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Plant Growth Promoting (PGP) Attributes of Stress Tolerant Rhizobial Isolates from Root Nodules of Pigeon Pea [*Cajanus cajan* (L.) Millspaugh] Growing in Haryana, India

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A B S T R A C T

A set of forty nine abiotic stress tolerant rhizobia of pigeon pea plant were isolated from four districts of Haryana, India to study plant growth promoting attributes. It was observed that Indole-3-acetic acid (IAA) production was low and varied from 0.16 µg ml^{-1} to 9.50 µg ml^{-1}. However, only 35% rhizobial isolates were siderophore producer. Interesting results were obtained as 96% of rhizobial isolates form significant zone of P-solubilization on Pikovskaya's medium and their P-solubilization index (P-SI) varied from 1.2 to 3.7. Among the forty nine rhizobial isolates, 49% of the isolates showed growth on 1-aminocyclopropane-1-carboxylate (ACC) supplemented plates while most of the isolates showed growth on ammonium sulphate plates. *Rhizobium* with the plant growth promoting (PGP) attributes have potential for increased tolerance to high salt, water potential, pH and temperature stresses, therefore could enhance production of food and forage legumes in semiarid and arid regions of the world.

Introduction

Pigeon pea (*Cajanus cajan* (L.) Millspaugh) is one of the important pulse crop and a very popular food in developing tropical countries. India is a principal pigeon pea growing country contributing nearly 90% of total world's production. Pigeon pea is able to associate with a large diversity of indigenous rhizobia in soil, reaching more than 150 kg of fixed N per ha^{-1} $year^{-1}$ (Peoples *et al.*, 1995). Seed inoculation of pulse crops with effective *rhizobium* strains prior to sowing is a recommended practice as it improves nodulation and nitrogen fixation, which in turn is translated into enhanced growth and grain yield. The mechanisms of plant growth promotion known to be employed by bacterial endophytes are similar to the mechanisms used by rhizospheric bacteria, e.g., the acquisition of resources needed for plant growth and development (Santoyo *et al.*, 2016). Various free living soil bacteria that are capable of exerting beneficial effects on plants and can lead to increased yields of a wide variety of crops are known as plant growth promoting rhizobacteria (PGPR) showing several plant growth promoting activities (Glick *et al.*, 1994). Direct plant growth promoting activities include production of indole-3-acetic acid (IAA), siderophore production, phosphate

solubilization (Arora *et al.*, 2001) and as the biological control agent for phytopathogens such as *Macrophomina phaseolina*, *Fusarium oxysporum*, *F. solani*, *Rhizoctonia solani*, *Pythium* sp. etc. by producing secondary metabolites such as antibiotics, hydrogen cyanide *(*HCN) and phytoalexins (Deshwal *et al.,* 2003). Plant growth promoting (PGP) rhizobial strains may use one or more of these mechanisms in the rhizosphere can be a significant component of management practices to achieve the attainable yield (Cook, 2002).

Indole-3-acetic acid (IAA) is an important naturally occurring auxin with broad physiological effects on plants (Davies, 2010). Interactions between IAA-producing bacteria and plants lead to diverse outcomes on the plant, varying from pathogenesis to phytostimulation. Many plant growth promoting rhizobacteria (PGPR) including *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas* and *Rhizobium* produce IAA or related auxins (Taghavi *et al.*, 2009). Low concentration of IAA was found to promote plant growth, whereas high concentrations inhibited root growth, thus indicating that effect of IAA depends on the concentration (Keyeo *et al.,* 2011). Tryptophan (L-trp) is physiologically a precursor of auxin biosynthesis in higher plants and microorganism. Rhizobia can be used as bioenhancer and biofertilizer for wheat production as it can uptake more nutrients (N, P and K) by producing IAA and subsequently increases the plant root system (Etesami *et al.,* 2009).

