pseudomonas* and *Azotobacter* species were rod shaped, motile and gram negative in reaction whereas *Bacillus* species were also rod shaped and motile but gram positive in reaction.

Table 1. Morphological cultural, and biochemical characteristics of rhizobacteria

Isolates	***Bacillus***	***Pseudomonas***	***Azotobacter***
Cell shape	Rod	Rod	Rod
Elevation	Umbonate	Raised	Raised
Texture	Mucoid	Mucoid	Mucoid
Margin	Irregular	Smooth	Entire
Color	Cream	light yellow	Tranparent opaque
Odour	Odourless	Odourless	Odourless
Size	Medium	Medium	Medium
Pigmentation	None	Fluorescent green	None
Motility	Motile	Motile	Motile
Gram reaction	Gram positive	Gram negative	Gram negative
Oxidase	+	+ (with in 10 sec)	+
Catalase	+	+	+
Citrate utilization	+	+	+
Methy red (MR)	-	-	+
Voges Proskauer (VP)	+	-	–
Nitrate reduction (NR)	+	+	+
Indole	-	-	+

Table 2. Qualitative analysis of indole acetic acid (IAA)

Bacterial isolates	**B2, P10, A3**	**B6, B11, P4**	**B3,B7,B8, P2, P8, A1, A7**	**B1, B10, P5, A9**
Intensity	+++	++	+	-

(+ : low; ++ : medium and +++ : high, - : absence of IAA production)

Table 3. Characterization of rhizobacteria for biocontrol and stress tolerant activities

Cultures	**NH_3**	**HCN**	**Chitinase activity**	**Protease activity**	**ACC deaminase Activity**
B1	-	-	-	+	+
B2	+	+	-	+	+
B3	-	-	-	+	+
B6	-	+	-	-	+
B7	-	-	-	-	-
B8	-	-	-	-	+
B10	-	-	-	-	-
B11	-	-	-	+	+
P2	-	-	-	+	-
P4	-	+	-	-	+
P5	-	-	-	+	-
P8	-	-	-	-	-
P10	-	+	-	+	+
A1	-	-	-	-	+
A3	+	+	-	+	+
A7	-	-	-	-	+
A9	-	-	-	+	+

Biochemical characteristics of rhizobacteria

On the basis of biochemical characterization, all isolates were found to be positive for catalase, citrate utilization and nitrate reduction. All rhizobacterial isolates were positive for oxidase test (Table 2). *Bacillus* and *Pseudomonas* species were positive for VP but negative for MR except *Azotobacter.*

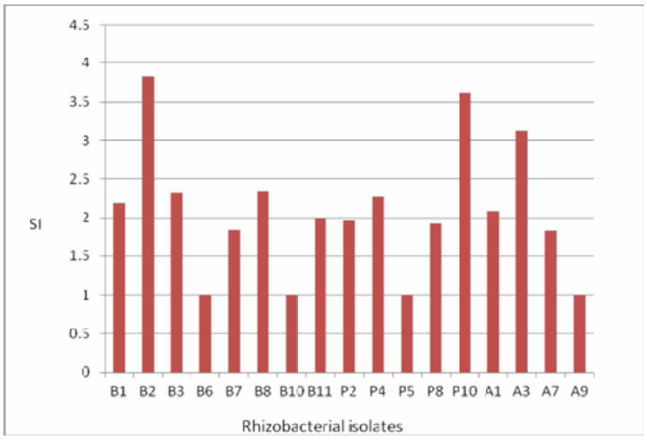

Fig 1. P Solubilisation Index (SI) for rhizobacterial isolates

Out of 48 isolates, 29.2% were *Bacillus* sp.(B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13 and B14.), 22.9% were *Pseudomonas* sp. (P1, P2, P3, P4, P5, P6, P7, P8, P9, P10 and P11) and 18.7% were *Azotobacter* sp. (A1, A2, A3, A4, A5, A6, A7, A8 and A9).

