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Isolation and characterization of plant associated rhizobacteria for plant growth promoting traits

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ABSTRACT

The use of plant growth promoting rhizobacteria (PGPR) in sustainable and eco-friendly management of plant growth promoting is gaining importance over the previous decades around the world. In the current research work, the isolation of the rhizobacteria were done using nutrient agar media following standard protocol for isolation of bacteria. We have isolated and characterized 32 rhizobacterial isolates from five different plant species and subjected to N_2-fixation, phosphorus solubilization and indole-3-acetic acid assay to identify potential PGPR. All the 32 rhizobacterial isolates showed at least one of the three major functionalities; *viz.* phosphorus solubilization, indole acetic acid production and N_2 fixation; considered for selection of PGPR when tested *in vitro*. Among the 32 isolates, 15 produced clear halo zones surrounding their colonies indicating phosphate solubilization with variable intensities. Among the fifteen, six bacterial isolates having high phosphate solubilization index (PSI) proved to be efficient phosphorus solubilizer in liquid medium. The bacterial isolate MQ2 solubilized maximum (0.697 µg mL^{-1}) phosphorus in liquid medium, followed by MQ3 and MQ1. Ten of the bacterial isolates were able to synthesize indole-3-acetic acid (IAA) in L-tryptophan supplemented media at varying capacity. The isolate OSn8 produced highest amount (6.204 µg mL^{-1}) of IAA followed by MQ5 and OSbr6, while the lowest amount of IAA (1.268 µg mL^{-1}) was produced by MQ1. All the isolated bacteria were tested positive for putative N_2-fixing ability with variation among the isolates as indicated by their growth in N_2-free medium. Considering the all three tested functionalities, the isolate MQ1 proved to be the best candidate as potential biofertilizer development. A consortium of isolated rhizobacteria comprising the best isolates from each category could be constructed to provide the best benefit to crops for maintaining yield and quality while decreasing agrochemical inputs.

Keywords: Plant growth promoting rhizobacteria (PGPR), Phosphorus solubilization, Indole-3-acetic acid, nitrogen fixation

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1 Introduction

Modern agriculture heavily dependent on agrochemical including fertilizers and pesticides to manage soil fertility and pest control, allowing a boast in cropping intensity and crop production to meet the increasing demand of food products. But the injudicious use of these agrochemicals for crop production has become a matter of concern as they decrease soil fertility and dismantle the environmental integrity (Jilani

et al., 2007; Rahman et al., 2015). Ecological imbalance fueled by the over-use of agrochemicals also leaves harmful contaminants and residues in soil water systems which in turn destroys or destabilizes the soil microbiome responsible for nutrient mineralization and recycling (Rahman et al., 2015). Over the years, the scientists are trying to reduce dependency on agrochemicals and looking for an effective alternative/amendment for crop production. The use of soil inhabiting microorganisms in crop production is being considered best options by growing numbers of plant scientists (Glick, 2012; Lwin et al., 2012; Majeed et al., 2015). Plant growth promoting rhizobacteria (PGPR) are diverse array of microorganisms which selectively colonize in the rhizosphere and stimulate the growth and development of plants (Kloepper, 1981; Bashan et al., 1993). The PGPR enhance the growth and development of plants by multiple direct and indirect mechanisms (Rahman et al., 2010; Backer et al., 2018). The direct mechanisms by which PGPR imparts their growth promoting functionalities include production of phytohormones, nitrogen (N_2) fixation, mineral phosphate and zinc solubilization, siderophore production etc., while production of antibiotics, extracellular polymeric substances (EPS), induced systemic resistance and production of defense related enzymes, competition for space and nutrient are considered as the principal indirect mechanisms of plant growth promotion by PGPR (Glick, 2012; Rahman et al., 2015; Majeed et al., 2015; Asha et al., 2015).

Indole-3-acetic acid (IAA) is considered as the one single molecule in plants which has profound role on plant growth and development (Patten and Glick, 1996) and considered as one of the direct mechanisms of plant growth promoting by rhizospheric microorganisms. IAA producing rhizobacteria releases enough auxin in the soil sufficient for developmental processes of plants and enable plants to fight against biotic and abiotic stresses (Spaepen et al., 2007; Glick, 2012; Costa-Gutierrez et al., 2020). L-tryptophan dependent IAA production is considered as the main pathway for bacterial IAA production in rhizosphere and it is now conformed that many soil microbiotas have the capacity to convert minute quantities of plant-derived L-tryptophan in the rhizosphere to IAA (Zhao, 2010; Rahman et al., 2010). Thus, plant microbiologists consider IAA production by rhizobacteria as one of the criteria for selecting PGPR.