Iron is also an important constituent of nitrogenase enzyme involved in BNF and siderophore plays an important role in solubilizing Fe (III). Siderophore chelate iron and supply to bacterial cell by outer membrane receptors. Rhizosphere inhabiting bacteria usually live in microcolonies where the transient concentration of available iron can vary greatly from that of bulk soil solution. Most of the microorganisms have evolved specific, high affinity mechanism to acquire iron by producing extra cellular siderophores (Deshwal *et al.,* 2003). Siderophore and their derivative have large application in agriculture as to increase soil fertility and biocontrol for fungal pathogen (Ali and Vidhale, 2013). Nitrogen content as well as iron content was found maximum on overall basis in high siderophore producing mutants. Improved iron scavenging properties of the rhizobia positively correlate with rhizosphere growth and nodulation effectiveness in groundnut and pigeon pea (O'Hara, 2001). There are three main kinds of siderophores known as hydroxamate, catecholate and carboxylate. A great variation is seen in siderophore structure produced by many bacteria.

It is undoubtedly clear that phosphorus is the second most important nutrient after nitrogen required for growth of plants. It is an essential element in all living systems. It is also important in several physiological processes of plants, especially in photosynthesis, carbon metabolism and membrane formation (Wu, 2005). Also, it plays the vital role in root elongation, proliferation, and its deficiency affects root architecture, seed development and normal crop maturity. Plants acquire phosphorus from soil solution in the form of phosphate anion. Despite the fact that the amount of phosphorus in the soil is generally quite high (often between 400 and 1,200 mg kg^{-1} of soil) most of this phosphorus is insoluble and therefore not available to support plant growth (Khan *et al.,* 2007). In addition, much of the soluble inorganic phosphorus that is used as chemical fertilizer is immobilized soon after it is applied and becomes unavailable to plants and is therefore wasted (Singh and Kapoor, 1994). Thus, low levels of phosphorus can affect symbiosis by

decreasing the supply of photosynthates to the nodule, which reduces the rate of bacterial growth and the total population of legume-nodulating microorganisms (Moreira *et al.*, 2010). It remains in a precipitated form in the soil as mono or orthophosphate or is absorbed by Fe or Al oxides through legand exchange. Generally, the phosphate solubilizing microorganisms (PSM) play a very important role in phosphorus nutrition by exchanging its availability to plants through lowering the soil pH by the microbial production of organic acids and mineralization of organic phosphorus by acid phosphatase (Baby *et al.*, 2016). The bacterial P solubilization activity is due to secretion of organic acids. The PSB and plant growth promoting (PGP) rhizobacteria together could reduce phosphorus fertilizer application by 50% without any significant reduction of crop yield (Jilani *et al.*, 2007). The ability to P-solubilization is found even among leguminosae nodulating bacteria (LNB), such as *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and other non-specified LNB species (Hara and Oliveira, 2004).

Among the different phytohormones ethylene is an inhibitor of the nodulation of legumes by *Rhizobia* sp. (Drennan and Norton, 1972). The microbial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase cleaves ACC, the immediate precursor of ethylene in plants to ammonia and α-ketobutyrate, both of which are readily metabolized by most soil bacteria. A significant positive correlation was observed between *in vitro* ACC-deaminase activity of bacterial cells and root elongation (Arshad *et al.*, 2007). The ability of plant growth-promoting (PGP) rhizobia that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase to lower plant ethylene levels, often a result of various stresses is a key component in the efficacious functioning of these bacteria. By lowering the local ethylene content in plants, ACC deaminase-producing bacteria can increase the extent of rhizobial nodulation in legumes such as pea, alfalfa, mungbean and chickpea. These bacteria not only directly promote plant growth but also protect plants against flooding, drought, salt, flower wilting, metals, organic contaminants and both bacterial and fungal pathogens (Glick, 2014).

Hence, present study evaluation of the rhizobial strains for plant growth promoting (PGP) attributes was undertaken in four districts (Hisar, Bhiwani, Mahendergarh and Rewari) of Haryana, India because legumes occupy a major position in the cropping system of this region since, these districts have the problem of water scarcity, low rainfall and high temperature.