In our study, three different genera were identified *viz. Bacillus, Pseudomonas* and *Azotobacter. Bacillus* (17.9%) was dominant group followed by *Pseudomonas* and *Azotobacter* in mungbean rhizosphere. Earlier studies have shown that *Bacillus* and *Pseudomonas* as dominant genera in the rhizosphere probably due to their ability to efficiently use nutrients in the root exduates. (Joseph *et al.* 2007, Joshi and Bhatt 2011). Ahmad *et al.* (2008) reported *Azotobacter* (65.2%) as a predominant group in the rhizospheric soil of different crops (mustard, barseem, wheat, sugarcane, brinjal, onion, cauliflower, cabbage and chickpea) as compared to *Pseudomonas* (12.5%) and *Bacillus* (13.8%). Similarly, Cattlen *et al.* (1998) reported *Pseudomonas, Burkholderia, Bacillus* and *Alcaligens* as predominant genera in rhizosphere of soybean. Dominance of these genera in rhizosphere of different crops is useful because of their significant ecological roles in soil and nutrient cycling (Gray and Smith 2005).

Plant Growth Promoting (PGP) activities

Phosphate solubilisation activity

Out of 34 rhizobacterial isolates, 57% of *Bacillus*, 45% of *Pseudomonas* and 44% of *Azotobacter* species showed P-solubilization and resulted into formation of sharp yellow phosphate solubilization zone on NBRIP medium Solubilization Index (SI) revealed variation from 1.0-3.0. Bacterial isolates B6, B10 and P5 showed SI index as 1.0 with solubilization zone as wide as the colony diameter (Fig. 1). Five isolates viz. B1 (2.19), B3 (2.33), B8 (2.34), P4 (2.28) and A1 (2.09) showed SI greater than 2.00. Three isolates i.e B2 (3.83), P10 (3.62) and A3 (3.13) with SI greater than 3.00 showed highest phosphate solubilization. This investigation has been found coherent with the result of Calvo *et al.* (2010) who reported *Pseudomonas, Bacillus* and *Azotobacter* as effective phosphate solubilizing genera. Kumar *et al.* (2012) also reported 12 out of 30 rhizobacteria belonging to genera *Acinetobacter Pseudomonas, Bacillus* and *Enterobacter* isolated from french bean rhizosphere and produced clear zones ranging from 4 to 20mm. Similarly, Sitepu *et al.* (2007) reported phosphate solubilizing bacteria with SI equal to 1 having solubilization zone as wide as the colony diameter and greater than 3.00 for bacteria with efficient phosphate solubilization on media containing calcium-triphosphate.

Indole acetic acid production

After 48h of incubation, the cellulose membrane (Whatman filter paper) was removed and treated with Salkowski's reagent. Observations showed that the membrane turned pink indicating the production of IAACellulose membrane placed on cultures of all the isolated bacterial species except B1, B10, P5 and A9 showed pink coloration but with varying intensity. Visual observation showed that B2, P10 and A3 cultures showed maximum colorations whereas B3, B7, B8, P2, P8, A1 and A7 cultures showed the least (Table 2 -)

Our results are in well agreement with findings of Joseph *et al.* (2007) who has reported the highest IAA production in all isolates of *Bacillus, Pseudomonas* and *Azotobacter* (100%) followed by *Rhizobium* (85.7%) in chickpea rhizosphere. Similarly, production of IAA has been reported in species of *Bacillus, Pseudomonas, Azotobacter, Azospirillium, Phosphobacteria, Glucanoacetobacter, Aspergillus* and *Penicillium* in rice (Ashrafuzzaman *et al*. 2009, Saharan and Nehra 2011). *B. megaterium* from tea rhizosphere also produced IAA and played an important role in plant growth promotion (Chakraborty *et al.* 2006). Variation in IAA production with different isolates in present study was well supported with findings of Ashrafuzzaman *et al.* (2009) who also revealed that IAA production by PGPR can vary among different species and strains and is also influenced by culture condition, growth stage and substrate availability. IAA functions as an important signal molecule in the regulation of plant development and indirectly by influencing bacterial amino cyclopropane-1-carboxylate (ACC) deaminase activity (Ryu and Patten 2008, Wahyudi *et al.* 2011). Role of bacterial IAA in different plant-microbe interactions 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). Enzyme indolepyruvic decarboxylase (IPDC) is the principal enzyme which determines IAA biosynthesis and stimulates the development of the root system of the host plant (Erturk *et al.* 2010).

