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Glucose dehydrogenase gene containing phosphobacteria for biofortification of Phosphorus with growth promotion of rice

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ABSTRACT

Phosphorus (P) is an essential plant nutrient, but often limited in soils for plant uptake. A major economic constraint in the rice production is excessive use of chemical fertilizers to meet the P requirement. Bioaugmentation of phosphate solubilizing rhizobacteria (PSB) can be used as promising alternative. In the present study 11 mineral PSB were isolated from Basmati rice growing areas of Pakistan. In broth medium, PSB solubilized tricalcium phosphate (27–354 μg mL^{-1}) with concomitant decrease in pH up to 3.6 due to the production of different organic acids, predominantly gluconic acid. Of these, 4 strains also have ability to mineralize phytate (245–412 μg mL^{-1}). Principle component analysis showed that the gluconic acid producing PSB strains *(Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7) have pronounced effect on grain yield (up to 55%), plant P (up to 67%) and soil available P (up to 67%), with 20% reduced fertilization. For simultaneous validation of gluconic acid production by MR5 and MR7 through PCR, new specific primers were designed to amplify *gcd*, *pqqE*, *pqqC* genes responsible for *glucose dehydrogenase* (*gcd*) mediated phosphate solubilization. These findings for the first time demonstrated *Acinetobacter soli* as potent P solubilizer for rice and expands our knowledge about genus specific *pqq* and *gcd* primers. These two *gcd* containing PSB *Acinetobacter* sp. MR5 (DSM 106631) and *Pseudomonas* sp. MR7 (DSM 106634) submitted to German culture collection (DSMZ), serve as global valuable pool to significantly increase the P uptake, growth and yield of Basmati rice with decreased dependence on chemical fertilizer in P deficit agricultural soils.

1. Introduction

Rice is grown on 2.7 million hectares in Pakistan (Pakistan economic survey 2016-17). Among different rice varieties grown in the country, Basmati rice is treasured around the world for its blessed characteristics of long slender grains, delicious taste and superior aroma. Adequate phosphorus (P) nutrition is critical for proper growth of rice plant in particular in the early vegetative growth stages because it promotes root development, tillering, early flowering and ripening. A substantial amount of P occurs in soil, but the major part of soil P is present in the form of insoluble phosphates (Vassilev and Vassileva, 2003), while the concentration of bio-available P in soil is often very low. Therefore, to meet the plant P nutritional needs, chemical phosphate fertilizers have been widely applied and profitable rice growing requires fertilization of about 50–60 kg mineral P $hectare^{-1}$ (Khan et al., 2010a,b). However, the efficiency of applied P fertilizer throughout the world is only 10 to 25% due to its rapid and strong adsorption in the soils (Mohammadi, 2012). This immobilization is due to the high reactivity of phosphate anions with cations, i.e. iron or aluminum in acidic soils (Norrish and Rosser, 1983) whereas, calcium in neutral to alkaline soils (Lindsay et al., 1989). The continuous accumulation of applied P fertilizers leads to environmental pollution and issues related to human health (Killebrew and Wolff, 2010). Therefore, there is an urgent need to increase the availability of adsorbed P from agricultural soils to the plants (Sharma et al., 2013).

Phosphate solubilizing bacteria (PSB) in the crop rhizosphere imparts a vital eco-physiological role in liberating phosphate from inorganic compounds by secreting organic acids (Buch et al., 2009).

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These organic acids chelate the cations of Ca, Fe and Al that are bound to phosphate through their hydroxyl and carboxyl groups and thus convert P into soluble forms that are available for plant uptake. The efficiency of PSB for P solubilization depends upon the type and quantity of organic acids produced (Khan et al., 2007). In particular, gluconic acid is involved in the release of P from phosphate minerals and other inorganic phosphates. The synthesis of gluconic acid from glucose via the direct oxidation pathway is mediated by glucose dehydrogenase (GDH which is one of the quinoproteins), an enzyme encoded by the *gcd* gene, and its respective cofactor, pyrroloquinoline quinone (PQQ), encoded by the *pqq* operon (Sharma et al., 2013). This operon consists of six core genes pqqA, B, C, D, E, and F. In addition to the production of organic acids for P solubilization, some microbes also produce phytase enzyme for the release of P from organic compounds that are mostly present as phytates (Richardson et al., 2001). Hence, phytate degradation by microbial phytases compensates the inability of plants to acquire phosphorus directly from phytate.

With growing awareness about health hazards and environmental challenges associated with application of chemical fertilizers, there is an urgent need to select crop- and soil-specific efficient PSB for application as biofertilizers. For instance, several studies suggested that sustainable crop production can be achieved by inoculating PSB along with reduced amount of chemical fertilizers without elucidation of genetic basis involved in mineral P solubilization (Khan et al., 2010a,b; Sarkar et al., 2012). Previously, wheat and potato rhizosphere associated PSB have been isolated from Himalayan region and arid zones of Pakistan (Tahir et al., 2013; Naqqash et al., 2016), but to the best of our knowledge no research work has been conducted on native PSB from Basmati rice rhizosphere in the major rice growing areas of Pakistan. Most of the rice fields are deficient in available P where chemical phosphorus as Di-ammonium phosphate (DAP) is being applied to meet the plant requirement (Wakeel et al., 2017). For low-cost P biofortification of rice from unexplored aromatic Basmati rice growing areas, the present study aimed to i) isolate native P solubilizing bacterial strains from the rice rhizosphere; ii) to assess the effects of PSB strains on rice germination, root architecture, growth, yield and plant P contents as well as on the soil P mobilization; iii) to determine the survival and colonization of promising PSB in the rhizosphere; iv) To elucidate the genetic basis of gluconic acid production; supposed to play a predominant role in P solubilization, gene specific primers were designed to amplify genes encoding for GDH (*gcd*) and PQQ (pqq*C,* pqq*E*) of the most promising, rhizosphere-dwelling bacterial strains.

2. Materials and methods

2.1. Soil sampling

Approximately 100 g rice rhizosphere soil was collected from six different sites of Punjab rice belt in Faisalabad district (31°23′44.0″N 73°01′37.3″E), Gujranwala district (32°13′21.5″N 74°13′02.7″E), Hafizabad district (32°04′05.4″N 73°40′34.3″E), Kala Shah Kaku, district Sheikhupura (31°43′34.2″N 74°17′07.8″E), another site at Sheikhupura district (31°42′42.6″N 73°59′07.5″E) and Sialkot district (32°27′24.2″N 74°24′35.0″E). The soil particles adhered to roots in 0–20 cm depth were collected from five different plants of each sampling site, mixed and homogenized to obtain one composite sample from each site. The soil samples were transferred to the laboratory and stored at 4 °C.

2.2. Isolation and identification of phosphate solubilizing bacterial strains

For the isolation of phosphate solubilizing bacteria, Pikovskaya's agar medium (pH 6.8–7.0) containing insoluble tricalcium phosphate (TCP) as the sole P source was used (Pikovskaya, 1948). Agar plates were inoculated with soil suspensions and incubated at 28 ± 2 °C for 7 days. Isolation of PSB was conducted by a plate screening assay based on visual detection of clear halo zone formation, and the eleven strains showing the largest halo zones were used for further studies. Colony morphology, cell morphology, and motility of the bacterial isolates were studied using light microscopy. For phylogenetic classification, total genomic DNA was extracted with the CTAB method (Wilson, 2001) and 16S *rRNA* genes were amplified according to the protocol described by Ayyaz et al. (2016) using full length forward "PA-5′ AGACTTTGATCCTGCTCAG 3′-" and reverse "PH -5′ AAGGAGGTGAT CCAGCCGCA 3′-" primers (Edwards et al., 1989). The resulting nucleotide sequences were aligned and used for the construction of a maximum likelihood-based phylogenetic tree according to Hakim et al. (2018). The obtained sequences were deposited in GenBank under accession numbers LT629133-34, LT629136, LT629139-44, LT629146 and LT909525. The strains were submitted to the NIBGE Biotech Resource Centre (NBRC), Pakistan with accession numbers NBRC447-457. The two most efficient PSB strains *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 were additionally submitted to the German culture collection (DSMZ) with accession numbers DSM 106631 and 106634, respectively.