Phosphorus (P) is an important essential plant nutrient, deficiency of which limits plant growth and crop yield seriously. Only a small portion of applied phosphorus containing fertilizers are up taken by plants, while the remainder is fixed in most soils. The PGPR plays an important role in mobilizing the fixed pool of inorganic phosphates along with organic phosphate pool (Gaind and Gaur, 1989; Khan et al., 2010; Taher et al., 2019) thereby reducing amount of phosphatic fertilizer for crop production. PGPR employs a number of mechanisms including production of organic acids and phosphatase enzymes to solubilize phosphate. Besides IAA production and phosphate solubilization, N_2-fixing ability of PGPR is also considered as one of the important mechanisms of plant growth promotion and used as criteria for PGPR selection. N_2-fixation by rhizobacteria in leguminous plants including lentil, soybean etc. are well-documented and credited to symbiotic N_2-fixing bacteria (Rashid et al., 2009; Islam et al., 2007, 2013). N_2 fixation by non-symbiotic rhizobacteria in crops other than legume is gaining attention (Franche et al., 2008; Islam et al., 2013; Díez-Méndez and Menéndez, 2020). In Bangladesh, the use of PGPR in crop production is very limited, partly due to the unavailability of potential PGPR based biofertilizer to the farmers and also lack of farmer's willingness to reduce the use of conventional fertilizers. Therefore, the current research was designed to isolate rhizobacteria from different plant sources and their functional characterization as potential agriculturally important microorganism that can be used for biofertilizer development.

2 Materials and Methods

Plant samples were collected from different areas of Agronomy Field Laboratory, and undisturbed campus soils of Bangladesh Agricultural University, Mymensingh, Bangladesh. A total of five (5) plant species comprising eight (8) samples (Table 1) including their roots and rhizospheric soil were collected. After removing extra soil by vigorous shaking, plant roots were cut off using surface sterilized scissor and kept in labeled sterile test tubes containing 10 mL of sterilized distilled water and immediately brought to the laboratory for isolation of rhizobacteria.

2.1 Isolation of rhizobacteria

The collected plant samples were shaken vigorously in test tubes for few minutes to mix well. A dilution series of up to 10^{-3} were prepared for each sample with sterilized distilled water to facilitate bacteria isolation. Nutrient broth agar (NBA) medium (pH 7.0) was used to isolate the bacteria and prepared according to the manufacturer's instruction (Asha et al., 2015; Glick, 2012). The 10 mL of bacterial suspension were inoculated into the medium with the help of a glass spreader and plates were incubated in microbial incubator for 24 hours at 28±2 °C (Asha et al., 2015). Then the bacterial colonies were picked with sterile toothpick based on size, shape and color and repeatedly inoculated on NBA media until obtaining a pure culture of bacterial isolate. Finally, pure colonies of bacterial isolates were maintained on NBA plates for regular use and preserved in 30% glycerol for long time storage at low temperature refrigerator (−20 °C).

Table 1. Plant species collected for isolation of rhizobacteria and location of sampling

Local name	Scientific name	N†	Sampling location
Rice	*Oryza sativa*	3	Bangladesh Agricultural University
Shama	*Echinochloa crusgalli*	1	(24°43′10.0″N, 90°25′39.5″E)
Soybean	*Glycine max*	1	
Fern	*Pteris* spp.	2	
Sushni Shak	*Marsilea quadrifolia*	1	

† Number of samples

2.2 Screening of IAA producing bacteria

Modified Winogradsky's mineral solution was used as a media for screening of IAA producing rhizobacteria and the media was prepared as described in Rahman et al. (2010). The medium was supplemented with 100 mg L^{-1} L-tryptophane and the pH of the solution was adjusted to 6.0-6.2 with 0.1M HCl and 0.1M NaOH. 30 mL of liquid medium were inoculated with a loopful of overnight grown bacteria and incubated at room temperature in a horizontal shaker (JSOS-500 JSR, Korea) at 120 rpm for 72 hours under dark condition. After 3 days, the culture media were centrifuged at 10,000 rpm for 10 minutes to obtain cell free supernatant. IAA production was qualitatively and quantitatively determined by Salkowski reagent method (Rahman et al., 2010). After centrifugation, the supernatant was decanted and pH was adjusted to 2.5 to 3.0 with 2 M HCl. Then 2 mL of supernatant and 2 mL of Salkowski's reagent (2% of 0.5 M $FeCl_3$ solution in 35% of $HClO_4$) were taken in the test tube and kept in dark condition for 30 minutes. Development of pink to reddish color was taken as the indication of IAA production. Quantitative determination of IAA was done by the colorimetric method using UV/VIS spectrophotometer at 535 nm wavelength (Lwin et al., 2012). A calibration curve was prepared using standard solution of pure IAA.