Biocontrol activities

NH_3 and HCN production

Development of yellow-brown color was observed after addition of Nessler's reagent indicating a positive test for ammonia production. Only two isolates B2 and A3 were able to produce ammonia (Table 3 -) whereas no ammonia production was observed with *Pseudomonas* isolates. Ammonia production indirectly influenced the plant growth and directly involves in biocontrol activities. Our results are well corroborated with findings of Mishra *et al.* (2010) who revealed *B. subtilis* strain MA-2 and *Pseudomonas fluorescens* strain MA-4 was efficient in ammonia production as compared to *Azotobacter* isolates and significantly increased biomass of medicinal and aromatic plant such as *Geranium*. In present study, ammonia production was recorded with 11.3% of the rhizobacterial isolates in mungbean rhizosphere. On the contrast, Samuel and Muthukkaruppan (2011) detected ammonia production in 95% of the isolates from the rhizosphere of rice, mangrove and effluent contaminated soil influencing plant growth promotion. Similarly, Joseph *et al.* (2007) revealed the production of ammonia commonly, detected in the isolates of *Bacillus* (95%) followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%).

Five isolates (B2, B6, P4, P10 and A3) among selected isolates were found positive for HCN production from mungbean rhizosphere (Table 3). Similarly, rhizobacteria belonging to genera *Pseudomonas, Bacillus,* and *Azotobacter* from rhizosphere of rice, mangrove, chickpea, frenchbean and effluent contaminated soil showed HCN production (Joseph *et al.* 2007; Samuel and Muthukkaruppan 2011, Kumar *et al.* 2012). HCN production by rhizobacteria has been postulated to play an important role in the biological control of pathogens (Kumar *et al.* 2012).

Chitinase and Protease production

Production of fungal cell wall degrading enzymes was analysed because this is an important mechanism of fungal inhibition. All the selected isolates were negative for chitinase production on chitin agar, whereas 52.9% of selected isolates were positive for protease production on skimmed milk agar (Table 3). Similarly, Chaiharn *et al.* (2008) reported 14 rhizosphere isolates (6%) positive for cellulase, a fungal cell wall degrading enzyme, detected chitinase activity in fifteen isolates (6%) and found eleven isolates (5%) producing halo zones on skim milk agar that showed protease activity. The screening of isolates for protease production on skim milk agar plate according to Cattelan *et al.* (1999) showed that *Pseudomonas* isolates from soybean rhizosphere were positive for the proteolytic activities which supported the work done by our study. Ruchi *et al.* (2012) also reported the proteolytic activity by 26 rhizobacterial isolates from the rhizosphere of both apple and pear growing in normal and replant sites. Evidence for suppression of soil borne plant pathogens by both antibiotic and lytic enzyme producing *Bacillus* strains have recently been described (Kumar *et al.* 2012).

Stress tolerant activity

ACC deaminase activity

Seventy percent of the selected isolates showed the growth on plates containing DF minimal medium with ACC as a sole nitrogen source indicating the presence of ACC deaminase activity (Table 3). Out of 17 selected isolates, 100% of *Azotobacter* sp. detected ACC deaminase activity followed by 75% of *Bacillus* sp. and 40 % *Pseudomonas* sp. Similarly, several bacterial species belong to different genera such as *Azospirillum, Agrobacterium, Achromobacter, Burkholderia, Enterobacter, Pseudomonas* and *Ralstonia* have been reported to possess variable ACC-deaminase activity in maize and pea rhizosphere (Nadeem *et al.* 2007; Arshad *et al.* 2008). It is highly likely that rhizobacteria promoted root growth by lowering ethylene levels in plant and/or in the vicinity of roots because of their ACC-deaminase activity. Many researchers have reported better root growth in plants inoculated with bacteria containing ACC-deaminase (Glick *et al.* 1995, Mayak *et al*. 2004, Shaharoona *et al.* 2006). Rhizobacteria containing ACC-deaminase can facilitate plant growth to overcome harmful effects of ethylene under water stress conditions. Inoculation with rhizobacteria containing ACC-deaminase increased the root length of the wheat seedlings ranging from 21 to 23% over uninoculated control (Bangash *et al*. 2013).

This study showed that three rhizobacterial isolates (B2, P10 and A3) were found most promising for multiple activities (PGP traits, biocontrol and stress tolerant activities) and have potential to be examined for their capability in enhancing plant growth. A better understanding on their diversity in the rhizosphere along with their colonization ability and mechanism of action should facilitate their application as a reliable component in the management of sustainable agricultural system.

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