2.3. Screening of PSB for phosphate solubilization efficacy and production of organic acids

To estimate the relative TCP solubilization activities, bacterial strains were grown on Pikovskaya (Pikovskaya, 1948) and National Botanical Research Institutes Phosphate (NBRIP) agar media (Nautiyal et al., 1999). Solubilization indices were determined using the following formula (Paul and Sinha, 2017).

$$\text{Solubilization Index (SI)} = \frac{\text{Halo zone diameter (including colony)}}{\text{Colony diameter}}$$

For quantification of released P, PSB strains were grown in 50 mL Pikovskaya and NBRIP broth media in triplicate. Inoculated broth cultures were incubated at 28 ± 2 °C on a shaker (Kottermann 4020, Arnsberg, Germany) at 80 rpm. After 7 days, cultures were centrifuged at 4000 rpm in refrigerated centrifuge for 10 min at 4 °C and pH of filtrate was analyzed. Liberated P was determined quantitatively using Vanado-molybdate method (Murphy and Riley, 1962). Pikovskaya filtrates were further used for quantification of organic acids (gluconic, lactic, malic, acetic, citric, and succinic acid) on High Performance Liquid Chromatography (HPLC) as described previously (Tahir et al., 2013). Uninoculated media were considered as controls and respective P values were subtracted from the P concentrations in the inoculated samples.

Furthermore, the strains were grown on phytate screening medium to assess their efficacy for organic P mineralization (Howson and Davis, 1983) and quantification of released inorganic P from organic P was assessed by same protocol describe above (Murphy and Riley, 1962).

Primer designing and PCR amplification of *pqqC, pqqE* and *gcd* genes responsible for gluconic acid production in *Acinetobacter* and *Pseudomonas* strains

Glucose dehydrogenase gene *gcd* and pyrroloquinoline quinone genes *pqqE* and *pqqC* (pqq operon genes involved in biosynthesis of pqq cofactor) were amplified from genomic DNA of *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7. The following primer pairs were used for amplification of *Acinetobacter* sp. MR5 genes: *pqqEF* 5′ GATCGATCTG CGCTCGGTAG 3′ and *pqqER* 5′ GTGGAGAGCCTTTACTGCGG 3′, *pqqCF* 5′ CCTGACCCAAACGGCTTCTG 3′ and *pqqCR* 5′ CACTGCGTGATAAG GGCTGC 3′, as well as *gcdF* 5′GGCGTTCGACCCACGTAATC 3′ and *gcdR* 5′ CCATCGGGCCGGTATCTTCA 3′. These primers were designed from available nucleotide sequences of the *pqqC*, *pqqE* and *gcd* genes of *Acinetobacter soli* strain GFJ2 (GenBank accession no. CP016896). Instead, the genome sequence of *Pseudomonas putida* KT2440 (GenBank accession no. NC_002947) was used for primer design of *pqqEF* 5′ CGCCACGTGCCAGTTCTATG 3′ and *pqqER* 5′ TAGCCACGGAAGCGG

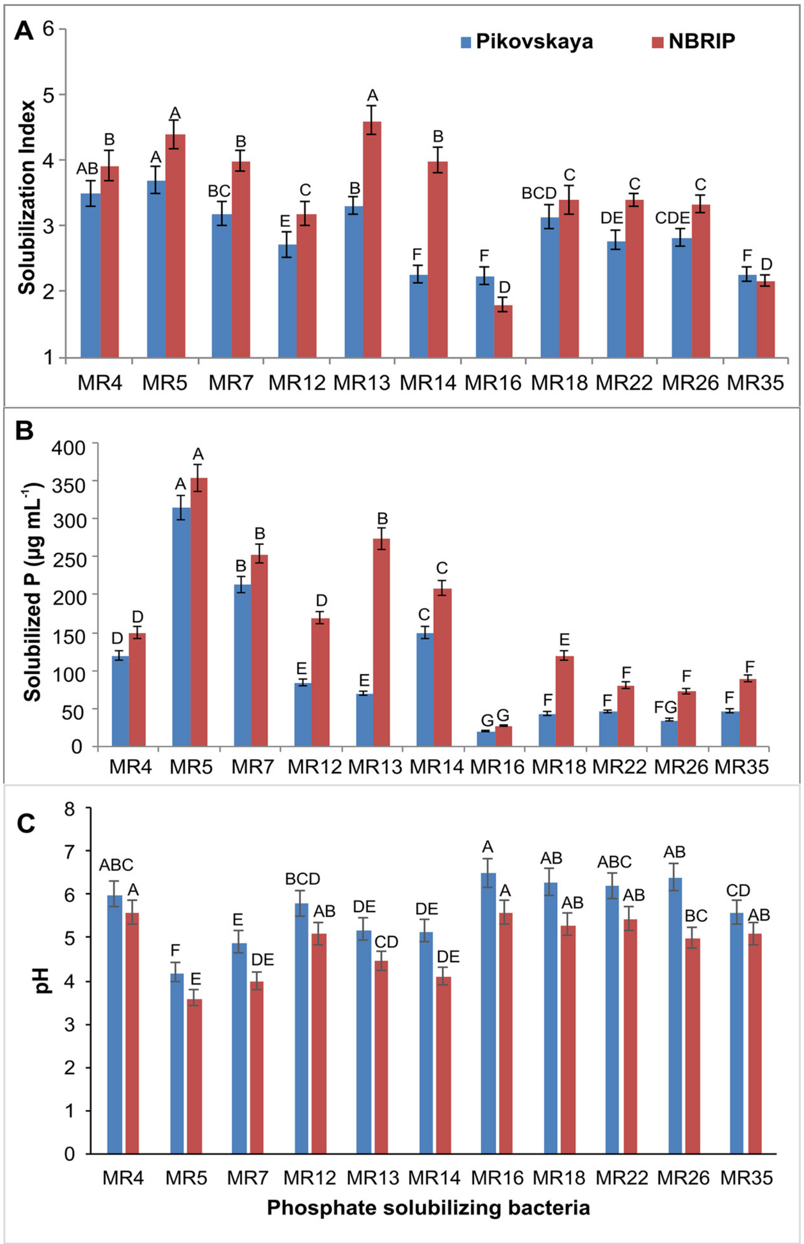


Fig. 1. Mineral phosphate solubilization activities of the PSB (A) P solubilization indices were calculated from halo zone formation on Pikovskaya's and NBRIP agar media and (**B**) based on the amount of solubilized P in liquid media. Mean concentrations (n = 3) of soluble P (µg mL^{-1}) after growth for seven days in Pikovskaya and NBRIP broth media. Bars represent the standard errors. (**C**) pH of the media after the incubation (initial pH = 7). Values with the same letter in one and the same medium differ non-significantly at $p \geq 0.01$ according to LSD.

TTG 3′ for *Pseudomonas* sp. MR7. PCR conditions were 32 cycles of 60 s at 95 °C, 60 s at 56 °C and 120 s at 72 °C. The *gcd* gene in *Pseudomonas* strain MR7 was amplified using the previously reported primers *gcdF* 5′ GACCTGTGGGACATGGACGT 3′ and *gcdR* 5′GTCCTTGCCGGTGTAGSTCATC 3′ as described by Chen et al. (2016). Amplified PCR products were separated on 1% agarose gel. Purified PCR products were commercially sequenced by Macrogen Inc. (Seoul, South Korea). Sequence data was aligned with the help of online program BLASTX and compared with *Pseudomonas* and *Acinetobacter gcd* or *pqq* gene sequences, respectively, available at NCBI. The phylogenetic relationships were studied by maximum likelihood method using protein sequences with help of MEGA7 software (Kumar et al., 2016). These sequences of *gcd, pqqE* from *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 and *pqqC* from *Acinetobacter* sp. MR5 were deposited to the NCBI with the accession number MH622199-622202, LT908483, respectively.