Materials and Methods

Isolation of rhizobia

Root nodulating bacteria were isolated from healthy pink nodules of pigeon pea plants (Paras variety) grown in farmer's field as well as pot house from Hisar, Bhiwani, Mahendergarh and Rewari districts of Haryana, India in July and August. The area has an average temperature range of l0° C (in winters) to 45°C (in summers). The method of Vincent (1970) was followed for isolation of root nodulating bacteria. Pigeon pea seedlings were uprooted carefully and root nodules were collected and washed with sterile distilled water followed by surface treatment with 95% ethanol (2 ml) and further rinsing with sterile distilled water. Properly washed nodules were surface sterilized quickly (2 to 3 min) with 0.1% mercuric chloride ($HgCl_2$) and again cleaned for at least 5-7 times with sterile distilled water so as to remove the traces of $HgCl_2$. The nodules were crushed in a half filled culture tube with saline water (0.85% NaCl) with the help of sterile glass rod. A milky bacterial suspension obtained

was serially diluted and streaked on sterile yeast extract mannitol agar (YEMA) plates (Vincent, 1970). The inoculated plates were incubated at 28 ± 2°C for 24-48 h and observed for specific features of rhizobia. Fourty nine rhizobial isolates obtained from pigeon pea were named as pigeon pea (PP) (H- Hisar, B- Bhiwani, M- Mahendergarh and R-Rewari, respectively) and maintained separately on YEMA slants at 4°C for further study.

Estimation of Indole-3-Acetic Acid (IAA) production

IAA was estimated by Salkowski's method (Tang and Bonner, 1974).

Reagents

Salkowski's reagent- 1 mL of 0.05 M $FeCl_3$ in 50 mL of 35 per cent of perchloric acid ($HClO_4$).

IAA stock solution 100 mg mL-1 in 50 per cent ethanol.

Selected rhizobial isolates were inoculated in 25 mL of YEM broth supplemented with 0.1 g L-1 DL-tryptophan. These flasks were incubated at 28+2^{o}C in a shaking BOD incubator.

After 4 days of incubation, 2 mL of culture broth was centrifuged at 7,000 rpm for two minutes and then IAA was determined in culture supernatant by following method: To 2 ml of supernatant, an equal volume of Salkowski's reagent was added.

The contents were mixed by shaking and allowed to stand at room temperature for 30 minutes for development of pink colour which was estimated colorimetrically at 500 nm using spectrophotometer. Indole- 3- acetic acid was used as a standard.

Siderophore production

Siderophore production was determined on chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987).

The log phase culture of bacterial strains spotted separately on CAS medium and plates were incubated at 28 ± 2°C for 48 h. Formation of orange to yellow halo around the colonies showed the production of siderophore.

Phosphate solubilization

Phosphate solubilization ability of pigeon pea rhizobial strains were detected by spotting separately on Pikovskya's agar plates. Plates were then incubated at 28 ± 2°C for 3 d, and observed for the clearing zone around the colonies (due to the solubilization of inorganic phosphate by bacteria) (Pikovskya, 1948). Zone of solubilization was measured and colony size was also measured and these values were used to calculate solubilization index (SI) by the following formula.

SI = Colony diameter + Halozone diameter/Colony diameter

ACC utilization

The medium plates were prepared with minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC (Penrose and Glick, 2003). Log phase grown culture of the pigeon pea rhizobial isolates was spotted on the medium plates. The growth of bacterial isolates on ACC supplemented medium plates was recorded after 2-5 days of incubation at 28 ± 2°C. The bacterial cultures showing good growth on ACC supplemented medium plates and capable of utilizing ACC as nitrogen source, were selected. The growth on minimal medium plates supplemented with ammonium sulphate was used as control.