2.3 Screening of PSB

Screening of PSB was done using Pikovskaya's agar medium (Pikovskaya, 1948). The medium was prepared according to Asha et al. (2015) with adjusted pH at 7.0 before sterilization. Bacterial isolates were tested in triplicates by plate assay for observing mineral phosphate solubilization activities. Each isolate was inoculated in Pikovskaya's agar medium containing tricalcium phosphate and incubated at 28±2 °C for 6 days. A clear halo zone around the bacterial colony was considered as the indication of mineral phosphate solubilization (de Freitas et al., 1997). The diameter of the colony and diameter of the clear halo zone were measured with scale after 6 days of inoculation. Finally, phosphate solubilizing index (PSI) was calculated by method as suggested by Premono et al. (1996) (Fig. 1).

2.4 Determination of P solubilized by potential rhizobacteria

Quantification of phosphorus solubilized by potential rhizobacteria was done using Pikovskaya's broth medium. The bacteria used in this assay were selected on the basis of PSI determined in previous assay. The 6 selected bacteria were inoculated into Pikovskaya's broth media containing tricalcium phosphate with sterile inoculating loop and incubated at room temperature for 6 days on horizontal shaker (JSOS-500 JSR, Korea) at 120 rpm. After 6 days, the pH of the media was recorded using pH meter. Bacterial cultures were then centrifuged in a falcon tube at 6000 rpm for 10 minutes and the cell-free supernatant were collected in glass bottle for determination of phosphate content. Determination of phosphate solubilization content from the liquid media was done by developing phosphomolybdate blue complex with stannous chloride ($SnCl_2.2H_2O$). To form molybdophosphoric blue complex, $SnCl_2.2H_2O$ was used as a reducing agent. Exactly 2 mL of cell free supernatant was taken in 100 mL volumetric flask. Then sulphomolybdic acid and stannous chloride solution were added 4 mL and 6 drops, respectively. The volume of the solution was made up to the mark with distilled water and shaken thoroughly. After shaking, the solution allowed to stand for 5 minutes for color development. Finally, a spectrophotometer (Model-T60, PG Instruments, UK) was used for measuring the intensity of blue color (absorbance) at 660 nm wave length along with standard series solutions (Schroth and Hancock, 1982). Finally, the quantity of soluble phosphate was calculated by the regression equation of standard curve (Kumar et al., 2012). The values of phosphate solubilization was expressed in μg mL^{-1}.

2.5 Determination of total titratable acidity of PSB grown liquid medium

For determining titratable acidity, 10 mL of cell-free supernatant was taken in volumetric flask. Here, 2 drops of the phenolphthalein indicator were added. This content of the volumetric flask was titrated against standard 0.1 M sodium hydroxide (NaOH). During the addition of 0.1 M NaOH, the flask was

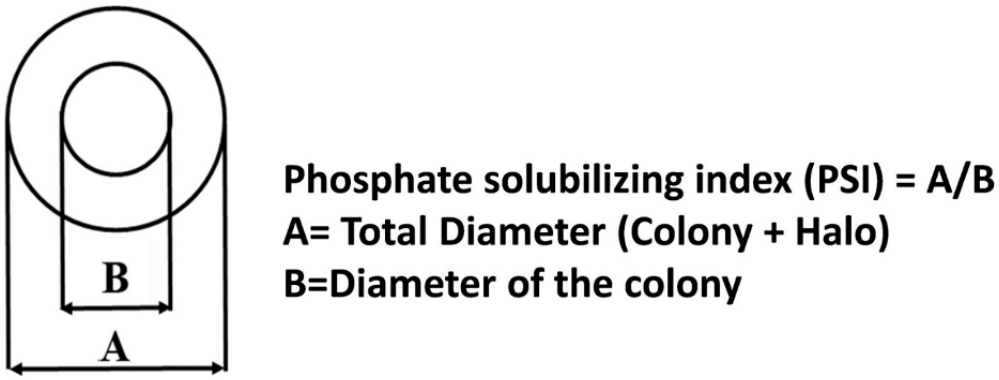

Figure 1. Equation for calculating phosphate solubilization index (Premono et al., 1996)

shaken thoroughly. The initial and final readings of burette were noted down for observing the differences and calculated the volume of NaOH used. The percentage of acidity in the PSB grown liquid media was calculated on the basis of the following relation (Sadler and Murphy, 2010).

$$W = \frac{M \times V \times 192.43}{3} \quad (1)$$

where, W = weight of acid (g), M = molarity of NaOH used (0.1), and V = volume of NaOH used (L).

$$TA\ (\%) = \frac{W \times 100}{W_s} \quad (2)$$

where, TA = % of total acidity, and W_s = weight of sample (g).

2.6 Screening of N_2-fixing bacteria

The isolates were grown in modified Winogradsky's N-free agar medium (Winogradsky's medium without tryptophan and yeast extract) to study N_2-fixing ability of the bacteria (Hashidoko et al., 2002). Then the culture plates were kept in microbial incubator at 28 °C for 48 hours. Growth of bacterial colonies on the N-free media were the indication of N_2-fixing ability of rhizobacteria.