2.4. Determination of plant beneficial traits of PSB

Potential for plant growth promotion by PSB was evaluated by standard protocols. IAA production levels were quantified by HPLC as described by Ayyaz et al. (2016) and Tien et al. (1979), while nitrogenase activity was analyzed by acetylene reduction assay using gas chromatography (Hardy et al., 1968). Siderophore production was evaluated on succinate medium and quantified by spectrophotometer (Olsen and Sommers, 1982), whereas 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activity was observed in vials containing 30 µL of 0.5 M ACC as the sole nitrogen source in 5 mL salt minimal medium (Penrose and Glick, 2003).

Table 1
Organic acid produced by phosphate solubilizing bacterial isolates.

Phosphate solubilizing bacteria	Site of isolation	Organic acids produced during phosphate solubilization (μg mL^{-1})						
		Acetic acid	Citric acid	Gibberellic acid	Gluconic acid	Malic acid	Oxalic acid	Succinic acid
Enterobacter sp. MR4	Kala Shah Kaku	12 ± 2^{G}	0.9 ± 0.8^{F}	ND	5 ± 1^{G}	9.5 ± 1.5^{D}	1 ± 0.36^{DEF}	14.5 ± 1.6^{D}
Acinetobacter sp.MR5	Kala Shah Kaku	180 ± 11^{A}	81 ± 6^{A}	5.2 ± 0.3^{F}	117 ± 9^{B}	30 ± 0.5^{B}	10 ± 1.3^{AB}	68 ± 5^{A}
Pseudomonas sp. MR7	Kala Shah Kaku	144 ± 7^{B}	18 ± 2^{B}	17.0 ± 1.8^{B}	130 ± 11^{A}	71 ± 6^{A}	8 ± 1^{BC}	62 ± 8^{A}
Pseudomonas sp. MR12	Gujranwala	29 ± 2^{EF}	4.5 ± 1^{E}	6.1 ± 1.0^{EF}	19 ± 3^{EF}	3.5 ± 1.5^{EF}	2.5 ± 0.9^{DE}	28 ± 2^{C}
1. Klebsiella sp. MR13	Sheikhupura	60 ± 6^{C}	12.5 ± 1.5^{C}	32.5 ± 2.5^{A}	52 ± 1.8^{C}	ND	6 ± 1^{C}	ND
Pseudomonas sp. MR14	Kala Shah Kaku	40 ± 5^{DE}	7 ± 2^{DE}	2.3 ± 0.5^{G}	45 ± 7^{C}	6.0 ± 0.7^{DE}	8 ± 0.8^{BC}	49 ± 2^{B}
Bacillus sp. MR16	Faisalabad	61 ± 4^{C}	ND	13.4 ± 1.2^{C}	25 ± 3^{DE}	5.0 ± 0.7^{DEF}	1 ± 0.7^{DEF}	13.5 ± 2^{DE}
Bacillus sp. MR18	Kala Shah Kaku	43 ± 2^{D}	ND	16.5 ± 1.3^{B}	33 ± 2^{D}	4.9 ± 0.2^{DEF}	11.2 ± 2^{A}	7.5 ± 3^{EF}
Pseudomonas sp. MR22	Faisalabad	156 ± 14^{B}	ND	11.5 ± 1.5^{CD}	32 ± 2^{D}	23.5 ± 1.6^{EF}	ND	ND
Rhizobium sp. MR26	Gujranwala	20 ± 5^{FG}	11.5 ± 2.2^{CD}	9.8 ± 1^{DE}	20 ± 4^{EF}	0.5 ± 0.2^{C}	3 ± 0.4^{D}	6 ± 1^{F}
Bacillus sp. MR35	Sialkot	30 ± 4^{DEF}	ND	6.5 ± 1.2^{EF}	13 ± 3^{FG}	2.9 ± 0.7^{EF}	1 ± 0.7^{EF}	13.5 ± 2^{DE}

All values represent means ± standard errors of three replicates and values with different letters indicate statistical difference according to LSD at $p < 0.01$. ND: non-detectable.

2.5. Effects of bacterial inoculation on rice seedlings

To study the impacts of the all (11) isolates on rice seed germination, seedling growth and vigor, a growth room experiment was conducted according to Kumar et al. (2012). Seeds of the rice variety "Super Basmati", obtained from the Agriculture Biotechnology Division (NIBGE, Pakistan), were rinsed with sterile distilled water, surface sterilized with 2% Na-hypochlorite for five minutes and washed 3–4 times with sterile distilled water. Surface-sterilized seeds were dipped in bacterial cultures (1×10^{8} colony forming units mL^{-1} of LB medium) for 20 min, separately. Uninoculated seeds dipped in LB medium were used as control. The treated 22 seeds were put on water agar (0.25%) plates of 14 cm diameter, using triplicate for each isolate and the plates were randomly placed in a growth room. Growth conditions were 28/23 °C day/night, 16 h day length and 20,000 lx light intensity, and the plants were grown for seven days. Seed germination was recorded after 2–3 days, when the radicle length was half of the seed length. The experiment was conducted twice. At the end of the experiment, root and shoot lengths were measured and plant vigor index was calculated by using the following formula (Islam et al., 2015).

$$\text{vigor index} = \%\text{Germination} \times \text{Total seedling length (mean of root and shoot length)}$$

Three plants were selected from each replicate for studying root growth parameters including total length of the root system (cm), projected area (cm^2), surface area (cm^2), average root diameter (mm), length per volume ($cm\ m^{-3}$), as well as numbers of tips and crossings using Rhizoscanner (EPSON Perfection V700 Photo, Suwa, Nagano Prefecture, Japan), equipped with Win RHIZO software (Regent Instruments Inc., Canada).

2.6. Effects of PSB on rice yield and P contents

A pot experiment was conducted to study the effects of PSB inoculation on rice yield and P contents. Nine bacterial strains that showed positive effects on rice germination and seedling vigor in the plate assay were used, i.e. *Enterobacter* sp. MR4, *Acinetobacter* sp. MR5, *Pseudomonas* spp. MR7, MR14, MR22, *Klebsiella* sp. MR13, *Bacillus* spp. MR16, MR18 and *Rhizobium* sp. MR26. The experiment was conducted under natural light and temperature conditions in net house during the rice growing season at NIBGE, Faisalabad. Pots (18 cm diameter) were filled with 12 kg soil (non-sterile, loamy, pH 7.9, EC 2.1 mS cm^{-1}, organic matter 0.85%, soil total P 40 mg kg^{-1}, available P 1 mg kg^{-1} total soil N 0.6 g kg^{-1}, available NH_4 0.4 g kg^{-1}, NO_3 100 mg kg^{-1}). Basal nutrients N (50 mg kg^{-1} soil), K (120 mg kg^{-1} soil) and P (150 mg kg^{-1} soil for the control and 80% of the recommended dose, i.e. 120 mg kg^{-1} soil, for the inoculated pots) were applied as urea, potassium chloride and diammonium phosphate, respectively. The experiment was designed with 10 treatments including the nine strains and an uninoculated control with eight replicates per treatment. These were arranged in a completely randomized design in the net house. Seed surface-sterilization and inoculation were performed according to the above section. Twelve seeds were sown in each pot at equal distances and watered daily. Seven days after germination the pots were thinned to six plants per pot. Four pots of each treatment were harvested after 30 days, and the remaining 4 pots of each treatment were harvested at maturity. From the harvested plants, shoot length, root length, plant height, no. of tillers, straw weight and grain weight were measured. Plant P contents were determined from 1 g of ground sample of whole plants taken after 30 days, but separately for seeds and for straw from plants harvested at maturity. In each case, plant material from three plants was used and P analysis was performed in triplicates using tri-acid digestion method as described by Tendon (1993). The soil of each treatment was studied for total cultivable bacterial population (Kirk et al., 2004) and soil available P contents were measured by using molybdate blue method after extraction of P from 2 g of air-dried soil with the bicarbonate method (Ziadi et al., 2013).