Results and Discussion

Isolation of rhizobia

Forty nine root nodulating bacteria were isolated from nodules of pigeon pea plants (Paras variety) grown in farmer's field as well as pot house from Hisar, Bhiwani, Mahendergarh and Rewari districts of Haryana, India in July and August. The area has an average temperature range of l0°C (in winters) to 45°C (in summers). Fourty nine rhizobial isolates obtained from pigeon pea were named as pigeon pea (PP) (H- Hisar, B-Bhiwani, M- Mahendergarh and R-Rewari, respectively) and maintained separately on YEMA slants at 4°C for further study. Similarly, Dhull and Gera (2017) isolated 158 clusterbean [*Cymopsis tetragonoloba* (L.) Taub.] rhizobia from semi-arid regions of Haryana, India.

Screening of rhizobial isolates for indole-3-acetic acid (IAA) production

All the rhizobial isolates were screened for the production of IAA and maximum IAA production of all the pigeon pea rhizobial isolates was recorded after 4 days of incubation at 28 ± 2°C as most of the researchers have also observed that maximum IAA was synthesized on 3rd day of the growth and remains constant up to 7th day whereas Sridevi and Mallaiah (2008) reported maximum IAA production after 72 h of incubation.

Pigeon pea rhizobial strains were found to be low IAA producers. Their IAA production varied from 0.16-9.50 µg ml^{-1}. Maximum IAA was produced by rhizobial isolate PPB-23B (9.50 µg ml^{-1}) while minimum by PPR-4B (0.16 µg ml^{-1}) (Fig. 1 and 2A). Similar results were also reported by Khalid *et al.*, (2004) who categorise the *in vitro* production of IAA by rhizobacteria in three principal groups: lower producers (1 to 10 µg/ml), medium producers (11 to 20 µg/ml) and higher producers (21 to 30µg/ml). Keyeo *et al.*, (2011) also reported that different bacterial strains have been found to produce IAA in varying amounts.

These results are in contrast to Padder *et al.*, (2017) who reported that out of 100 rhizobacterial isolates from soil samples of *Dalbergia sissoo* 80% isolates were IAA producers.

Screening of rhizobial isolates for siderophore production

The ability to synthesis siderophores was restricted to very few isolates as out of forty nine pigeon pea rhizobial isolates only 35% pigeon pea rhizobial isolates were able to produce siderophore out of which 8% high siderophore producer (HSP), 10% moderate siderophore producer (MSP), and 17% low siderophore producer (LSP) respectively and 65% of the isolates did not produce siderophore on the 7th days of incubation at 28 ± 2°C in BOD (Table 1 and Fig. 2B).

Similarly, Dhull *et al.*, (2016) reported that 33% of clusterbean rhizobial isolates were able to produce siderophore. These results are in agreement with Arora *et al.*, (2001) who reported that the ability to synthesize siderophore by rhizobia is restricted to a limited range of strains rather than wide distribution.

Similarly Jenifer *et al.*, (2013) reported that out of the 11 isolated cultures from rhizospheric soil, 4 cultures namely C2, C3, C8 and C11 were found to produce siderophore. While opposite result are reported by Duhan (2013) that on screening of 25 mutants for hydroxamate type of siderophore production 23 mutants were siderophore positive.

Table.1 Siderophore production by different pigeon pea rhizobial isolates

Category	Rhizobial isolates	Siderophore production
1.	PPH-4A, PPH-5A, PPH-8A, PPH-8C, PPH-9B, PPH-10A, PPR-4B, PPR-7B, PPB-7A, PPB-8A, PPB-8B, PPB-13, PPB-14, PPB-21B, PPB-22A, PPB-22B, PPB-25A, PPB-25C, PPB-30B, PPB-32C, PPB-33A, PPB-34B, PPB-34C, PPB-34D, PPB-37C, PPB-38B, PPM-14, PPM-21A, PPM-21B, PPM-22A, PPM-23A, PPM-37D	-
2.	PPH-1B, PPH-2B, PPR-2, PPB-8, PPB-35A, PPM-33C, PPM-35A, PPM-37A	+
3.	PPB-1, PPB-3, PPB-23B, PPB-26A, PPB-27B	+ +
4.	PPH-2A, PPH-8E, PPH-10B, PPM-30A	+ + +