3 Results

3.1 Isolated rhizobacteria

A total of Thirty-Two bacterial strains were isolated from five species of plants depending on morphological characteristics and each of them were subjected to gram reaction and catalase test. Code names were given to the bacterial strains according to their origin on the basis of morphological characters (Table 2). The morphological features of the isolates along with the results of gram reaction and catalase test are given in Table 2. Most of the isolated bacteria were whitish and cream in color with few producing yellow and red pigments. Gram reaction test revealed that 18 of the isolated bacteria were Gram positive and the rest 14 were gram negative. None of the isolated bacteria were catalase positive.

3.2 IAA production

The ability of the isolates to produce IAA were tested using modified Winogradsky mineral media supplemented with L-tryptophan. About 34.71% of the isolated bacteria (10 out of 32) were able to produce IAA in liquid media in varying quantities as indicated by pink to reddish pink color development when treated with Salkowsky's reagent, while 64.29% failed to do so (Fig. 2A). Four bacterial isolates assumed to be strong IAA producer; three of which were isolated from rice rhizosphere [OS29(3), OSbr(6) and OSn(8)] samples and one, MQ5 was isolated from Sushni Shak. The other 6 of the IAA isolates were slight to medium IAA producer. The rest 18 bacterial isolates produced no color which was the indication of no IAA production (Table 2 and Fig. 2B). The quantity of IAA produced by bacterial isolates were also determined using the liquid medium. The quantity of IAA produced by the rhizobacterial isolates ranged from 1.268 μg mL^{-1} to 6.204 μg mL^{-1} in Salkowski reagent positive isolates. Highest quantity of IAA (6.204 μg mL^{-1}) was produced by OSn8 isolated from rice rhizosphere followed by MQ5 (5.643 μg mL^{-1}), while the lowest (1.268 μg mL^{-1}) was produced strain MQ1 isolated from Sushni Shak (Table 3).

3.3 Isolates of PSB

All the isolated rhizobacteria were subjected to tricalcium phosphate amended medium to assess their phosphate solubilization capacity. The results of plate assay revealed that about 46.87% of the rhizobacteria (15 out 32 isolates) were able to produce clear halo zones surrounding their (Fig. 3B) colonies indicating their ability to scavenge phosphorus from unavailable sources. While 53.13% of bacteria proved

Table 2. List of rhizobacteria isolated from different plant sources, their morphological characters and results of gram reaction

Source	Bacteria	Colony color	Elevation	Shape	Gram reaction
Oryza sativa (BRRIdhan 29)	OS29(1)	Whitish	Non-raised	Round	(−)ve
	OS29(2)	Whitish	Non-raised	Round	(−)ve
	OS29(3)	Yellow	Raised	Round	(−)ve
Oryza sativa (Bashi Raj)	OSbr4	Whitish	Raised	Round	(+)ve
	OSbr5	Cream	Raised	Round	(−)ve
	OSbr6	Cream	Non-raised	Irregular	(−)ve
	OSn7	Whitish	Raised	Round	(+)ve
	OSn8	Cream	raised	Round	(−)ve
Echinochloa crusgalli	EC1	Dark yellow	Non-raised	Irregular	(+)ve
	EC2	Whitish	Non-raised	Round	(+)ve
	EC3	Yellow	Raised	Irregular	(−)ve
	EC4	Whitish	Non-raised	Round	(+)ve
	EC5	Yellow	Raised	Round	(+)ve
	ECL1	Cream	Raised	Round	(+)ve
Pteris spp.	Fr1	Whitish	Non-raised	Round	(−)ve
	Fr2	Whitish	Non-raised	Irregular	(+)ve
	Fr3	Cream	Non-raised	Round	(+)ve
	Fr4	Whitish	Non-raised	Round	(+)ve
	Fr5	Cream	Non-raised	Irregular	(+)ve
	Fr6	Cream	Raised	Irregular	(+)ve
	Fr7	Cream	Non-raised	Irregular	(+)ve
Glycine max	GM1	Whitish	Non-raised	Round	(+)ve
	GM2	Cream	Non-raised	Round	(+)ve
Marsilea quadrifolia	MQ1	Reddish pink	Raised	Round	(−)ve
	MQ2	Cream	Non-raised	Round	(−)ve
	MQ3	Cream	Raised	Round	(+)ve
	MQ4	Cream	Non-raised	Irregular	(+)ve
	MQ5	Cream	Raised	Round	(−)ve
	MQ6	Whitish	Raised	Irregular	(−)ve
	MQL7	Whitish	Raised	Round	(−)ve
	MQL8	Whitish	Raised	Irregular	(−)ve
	MQL9	Cream	Non-raised	Irregular	(+)ve

Table 3. Qualitative and quantitative assay for IAA production by rhizobacterial isolates from different plant sources