2.7. Root colonization potential and re-isolation of inoculated PSB

Strains *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 were studied for root colonization using fluorescence in situ hybridization in combination with confocal laser-scanning microscopy (FISH-CLSM). The total bacterial population was labelled with FLUOS (InteractivaBiotechnologie GmbH, Ulm, Germany). Cy3-labelled probes specific for gamma-proteobacteria (γ) and *Pseudomonas* species (PSM_G) were used for the detection of bacterial populations of *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7, respectively (Braun-Howland et al., 1993; Sharafat et al., 2018). Three plants from each treatment were taken without damaging the roots and processed using the protocol described by Hofmann et al., 2014. The roots were observed under confocal laser scanning microscope (Olympus FV1000, Japan) equipped with an argon laser for excitation of FLUOS at 488 nm and Cy3 at 514 nm wavelengths. Bacterial cells were localized using a 100x water immersion objective. FLUOS-labelled oligonucleotide probes showed green fluorescent signals for total bacterial population. Cy3-labelled oligonucleotide probes showed red fluorescent signals for *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7. Images were recorded using FV10-ASW 1.7 software.

Both strains were re-isolated from the inoculated soil after rice growth. Rhizospheric soil (5 g) was taken from five plants of each treatment, mixed thoroughly and PSB were re-isolated on LB agar plates by serial dilutions method (Somasegaran and Hoben, 1994). The plates

Table 2
Effect of PSB inoculation on root morphological traits of rice variety Super Basmati.

Treatments	Total root length (cm)	% G	Vigor index	Root diameter (mm)	Root volume (cm^3)	Projection area (cm^2)	Surface area (cm^2)	No. of tips	No. of forks	No. of crossings
Enterobacter sp. MR4	22.2 ± 2.1^{F}	94 ± 3^{BC}	1006 ± 34^{G}	0.24 ± 0.03^{BCD}	0.0090 ± 0.0001^{CDE}	0.47 ± 0.25^{EFG}	1.58 ± 0.09^{EF}	161 ± 10^{F}	104 ± 21^{E}	14 ± 4^{G}
Acinetobacter sp. MR5	24.4 ± 3.6^{EF}	100 ± 2^{A}	960 ± 12^{H}	0.31 ± 0.02^{A}	0.0126 ± 0.0001^{B}	0.74 ± 0.04^{CD}	2.32 ± 0.21^{CD}	123 ± 9^{H}	115 ± 9^{DE}	12 ± 3^{H}
Pseudomonas sp. MR7	39.0 ± 2.8^{BC}	100 ± 2^{A}	1400 ± 23^{B}	0.24 ± 0.03^{BCD}	0.0190 ± 0.0001^{A}	0.93 ± 0.05^{BC}	2.93 ± 0.14^{B}	236 ± 12^{D}	185 ± 18^{B}	29 ± 2^{BC}
Pseudomonas sp. MR12	54.6 ± 5.2^{A}	80 ± 3^{E}	1328 ± 27^{C}	0.20 ± 0.01^{D}	0.0063 ± 0.0001^{CD}	1.08 ± 0.05^{A}	3.39 ± 0.14^{A}	390 ± 14^{A}	267 ± 24^{A}	54 ± 6^{A}
Klebsiella sp. MR13	30.0 ± 2.7^{DE}	100 ± 1^{A}	1030 ± 38^{F}	0.30 ± 0.04^{A}	0.0100 ± 0.0001^{BCD}	0.88 ± 0.06^{BC}	2.76 ± 0.13^{BC}	165 ± 8^{F}	149 ± 12^{CD}	$20 \pm {}^{F}$
Pseudomonas sp. MR14	43.6 ± 4.4^{B}	97 ± 1^{AB}	1610 ± 45^{A}	0.22 ± 0.01^{D}	0.0103 ± 0.0001^{BCD}	0.95 ± 0.03^{AB}	2.99 ± 0.17^{B}	316 ± 20^{B}	205 ± 23^{B}	30 ± 2^{B}
Bacillus sp. MR16	13.4 ± 2.3^{G}	78 ± 3^{E}	562 ± 8^{L}	0.28 ± 0.03^{AB}	0.0083 ± 0.0001^{DEF}	0.38 ± 0.02^{EFG}	1.18 ± 0.07^{FG}	47 ± 7^{I}	40 ± 11^{F}	6 ± 1^{IJ}
Bacillus sp. MR18	10.2 ± 2.7^{G}	88 ± 1^{D}	906 ± 19^{J}	0.27 ± 0.02^{ABC}	0.0053 ± 0.0001^{G}	0.27 ± 0.02^{G}	0.84 ± 0.04^{G}	34 ± 3^{I}	16 ± 32^{F}	7 ± 2^{I}
Pseudomonas sp. MR22	11.9 ± 1.9^{G}	94 ± 5^{BC}	679 ± 54^{K}	0.28 ± 0.01^{AB}	0.0073 ± 0.0001^{EFG}	0.34 ± 0.02^{FG}	1.05 ± 0.05^{G}	38 ± 2^{I}	37 ± 8^{F}	5 ± 3^{J}
Rhizobium sp. MR26	27.0 ± 2.8^{EF}	97 ± 2^{AB}	1193 ± 44^{E}	0.24 ± 0.02^{BCD}	0.0110 ± 0.0001^{BC}	0.60 ± 0.04^{DE}	1.89 ± 0.11^{DE}	187 ± 9^{E}	140 ± 4^{CD}	27 ± 4^{CD}
Bacillus sp. MR35	24.0 ± 1.5^{EF}	70 ± 4^{F}	924 ± 27^{I}	0.22 ± 0.01^{CD}	0.0090 ± 0.0001^{CDE}	0.52 ± 0.02^{DEF}	1.64 ± 0.07^{E}	137 ± 11^{G}	143 ± 15^{CD}	23 ± 1^{E}
Control	35.0 ± 3.3^{CD}	91 ± 2^{CD}	1247 ± 8^{D}	0.22 ± 0.01^{CD}	0.0063 ± 0.0001^{FG}	0.60 ± 0.03^{DE}	2.43 ± 0.12^{C}	258 ± 5^{C}	171 ± 9^{BC}	26 ± 1^{D}

Rhizo-scanning was carried out on seven days old seedlings grown on 0.05% water agar plates 22 seeds on each replicate. Values represent means ± standard error of three biological replicates and means in each column followed by the same letter do not differ significantlyat $p \leq 0.01$ according to LSD. The experiment was repeated twice. % G: percent germination.

were then incubated at 28 ± 2 °C for 48 h and colonies were counted using colony counter (Color Q counter, Model UVD-3200). The colonies of inoculated strains were selected and transferred to LB agar plates. The colony and cell morphology were reconfirmed on the basis of colony characteristics and light microscopy, respectively. The re-isolated strains were also studied for other characteristics i.e. solubilization of TCP and Na-phytate, IAA production, and nitrogenase activity. BOX-PCR fingerprints of the two inoculated strains were compared with those of the re-isolated cultures using BOX AIR primer 5′ CTACGGCA AGGCGACGCTGACG 3′ (Louws et al., 1994; Basheer et al., 2016; Yasmin et al., 2017).

2.8. Statistical analysis

Data of *in vitro* studies and pot experiments was statistically analyzed by ANOVA and difference between the treatments were compared by least significant difference (LSD) test at 1% and 5% level of confidence, respectively using STATISTIX 10.0 software (Tallahassee, FL, USA). Principal component analysis was used on different root and yielding parameters using SPSS software Package Version 23.0 (SPSS Inc, USA).