[(No growth (-), Poor growth (+), Moderate growth (++) and Good growth (+++)]

Table.2 P-solubilization by different pigeon pea rhizobial isolates

Sr. No.	Rhizobial isolates	P-Solublization Index (P-SI)	Sr. No.	Rhizobial isolates	P-Solublization Index (P-SI)
1	PPH-1B	1.6	26	**PPB-23B**	**3.7**
2	PPH-2A	2.0	27	PPB-25A	3.5
3	PPH-2B	1.3	28	PPB-25C	2.5
4	PPH-4A	1.5	29	PPB-26A	2.6
5	PPH-5A	3.2	30	PPB-27B	2.6
6	PPH-8A	2.2	31	PPB-30B	2.0
7	PPH-8C	0.0	32	PPB-32C	1.8
8	PPH-8E	1.5	33	PPB-33A	3.0
9	PPH-9B	3.2	34	PPB-34B	2.0
10	PPH-10A	1.2	35	PPB-34C	1.4
11	PPH-10B	2.4	36	PPB-34D	2.2
12	PPR-2	1.7	37	PPB-35A	3.2
13	PPR-4B	2.3	38	PPB-37C	2.6
14	PPR-7B	2.5	39	PPB-38B	2.5
15	PPB-1	2.5	40	PPM-14	3.1
16	PPB-3	1.4	41	PPM-21A	1.2
17	PPB-7A	2.2	42	PPM-21B	1.3
18	PPB-8	3.0	43	PPM-22A	2.0
19	PPB-8A	3.1	44	PPM-23A	1.7
20	PPB-8B	0.0	45	PPM-30A	1.4
21	PPB-13	1.6	46	PPM-33C	1.4
22	PPB-14	1.3	47	PPM-35A	3.0
23	PPB-21B	2.4	48	PPM-37A	2.2
24	PPB-22A	3.0	49	PPM-37D	2.5
25	PPB-22B	2.2			

(P-solublization index (P-SI) = Zone diameter + Colony diameter/Colony diameter)

Table.3 ACC utilization by different pigeon pea rhizobial isolates

Category	Rhizobial isolates	Ammonium Sulphate (2 g/l)	ACC (3 mM)
1.	PPH-2A, PPB-8, PPB-21B, PPB-32C, PPB-33A, PPB-38B, PPM-22A, PPM-23A, PPM-35A	+ +	-
2.	PPH-4A, PPH-8C, PPH-10A, PPB-1, PPB-8A, PPB-8B, PPB-14, PPB-23B, PPB-25A, PPB-27B, PPB-34B, PPB-34C, PPB-35A, PPB-37C, PPM-14, PPM-21A	+ + +	-
3.	PPM-37A	+ +	+ +
4.	PPH-1B, PPH-2B, PPR-2, PPB-22A, PPB-22B, PPB-26A, PPB-30B, PPM-30A	+ + +	+ +
5.	PPH-5A, PPH-8A, PPH-8E, PPR-7B, PPM-21B, PPM-33C	+ + +	+ + +
6.	PPH-9B	+ +	+
7.	PPH-10B, PPR-4B, PPB-3, PPB-7A, PPB-13, PPB-25C, PPB-34D, PPM-37D	+ + +	+

[- (No growth), + (Poor growth), + + (Moderate growth), + + + (Good growth)]

Table.4 Most efficient pigeon pea rhizobial isolates

Rhizobial isolates	Biochemical characters
PPH-8E	I+P+S+A
PPR-2	I+P+S+A
PPB-26A	I+P+S+A
PPM-30A	I+P+S+A
PPM-33C	I+P+S+A
PPM-37A	I+P+S+A

[I= IAA (Indole-3-Acetic-Acid), S= Siderophore, P= P-Solubilization, A= ACC utilization]