Bacteria	Visual color	Intensity	Quantity of IAA (μg/mL)
OS29(1)	Colorless	−	−
OS29(2)	Colorless	−	−
OS29(3)	Reddish pink	+++	2.93±0.58
OSbr4	Colorless	−	−
OSbr5	Colorless	−	−
OSbr6	Reddish pink	+++	4.55±1.21
OSn7	Light pink	++	1.85±0.34
OSn8	Reddish pink	+++	6.20±1.89
EC1	Light pink	+	1.44±0.51
EC2	Light pink	+	1.30±0.38
EC3	Colorless	−	−
EC4	Colorless	−	−
EC5	Colorless	−	−
ECL1	Colorless	−	−
Fr1	Colorless	−	−
Fr2	Colorless	−	−
Fr3	Colorless	−	−
Fr4	Colorless	−	−
Fr5	Colorless	−	−
Fr6	Light pink	+	1.67±0.44
Fr7	Colorless	−	−
GM1	Light pink	+	1.47±0.48
GM2	Colorless	−	−
MQ1	Pink	++	1.27±0.21
MQ2	Colorless	−	−
MQ3	Colorless	−	−
MQ4	Colorless	−	−
MQ5	Reddish pink	+++	5.64±2.12
MQ6	Colorless	−	−
MQL7	Colorless	−	−
MQL8	Colorless	−	−
MQL9	Colorless	−	−

Development of pink to reddish pink color indicates IAA biosynthesis from L-tryptophan. The sign (−) denotes IAA non-producer isolate. The sign (+) denotes the intensity of IAA biosynthesis by the rhizobacteria; +++ strong producer, ++ medium producer and + low IAA producer isolate.

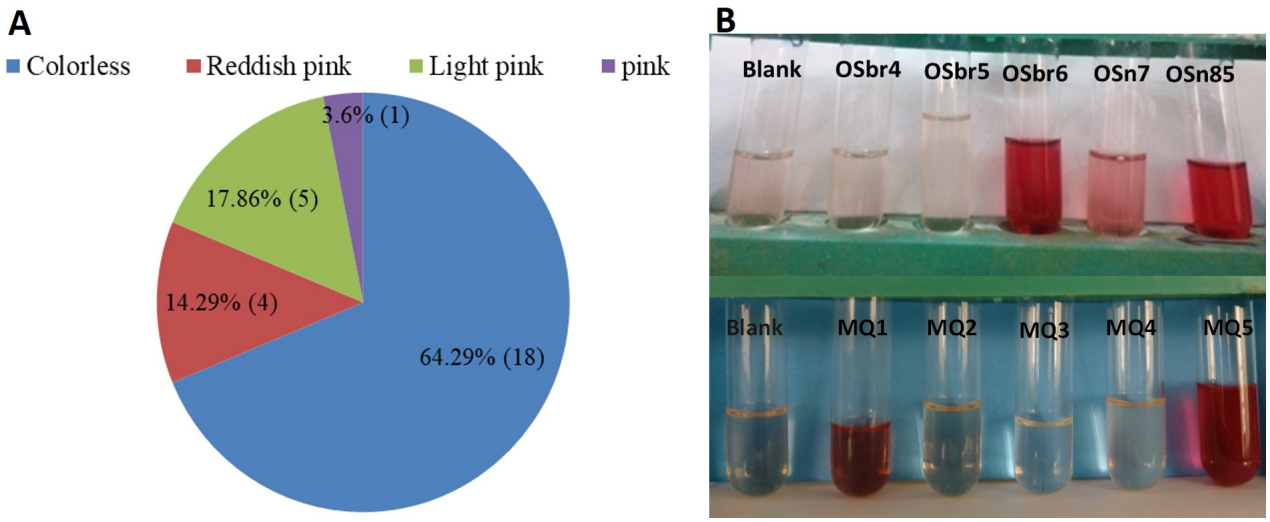

Figure 2. IAA biosynthesis from l-tryptophan by bacteria isolated from different plant species. (A): proportion of IAA producing and non-producing bacteria with their biosynthetic capacity. Reddish color denotes strong IAA producer, while light pink color denotes low IAA producer isolates. More than two-thirds of the rhizobacteria did not produce IAA in culture medium. (B). Salkowski reagent positive isolates develop reddish to light pink color in culture supernatant, an indication of IAA biosynthesis from l-tryptophan. Colorless tubes represent isolates with no ability to produce IAA