3. Results

3.1. Isolation and identification of phosphate solubilizing bacteria

Rice rhizosphere soil samples were collected from six different sites of Punjab rice belt for the isolation of PSB. Among the grown bacterial colonies, 11 strains were efficient P solubilizers (isolates MR4, MR5, MR7, MR12, MR13 MR14, MR16, MR18, MR22, MR26 and MR35) as indicated by halo zone formation on Pikovskaya agar medium and therefore were selected for further studies. On LB agar, the isolates MR4, MR5, MR7, MR13 and MR26 produced small round and gummy colonies with smooth shiny surface and off-white (MR4, MR5), white (MR13), yellowish (MR7) and light brown (MR26) colors, respectively. The isolates MR12, MR14 and MR22 produced large, gummy colonies with shiny silver-brown, brown, and shiny grayish-green color, respectively. The isolates MR16, MR18 and MR35 produced white, flat and shiny colonies. Light microscopy showed that the cells of all isolates were rod-shaped and motile. The isolates were identified on the basis of 16S rRNA gene analysis (Fig. S1). 16S rRNA gene sequencing of strains MR12, MR14, and MR22 revealed 100% sequence homology with *Pseudomonas aeruginosa* and sequences of MR7, MR4, MR5 showed 99% and MR13 100% homology with those of *Pseudomonas putida*, *Enterobacter cancerogenus*, *Acinetobacter soli and Klebsiella pneumonia*, respectively. The isolates MR16, MR18 and MR35 were identified as *Bacillus* on the basis of high (99%) sequence similarity. Strain MR26 showed 99% homology with *Rhizobium rhizoryzae.*

3.2. Estimation of phosphate solubilization efficiency

All isolated PSB showed P solubilization activity with solubilization indices (SI) ranging from 1.8 to 4.6 and from 2.2 to 3.7 on NBRIP and Pikovskaya's agar media, respectively (Fig. 1A). *Enterobacter* sp. MR4 (SI: 3.9 and 3.5), *Acinetobacter* sp. MR5 (SI: 4.4 and 3.7), *Pseudomonas* sp. MR7 (SI: 4 and 3.2) and *Klebsiella* sp. MR13 (SI: 4.6 and 3.3), showed the highest phosphate solubilization indices. P quantification in broth media inoculated with PSB showed significant ($p \leq 0.01$) increases of solubilized P along with significant pH decreases (Fig. 1B - C). The solubilized P ranged from 27 to 354 μg mL^{-1} with pH decline up to 3.6 in NBRIP medium and from 20 to 315 μg mL^{-1} with pH decline up to 4.2 in Pikovskaya medium, respectively, whereby maximum P release and acidification was observed for *Acinetobacter* sp. MR5.

In vitro released organic acids by bacterial strains during TCP solubilization were detected by HPLC (Table 1). Acetic acid (12–180 μg mL^{-1}) and gluconic acid (5–130 μg mL^{-1}) were produced by all the

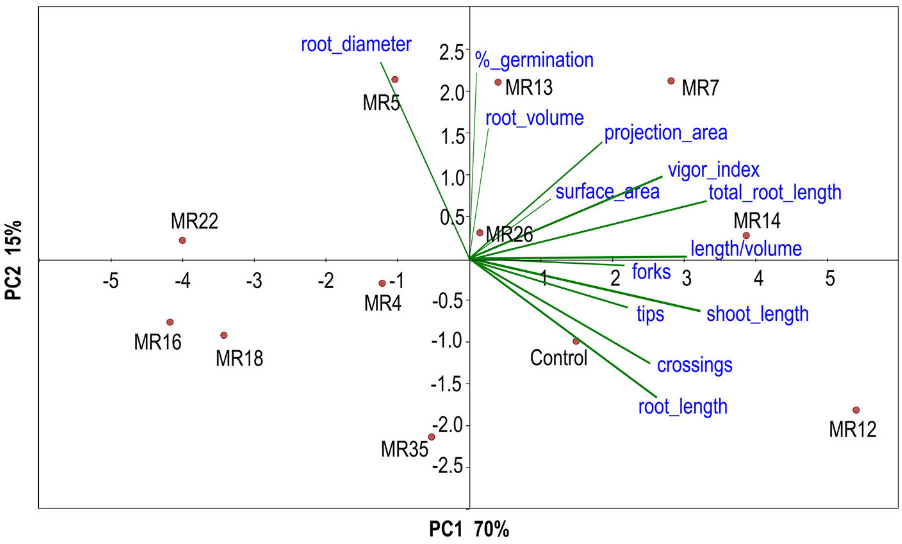


Fig. 2. Impacts of the PSB on early rice seedling growth. Principle component biplot of 7-days-old rice seedling parameters under controlled conditions. Distances between variables on the biplot represent Euclidean distances in multidimensional space. Projections drawn from a treatment on the variable vectors indicate its position along that vector.

tested strains. The production of other organic acids was restricted to some strains: citric acid (0.9–81 µg mL^{-1}) was produced by *Pseudomonas* spp. MR7, MR12 and MR14, *Acinetobacter* sp. MR5, *Enterobacter* sp. MR4, *Klebsiella* sp. MR13 and *Rhizobium* sp. MR26. Succinic acid (6–68 µg mL^{-1}) was produced by *Pseudomonas* spp. MR7, MR12 and MR14, *Acinetobacter sp.* MR5, *Rhizobium* sp. MR26, *Enterobacter* sp. MR4 and *Bacillus* spp. MR16, MR18 and MR35. All strains showed oxalic acid production (1–11 µg mL^{-1}) except *Pseudomonas* sp. MR22, gibberellic acid production (2.3–32.5 µg mL^{-1}) except *Enterobacter* sp. MR4 and malic acid production (0.5–71 µg mL^{-1}) except *Klebsiella* sp. MR13. The two most efficient P-solubilizing strains *Pseudomonas* sp. MR7 and *Acinetobacter* sp. MR5 (high solubilization in Fig. 1B) also produced significantly higher amounts of gluconic acid (130 µg mL^{-1} for MR7 and 117 µg mL^{-1} for MR5; Table 1) as compared to other tested strains (5–52 µg mL^{-1}).

P mineralization activity on phytate medium was determined qualitatively (halo zone 3–9.5 mm) and quantitatively (245–412 µg mL^{-1}). Maximum activity (zone diameter 9.5 mm; 412 µg mL^{-1}) was observed for *Klebsiella* sp. MR13 followed by MR7 (7.2 mm; 392 µg mL^{-1}), MR5 (5.5 mm; 300 µg mL^{-1}) and *Enterobacter* sp. MR4 (3 mm; 245 µg mL^{-1}).

PCR amplification of *pqqC, pqqE* and *gcd* genes responsible for gluconic acid in *Acinetobacter* and *Pseudomonas* strains using newly designed primers

The high amounts of gluconic acid produced by *Acinetobacter* strain MR5 and *Pseudomonas* strain MR7 strongly suggested the production of proteins responsible for gluconic acid production via the direct oxidation pathway, namely quinoprotein glucose dehydrogenase and the cofactor PQQ, encoded by *gcd* and *pqq* genes, respectively. In order to confirm this hypothesis, we used specific primers designed to amplify *pqqC*, *pqqE* and *gcd* genes. The results showed that bands of the expected size for *pqqC* (504 bp), *pqqE* (471 bp) and *gcd* (968 bp) for *Acinetobacter* strain MR5. Using *Pseudomonas* sp. MR7 as a template we also found positive amplification of *pqqE* (345 bp) and *gcd* (876 bp) genes. Partial sequencing of the PCR products confirmed that they correspond to the respective genes (Fig. 6A, Table 4).

The *gcd* genes amplified from *Acinetobacter* strain MR5 (GenBank accession MH622201) and *Pseudomonas* sp. MR7 (GenBank accession LT908483) showed 100% identity with *gcd* genes of *Acinetobacter soli* (WP004937792) and 95% with *Pseudomonas* strain (WP020191187) (Fig. 6B). As expected, phylogenetic analysis of the partial DNA sequence for the *pqqE* gene of *Acinetobacter* sp. MR5 (GenBank accession MH622199) and *Pseudomonas* sp. MR7 (GenBank accession MH622202) showed the highest identity (99%) with *pqqE* gene of *Acinetobacter soli* (GenBank accession WP004939059) and 97% identity with *Pseudomonas putida* (WP_099452708) (Fig. 6C). *PqqC* gene amplified from *Acinetobacter* strain MR5 showed 97% homology with that of *Acinetobacter soli* (WP055415721).