Fig.1 IAA production (µg mL^{-1}) by different pigeon pea rhizobial isolates

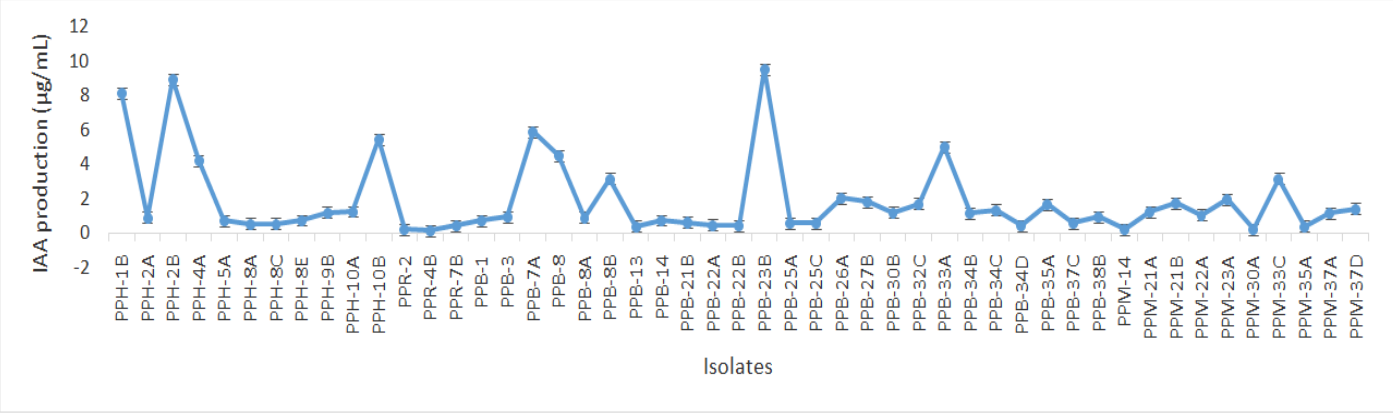


Fig.2 IAA production (A) and Siderophore production (B) by pigeon pea rhizobial isolates