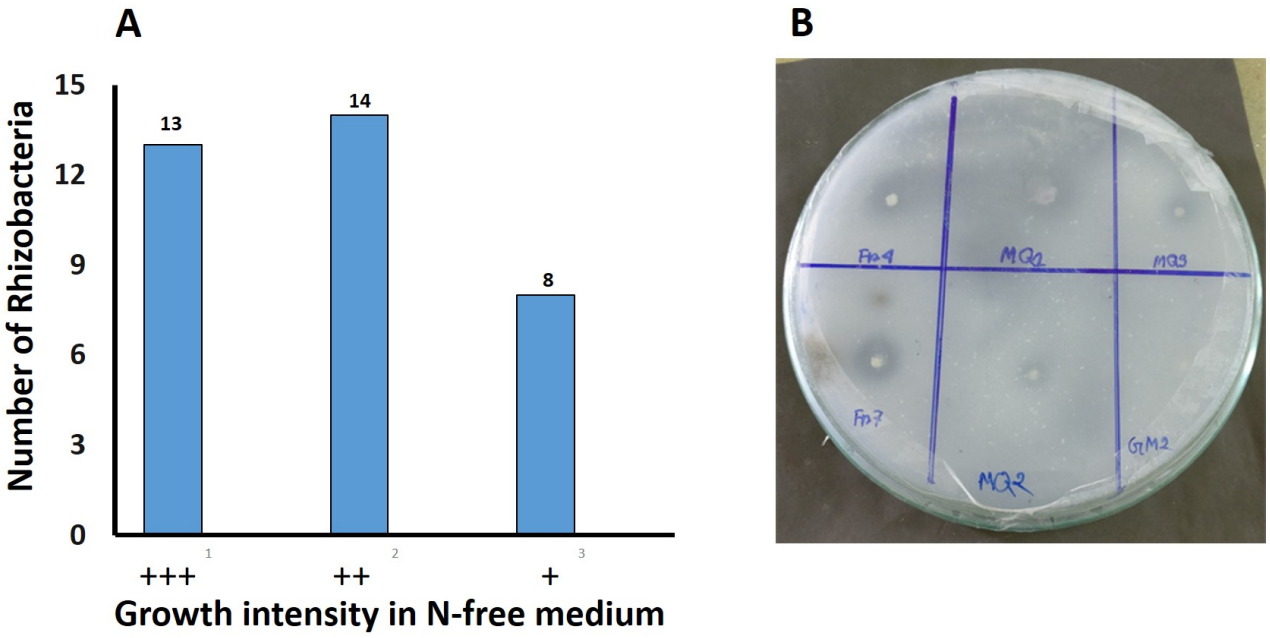

Figure 3. Assay results for nitrogen fixation and plate assay for P solubilization. (A): The putative nitrogen fixing ability of the rhizobacteria on N-free medium. All the bacteria were able to grow in N-deficient medium indicating their ability to grow in varying capacity. (B): Representative bacterial isolates developed clear halo zones surrounding the colony in Pikovskaya's agar medium containing tricalcium phosphate, an indication of phosphate solubilization

to be phosphorus non-scavengers. The phosphorus solubilization capacity of the isolates varies and the intensity of P solubilization were denoted as level 1 (+), level-2 (++) and level-3 (+ + +) phosphorus solubilizer (Table 4). The phosphorus solubilization index (PSI) of the isolates ranged between 1.1 to 11 for the 15 potential phosphate solubilizing bacteria (PSB). The highest PSI value (11) was recorded for the isolate MQ2 followed by MQ1 (PSI 8) both isolated from Sushni Shak. On the contrary, lowest PSI (1.1) were recorded for in OSn7, OSn8 and MQ4 isolates among the P solubilizers (Table 4).

Selected phosphate solubilizing rhizobacteria (6) were quantitatively evaluated using broth medium. The bacteria were selected on the basis of maximum PSI values and supported by the qualitative plate assay (Fig. 3B). Among the isolates, the highest quantity (0.697 μg mL^{-1}) of P was solubilized by MQ2 followed by MQ1 and MQ3 (Table 4). On the other hand, the lowest quantity of P (0.0278 μg mL^{-1}) was solubilized by Fr7 which was isolated from fern rhizosphere. The rest four isolates Fr4 (0.072 μg mL^{-1}), GM2 (0.038 μg mL^{-1}), MQ1 (0.260 μg mL^{-1}) and MQ3 (0.260 μg mL^{-1}) showed differential of phosphate solubilization in liquid media.

The reduction of pH in culture medium was also recorded to understand the mechanism of P solubilization. The pH of the culture media for isolate MQ2 dropped to 4.87 followed by MQ3 (pH 5.53). The pH of the culture media for other isolates studied also dropped from initial pH (7.0) of the culture media which corelates to their phosphate solubilizing capacity (Fig. 4A). Titratable acidity was measured for the determination of total acidity of the PSB liquid media with rhizobacterial isolates. In this study, titratable acidity of the six-phosphate solubilizing rhizobacteria was measured and found that the titratable acidity was highest (0.055%) for MQ2 isolate which was also highest P solubilizer (0.6972 μg mL^{-1}) (Table 4). The lowest percentage of acidity (0.0064%) was the acidity was obtained for Fr4 and Fr7 both of which are also slight P solubilizer (Fig. 4B).