3.3. Plant growth promoting traits of phosphate solubilizing isolates

The eleven bacterial strains produced IAA in a growth medium supplemented with tryptophan at different levels, ranging from 0.3 to 16 µg mL^{-1}. Maximum IAA production was detected for *Klebsiella* sp. MR13, followed by *Pseudomonas* sp. MR14 (14 µg mL^{-1}). Eight strains showed nitrogenase activity (6–73 nmol ethylene mg^{-1} protein h^{-1}). Maximum nitrogenase activity was shown by *Acinetobacter* sp. MR5. Four of the studied PSB (strains *Enterobacter* sp. MR4, *Acinetobacter* sp. MR5, *Pseudomonas* sp. MR7 and *Klebsiella* sp. MR13.) produced siderophores and that at similar levels, from 1.09 to 1.28 µg mL^{-1} culture medium. ACC deaminase activity was observed for *Enterobacter* sp. MR4, *Acinetobacter* sp. MR5, *Pseudomonas* spp. MR7, MR12, MR14 and *Klebsiella* sp. MR13 (Table S1).

3.4. Effects of PSB on seed germination and seedling vigor

Rice seeds were inoculated with the isolated strains to study their impact on seed germination and early growth of the rice variety “Super Basmati”. Most of the inoculated strains showed positive effect on seed germination as compared to uninoculated control (91%). *Acinetobacter* sp. MR5, *Pseudomonas* sp. MR7 and *Klebsiella* sp. MR13 showed 100% germination (Fig. S2). Maximum plant vigor index (1610) was observed in seedlings inoculated with *Pseudomonas* sp. MR14 followed by the *Pseudomonas* spp. MR7 and MR12. Maximum root diameter (0.31 mm) was observed for *Acinetobacter* sp. MR5 followed by *Klebsiella* sp. MR13 and maximum root volume (0.019 cm^3) was observed for *Pseudomonas* sp. MR7 followed by *Acinetobacter* sp. MR5 (0.0126 cm^3). Root scanning showed significant increases of root lengths (up to 28% compared to uninoculated control) in plants inoculated with *Pseudomonas* spp. (MR7, MR12, and MR14). Moreover, inoculation with *Pseudomonas* spp. significantly increased the root diameter (up to 41%), root length to volume ratio (up to 56%), root volume, as well as the numbers of tips, forks and crossing (Table 2).

The seedling parameters were subjected to a principle component analysis (PCA). Fig. 2 includes the first two components (PC1 and PC2) that explains 85% of the total variance. It is evident from the figure that root volume and seed germination rate were highly correlated and positively affected by *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7, and *Klebsiella* sp. MR13. Vigor index was correlated with shoot length and with most of the root parameters, e.g. projection area, length per volume, no. of forks and tips. These parameters were increased by

Table 3

Effects of inoculated PSB on soil available P, plant P contents and growth parameters of rice variety Super Basmati at the harvesting stage.

Phosphate solubilizing bacteria	Plant height (cm)	Shoot dryweight (g)	Root dry weight (g)	Number of tillers	Grain weight (g $plant^{-1}$)	Root length (cm)	Plant P contents %		Soil available P (mg kg^{-1})
							Straw	Grain	
Enterobacter sp. MR4	106 ± 7^{BC}	47 ± 3^{E}	59.3 ± 3.9^{B}	14.0 ± 0.7^{ABC}	34.4 ± 2.0^{AB}	21.0 ± 2.8^{E}	0.044 ± 0.008^{CD}	0.184 ± 0.005^{C}	2.46 ± 0.25^{DE}
Acinetobacter sp. MR5	113 ± 10^{AB}	59 ± 6^{B}	53.2 ± 2.7^{BC}	15.5 ± 0.8^{A}	37.4 ± 1.8^{A}	30.5 ± 0.7^{BC}	0.056 ± 0.008^{B}	0.226 ± 0.006^{A}	3.11 ± 0.31^{B}
Pseudomonas sp. MR7	117 ± 8^{A}	53 ± 3^{CD}	38.5 ± 1.9^{FG}	13.1 ± 1.1^{CD}	33.8 ± 1.7^{AB}	36.5 ± 1.8^{A}	0.07 ± 0.01^{A}	0.202 ± 0.01^{B}	3.90 ± 0.05^{A}
Klebsiella sp. MR13	113 ± 5^{AB}	68 ± 7^{A}	47.0 ± 2.4^{CDE}	15.0 ± 0.7^{AB}	37.2 ± 1.8^{A}	29.1 ± 1.6^{CD}	0.050 ± 0.003^{BC}	0.194 ± 0.017^{BC}	2.80 ± 0.15^{BC}
Pseudomonas sp. MR14	103 ± 6^{CD}	55 ± 4^{BC}	49.7 ± 3.3^{CD}	12.5 ± 0.6^{CDE}	35.1 ± 2.1^{AB}	33.5 ± 2.4^{AB}	0.039 ± 0.004^{D}	0.141 ± 0.01^{E}	2.23 ± 0.24^{E}
Bacillus sp. MR16	105 ± 7^{BCD}	35 ± 10^{F}	39.8 ± 0.9^{EFG}	14.2 ± 0.4^{ABC}	22.9 ± 0.9^{D}	33.2 ± 1.7^{AB}	0.056 ± 0.002^{B}	0.186 ± 0.013^{C}	2.62 ± 0.20^{CD}
Bacillus sp. MR18	115 ± 11^{A}	50 ± 4^{DE}	51.5 ± 4.5^{BC}	11.5 ± 0.5^{CD}	26.9 ± 0.7^{C}	26.3 ± 0.9^{D}	0.041 ± 0.003^{CD}	0.148 ± 0.008^{DE}	2.41 ± 0.06^{DE}
Pseudomonas sp. MR22	110 ± 7^{ABC}	65 ± 6^{A}	81.5 ± 7.1^{A}	13.5 ± 0.8^{ABCD}	37.1 ± 1.8^{A}	27.5 ± 1.3^{CD}	0.043 ± 0.007^{CD}	0.158 ± 0.01^{D}	2.95 ± 0.12^{B}
Rhizobium sp. MR26	109 ± 4^{ABC}	54 ± 3^{CD}	32.3 ± 3.1^{G}	11.0 ± 0.28^{D}	33.2 ± 1.8^{B}	18.0 ± 1.1^{F}	0.038 ± 0.001^{D}	0.136 ± 0.004^{E}	2.40 ± 0.01^{DE}
Control	97 ± 5^{D}	46 ± 3^{E}	41.6 ± 2.3^{DEF}	13.0 ± 0.6^{ABCD}	24.1 ± 2.2^{D}	21.5 ± 2.0^{E}	0.042 ± 0.002^{CD}	0.135 ± 0.008^{E}	2.33 ± 0.08^{E}

Values represent averages ± standard errors of four biological replicates (2 plants per pot). Values with different letters indicate statistical difference according to LSD at $p < 0.05$.The experiment was repeated twice.

inoculation with *Pseudomonas* spp. MR12 and MR14. *Acinetobacter* sp. MR5 and *Klebsiella* sp. MR13 inoculation of rice seedlings showed an additionally positive impact on root diameter. However, the PCA plot also showed that some strains (*Enterobacter* sp. MR4, *Bacillus* spp. MR16, MR18, and MR35, *Pseudomonas* sp. MR22) negatively affected seedling health and development.

3.5. *In planta evaluation of PSB for soil P mobilization and rice growth promotion in a pot experiment*

Out of 11 the PSB strains, nine strains with superior performance in the *in vitro* germination test were further studied for their effects on various rice growth parameters in a pot experiment under net house conditions. Data collected after 30 days showed significant improvements ($p < 0.05$) in root length, shoot length, plant dry weight and plant P content compared to uninoculated control plants (Table S2). Maximum growth promotion was observed in plants inoculated with *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7. Inoculation with *Acinetobacter* sp. MR5 showed significant increase of plant dry weight (71%), plant shoot length (40%) and plant P content (56%) compared to uninoculated control plants. *Pseudomonas* sp. MR7 significantly enhanced plant dry weight (52%), plant shoot length (24%), plant P content (39%), and root length (38%). All inoculated treatments, besides for *Bacillus* sp. MR18, showed a significant increase of cultivable rhizosphere bacteria, with a maximum increase over control by 77% in soils inoculated with *Klebsiella* sp. MR13.