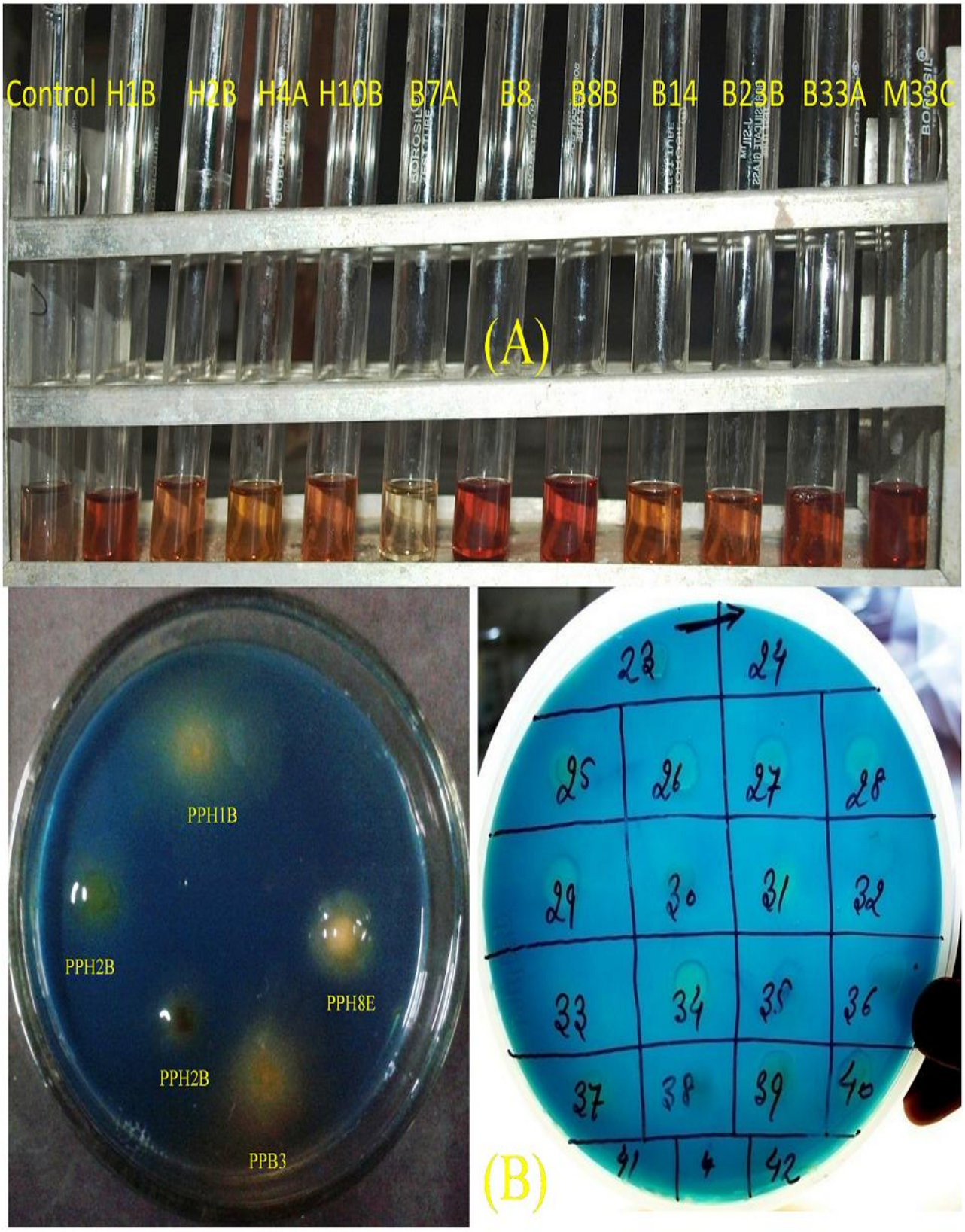
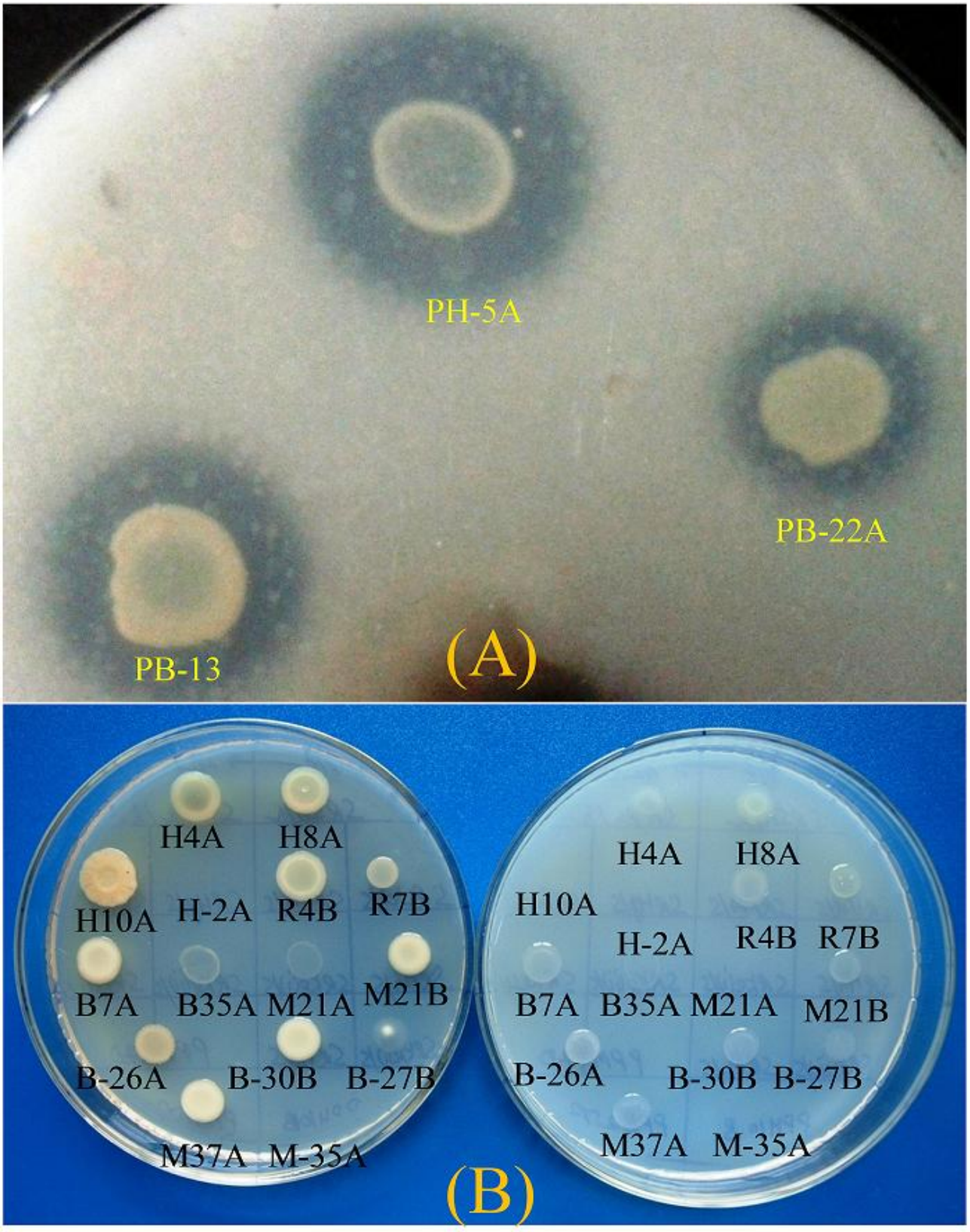