3.4 Screened out N_2-fixing bacteria

Growth of bacteria in N-free media was the indication of N_2-fixation by the bacteria. All the bacterial isolates are able to fix N_2. Among the 32 rhizobacteria about 13 bacteria (Fr3, Fr4, Fr5, Fr6, Fr7, GM1, GM2, EC1, MQ2, MQ3, MQL7, MQL8, and MQL9) showed high, 11 bacteria [OS29(2), OS29(3), OSbr6, OSn7, OSn8, EC2, Fr1, Fr2, MQ1, MQ4 and MQ5] showed medium and 8 bacterial isolates [OS29(3), OSbr4, OSbr5, EC4, EC5, Fr1, MQ6, and ECL1] showed slight ability to grow in N-free medium. Accordingly, the isolates were grouped into low, medium and high N_2 fixer (Fig. 3A).

4 Discussion

Diverse array of both beneficial and pathogenic microorganisms inhabits in the plant rhizosphere and surrounding areas due to the presence of abundant carbon resources excreted from plant as root exudates. A total of 32 rhizobacteria were isolated from different plant species comprising samples of both agronomic and non-agronomic plant species. Majority (18) of the rhizobacteria isolated were Gram positive and rest of them (14) were Gram negative (Table 2). Most of the isolated bacteria were whitish and cream in color with few producing yellow and red pigments (Table 2) shows the bacterial diversity in different plant species. The screening tests to find out potential plant growth promoting rhizobacteria revealed that about one third of the isolated rhizobacteria exhibits at least one plant of the major plant growth promoting traits i.e., IAA production, phosphate solubilization or N_2-fixation.

L-tryptophan dependent IAA production thought to be the major biosynthetic pathway for bacteria (Rahman et al., 2010). Indole-3-acetic acid is the dominant phytohormone produced by rhizobacteria and implicated in the growth promotion and developmental processes in plant. The IAA production assay using 32 isolated bacteria reported that 10 rhizobacterial isolates can bio-transform L-tryptophan in varying quantities, between 1.268 and 6.204 μg mL^{-1} (Table 3), which is much lower than other previously reported value (77 μg mL^{-1}) for IAA production by rhizobacteria (Majeed et al., 2015). The variation in rhizobacterial IAA production has previously been reported by bacteria isolated from various plant species like tomato, rice, maize, fern etc. (Majeed et al., 2015; Lwin et al., 2012; Asha et al., 2015). Rahman et al. (2010) found that half of the bacterial isolates among 69 showed positive colour reactions to Salkowski's reagent. IAA production by PGPR isolates may vary from different strains and species; and was additionally influenced by substrate availability, culture conditions and growth stage (Devi et al., 2015). Verma et al. (2015) also observed IAA producing ability of rhizobacteria (*Pseudomonas* spp., *Bacillus* spp. and *Acinetobacter* spp.) isolated from the rhizosphere of wheat.

Scavenging different nutrient elements via mineralization (to phosphate ion) of unavailable sources by PGPR is considered as one of the most important criteria for selection rhizobacteria for plant growth promotion (Vessey, 2003; Islam et al., 2007; Rahman et al., 2015). In this study, we found 6 bacterial isolates to be efficient P solubilizer when tricalcium phosphate was used as a source of phosphorus (Table 4). We also reported a drop in the pH of culture medium for the isolates that solubilized and released different quantities of phosphorus. Furthermore, percent of total titratable acidity in culture medium also in accordance with the results of P solubilization and

Table 4. In vitro qualitative and quantitative analysis of phosphate solubilization by rhizobacteria

Bacterial isolates	Intensity of PSB in solid medium	PSI after 6 days	Conc. of P (µg/mL)
OS29(1)	−	1.0	−
OS29(2)	+	1.5	−
OS29(3)	−	1.0	−
OSbr4	−	1.0	−
OSbr5	−	1.0	−
OSbr6	−	1.0	−
OSn7	+	1.1	−
OSn8	+	1.1	−
EC1	−	1.0	−
EC2	−	1.0	−
EC3	+	1.3	−
EC4	−	1.0	−
EC5	−	1.0	−
ECL1	−	1.0	−
Fr1	−	1.0	−
Fr2	−	1.0	−
Fr3	−	1.0	−
Fr4	++	7.5	0.07±0.00
Fr5	−	1.0	−
Fr6	−	1.0	−
Fr7	++	6.5	0.03±0.00
GM1	−	1.0	−
GM2	++	3.0	0.04±0.00
MQ1	++	8.0	0.26±0.05
MQ2	+++	11	0.70±0.19
MQ3	+	2.5	0.26±0.11
MQ4	+	1.1	−
MQ5	+	1.25	−
MQ6	−	1.0	−