These plant growth promoting effects, in particular of the strains MR5 and MR7, were also observed at plant harvesting stage (Table 3). Significant improvement over control was observed in plant height (16%), root length (42%), dry root weight (28%), number of tillers (19%) and grain weight (37.4g↑55%) in plants inoculated with *Acinetobacter* sp. MR5, whereas 21% increase in plant height, 40% (33.8 g) in grain weight and 70% in root length was observed in plants inoculated with *Pseudomonas* sp. MR7. The remaining seven strains showed less growth promoting effects, even though the maximum increases of shoot weight (48%) and root weight (96%) were recorded for the plants inoculated with *Klebsiella* sp. MR13 and *Pseudomonas* sp. MR22, respectively. However, the maximum plant P contents were also measured in grains (increase of 67% and 50%) and straw (increase of 33% and 67%) of plants inoculated with *Acinetobacter* sp. MR5 and with *Pseudomonas* sp. MR7, respectively (Table 3). P mobilization by the two strains *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 showed significant increases of 39% and 26% after 30 days as well as of 33% and 67% at plant harvesting, respectively (Tables S2 and Table 3), even though P fertilization was reduced by 20% in the inoculated treatments compared to the uninoculated controls.

The data collected at the harvesting stage were subjected to a principal component analysis (Fig. 3). The biplot demonstrated correlation between different plant parameters, which are partly related to inoculated strains. The first two components contributed about 65% towards variance plotted on x-axis (PC1 = 45%) and y-axis (PC2 = 20%). Plants inoculated with *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 had significant positive impacts on the number of tillers, the root length, soil available P and P contents of the rice plants. No studied parameters found to be negatively affected by inoculation with these potential strains.

3.6. *Root colonization and re-isolation of inoculated Acinetobacter sp. MR5 and Pseudomonas sp. MR7*

Root colonization was confirmed by confocal laser scanning micrographs of rice roots inoculated with *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7. The cells were hybridized with fluorescently-labelled probes and the micrographs demonstrated that the bacterial cells were tightly attached to the root cells (Fig. 4). All treatments including the uninoculated control showed bacterial root colonization.

Table 4

Primers details of genes responsible for Gluconic acid production.

PSB strains	Gene	Primer sequence	Detection of genes	Amplicon size	Accession number in NCBI	References
***Acinetobacter* sp. MR5**	*PqqE*	F "GATCGATCTGCGCTCGGTAG" R "GTGGAGAGCCTTTACTGCGG"	Yes	471	MH622199	This study
	PqqC	F "CCTGACCCAAACGGCTTCTG" R "CACTGCGTGATAAGGGCTGC"	Yes	504	MH622200	
	Gcd	F "GGCGTTCGACCCACGTAATC" R "CCATCGGGCCGGTATCTTCA"	Yes	968	MH622201	
***Pseudomonas* sp. MR7**	*PqqE*	F "CGCCACGTGCCAGTTCTATG" R "TAGCCACGGAAGCGGTTG"	Yes	345	MH622202	
	PqqC	F "GCAAATCCACCAGTCACGC" R "TAGGGTGGGCGGTTCAGT"	No	256	–	
	Gcd	F "GACCTGTGGGACATGGACGT" R "GTCCTTGCCGGTGTAGSTCATC"	Yes	876	LT908483	Chen et al., 2016

Gene encoding glucose dehydrogenase *(gcd)* for gluconic acid production and*pqqC, pqqE*. Gene sequence were deposited in NCBI Gene Bank.

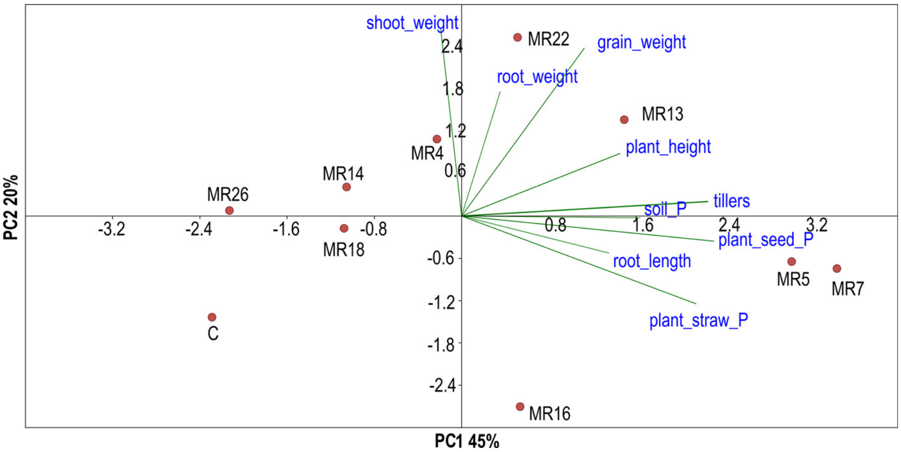


Fig. 3. Impacts of the PSB on rice at the harvesting stage. Principle component biplot of plant growth parameters at the harvesting stage of Basmati rice plants in a pot experiment implemented under net house conditions. Distances between variables on the biplot represent Euclidean distances in multi-dimensional space. Projections drawn from a treatment on the variable vector indicate its position along that vector.

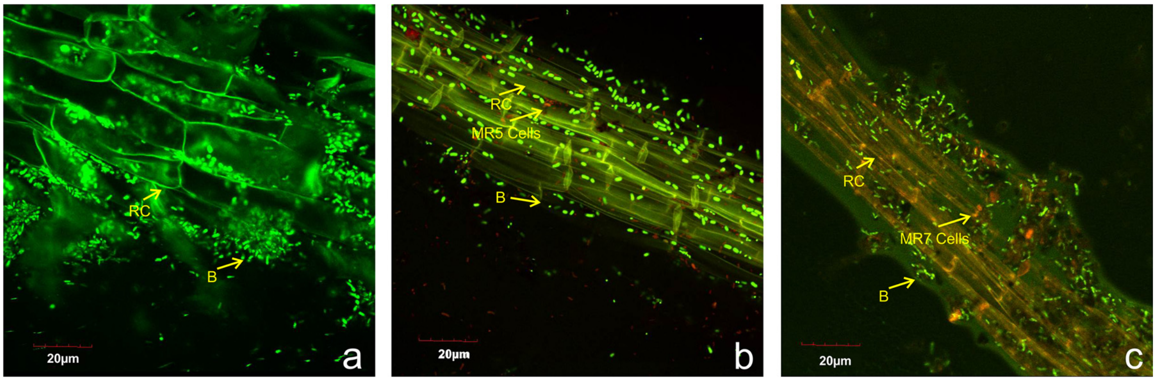
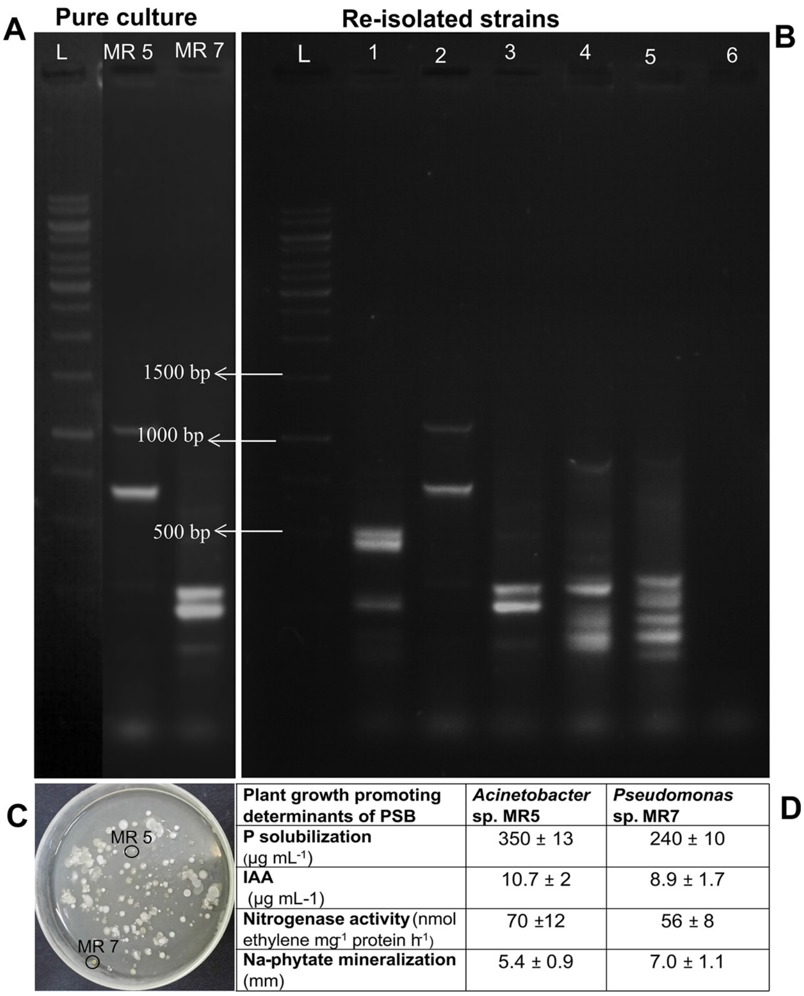