Fig.3 P-Solublisation (A) and ACC utilization (B) by pigeon pea rhizobial isolates



Screening of rhizobial isolates for P-solubilization

In the present work 96% of pigeon pea rhizobial isolates were able to form significant zone of P-solubilization on Pikovskya's medium and their P-solubilization index (P-SI) varied from 1.2 to 3.7 (Table 2 and Fig. 3A). The solubilization index varied from low to intermediate in different isolates was also observed by Marra, 2011. Similar results were reported by Alam *et al.,* (2002) that bacteria are more effective in phosphorus solubilization than fungi. While contrast result are reported by Jadhav (2013) that out of 10 rhizobial isolates from soybean crop only 3 isolates showed phosphate solubilization activity.

Screening of rhizobial isolates for utilization of 1-aminocyclopropane-1 carboxylate (ACC)

All the pigeon pea rhizobial isolates were screened for ACC utilization by spotting on ammonium sulphate (2 gl^{-1}) and ACC (3 mM) supplemented medium plate. Among all these isolates, 49% of the isolates showed growth on ACC supplemented plates and most of the isolates showed more growth on ammonium sulphate plates indicating that about half of the isolates possess ACC deaminase activity (Fig. 3B). On the basis of growth on medium plates, pigeon pea rhizobial isolates were divided in to 7 categories (Table 3). Almost similar results were observed by Khandelwal and Sindhu (2013) who reported that 38.9% *Pseudomonas* isolates showed good growth on ACC supplemented plates. Ma *et al.*, (2003b) also reported that 38.7% rhizobial strains possess ACC deaminase enzyme.

Six isolates namely PPH-8E, PPR-2, PPB-26A, PPM-30A, PPM-33C and PPM-37A (Table 4) were selected as most efficient pigeon pea rhizobial isolates on the basis of plant growth promoting (PGP) attributes which act as efficient biofertilizers for pigeon pea crop grown under semi-arid and arid regions in different regions.

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