Qualitative analysis was done using Pikovskaya's agar medium supplemented with tricalcium phosphate as insoluble source of phosphorus (P). Pikovskaya's broth medium was used to quantify the concentration of P released by selected rhizobacterial isolates (based on PSI value >2.0). The failure to produce clear halo zones around the colony on solid media is indicated by negative sign (−). The positive signs (+, ++ and +++) for solid media indicate the P solubilization ability of the isolates. The negative sign for the concentration of P in liquid medium indicates that these bacteria were excluded from quantitative P determination due to low PSI values. Here, (+), (++) and (+++) represent level 1, level 2 and level 3 phosphorus solubilizers, respectively

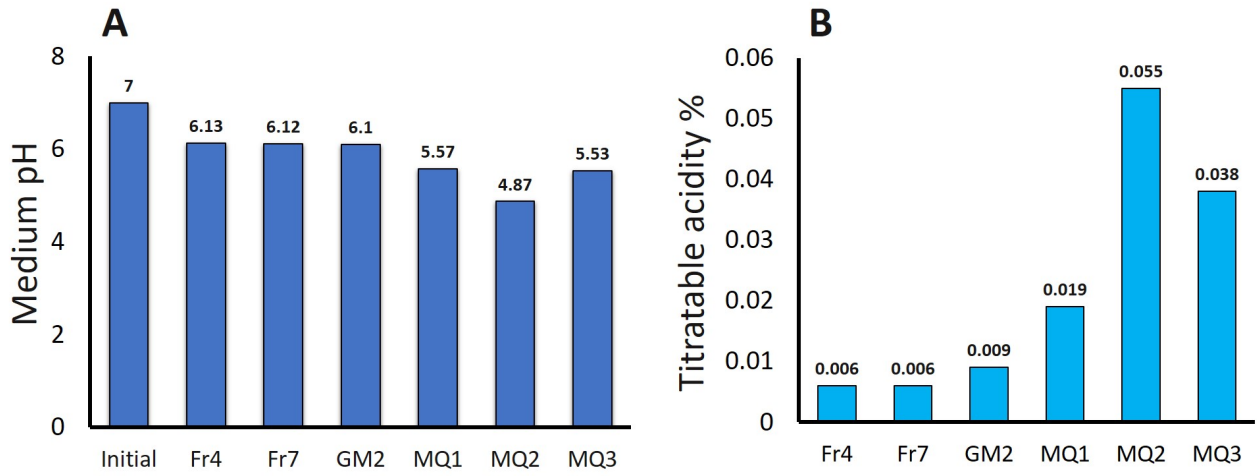

Figure 4. Reduction in medium pH due to inoculation of Rhizobacteria (A), and percent total titratable acidity (B). The results indicates that phosphorus was solubilized by the rhizobacteria due to the production of low molecular weight organic and inorganic acids

reduction in medium pH (Fig. 4A, B). The mechanism of microbial P solubilization varies among the bacterial species. In general, microbial phosphorus solubilization from inorganic pool of soil phosphorus is attributed to the production of different low molecular weight organic and inorganic acids like gluconic acid and 2-ketogluconic acid (Walpola, 2012) and release of chelating substance and carbonic acids (Vessey, 2003; Oteino et al., 2015).

The ability of N_2 fixation by PGPR is another major criterion for selecting rhizobacteria as components of microbial biofertilizer. Apart from the symbiotic N_2 fixing rhizobacteria, large number of free living and non-symbiotic rhizobacteria has been reported to have the ability to fix atmospheric N_2 and imparts plant growth promotion (Franche et al., 2008; Xu et al., 2018). In our study, all the rhizobacterial isolates were able to grow at varying capacity in N-free Winogrdsky's mineral media, an indication of putative N_2-fixation (Asha et al., 2015). Among the isolates, 13 isolates were designated as strong N_2-fixing bacteria due to their rapid and vigorous growth in N-free media (Fig. 3A). The presence of nitrogenase activity in bacteria is considered as the mechanism of N-fixation by free-living rhizobacteria (Rilling et al., 2018).

5 Conclusion

The search for multifunctional PGPR is gaining importance around the world to minimize the amount of chemical agrochemical inputs required for sustainable crop production. We isolated and characterized functionalities of 32 rhizobacteria from different plant species. We have reported the production of IAA, solubilization of mineral phosphate and N_2 fixing ability of the isolated rhizobacteria, all of which are considered as primary criteria for selecting PGPR. Based on the criteria studied for selecting potential PGPR, the isolate MQ1 proved to be the best over other isolated rhizobacteria and could be considered as potential PGPR for bioformulation. The application of these bacteria with plant growth promoting traits can be used to promote plant growth after evaluation of biofunctionalities under *in vitro* and *in vivo* conditions and detailed molecular characterization.

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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