Fig. 4. Bacterial colonization of rice roots. Pot experiment under net house conditions was conducted for 30 days. Confocal laser scanning micrographs of roots were recorded from (**A**) control treatments without inoculation and from the pots inoculated with (**B**) *Acinetobacter* sp. MR5 and (**C**) *Pseudomonas* sp. MR7 after hybridizing with fluorescently labelled probes. FLUOS-labelled oligonucleotide probes showed green fluorescent signals for total bacterial population and Cy3-labelled oligonucleotide probes showed red fluorescent signals for inoculated bacteria. B: bacteria, RC: root cell.

The Cy3-labelled GAM42a probe showed red cells belonging to gamma proteobacterial population in inoculated treatment of *Acinetobacter* sp. MR5 (4 A). Cy3-labelled PSM probe that detected several *Pseudomonas* species in inoculated treatment of *Pseudomonas* sp. MR7. On the root surface, besides our targeted, other *Pseudomonas* can also be found in great numbers colonizing the root hair zones of plants (4B).

To assess the survival of the inoculated strains *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7, the respective bacterial populations in the rice rhizospheres were studied after 30 days of rice growth. BOX-PCR of re-isolated, morphologically identical colonies confirmed the presence of both inoculated bacteria. DNA patterns obtained by the BOX primers from re-isolates were compared with BOX-PCR patterns of the original pure cultures (Fig. 5A, B). BOX-PCR pattern of the selected strains were identical and confirmed the identity of the re-isolated colonies using plate viable count (Fig. 5C). *In vitro* plant growth promoting traits P solubilization (MR5: 350 ± 13 µg mL^{-1}, MR7: 240 ± 10 µg mL^{-1}), production of IAA (MR5: 10.7 ± 2 µg mL^{-1}, MR7: 8.9 ± 1.7 µg mL^{-1}). Na-phytate mineralization (MR5: 5.4 ± 1.0 mm, MR7: 7.0 ± 1.1 mm) and nitrogenase activity (MR5: 70 ± 12 nmol ethylene mg^{-1} protein h^{-1}, MR7: 56 ± 8 nmol ethylene mg^{-1} protein h^{-1}) of the inoculated bacteria were comparable to those observed earlier in pure culture (Fig. 5D).



Plant growth promoting determinants of PSB	*Acinetobacter* sp. MR5	*Pseudomonas* sp. MR7
P solubilization (μg mL^{-1})	350 ± 13	240 ± 10
IAA (μg mL-1)	10.7 ± 2	8.9 ± 1.7
Nitrogenase activity (nmol ethylene mg^{-1} protein h^{-1})	70 ±12	56 ± 8
Na-phytate mineralization (mm)	5.4 ± 0.9	7.0 ± 1.1

Fig. 5. Confirmation of re-isolated colonies of inoculated PSB (A) Gel photograph showing BOX-PCR of pure culture obtained from the rhizosphere of rice, L :1Kb DNA ladder; MR5: *Acinetobacter* sp. MR5, MR7: *Pseudomonas* sp. MR7, (B) Gel photograph of re-isolated morphologically similar colony to confirm the inoculated PSB. L: 1Kb DNA ladder, 1: Non-specific colony. 2: Re-isolated colony similar to *Acinetobacter* sp. MR5; 3: Re-isolated colony of *Pseudomonas* sp. MR7, 4: Non-specific colony, 5: Non-specific colony, 6: -ve C, (C) Plate showing Re-isolated colonies (D) Plant growth promoting determinants of re-isolated phosphate solubilizing bacteria. To confirm both inoculated PSB: Phosphate solubilization was quantified using spectrophotometer, Indole acetic acid was detected quantitatively by HPLC, Organic P mineralization using Na- phytate as insoluble P source by halo zone formation on agar plate, nitrogenase activity was determined by GC, All values are an average of three biological replicates, ± represents standard deviation.

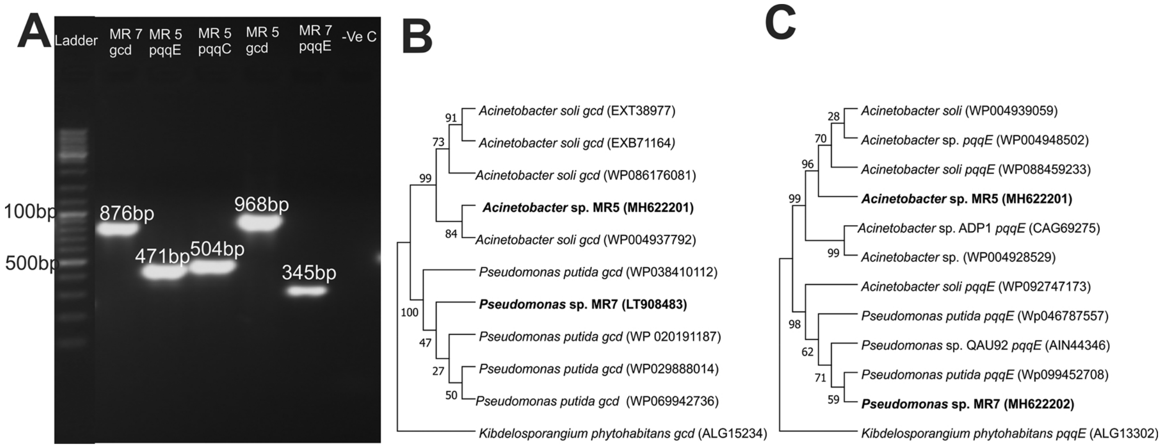


Fig. 6. *pqqE* and glucose dehydrogenase genes of *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7. (A) Gel photograph showing positive amplification of genes (B) The *pqqE* gene and (C) *gcd* gene phylogenetic trees, constructed by maximum likelihood method. Bootstrap node support values are ≥500 out of 1000.

4. Discussion

With the increasing awareness about economic, environmental, health and food security issues due to frequent application of chemical phosphatic fertilizers, the use of indigenous PGPR has emerged as a sustainable approach for increasing soil fertility. Due to their potential to increase the available P in the soil for plant uptake, bio-inoculants containing native PSB have been proposed for use in integrated nutrient management systems (Khan et al., 2010a,b; Richardson et al., 2011). PSB have been successfully isolated from rice in China, India and Bangladesh (Chung et al., 2005; Panhwar et al., 2009, 2012; Sarkar et al., 2012). However, any detailed information has not been reported so far on PSB associated with “Basmati rice” of Punjab rice belt which is a well-known area for the production of aromatic rice in Indo-Pak sub-continent. Therefore, the present study was designed to select PSB from the rhizosphere of Basmati rice for better plant P uptake and grain yield with reduced inputs of agrochemicals and less consequences for the environment.

We used a sequential selection procedure for phosphate solubilizing efficiency of isolated bacteria. The first step led to the selection of eleven P-solubilizing bacterial isolates from the rice rhizosphere. Different genera have already been reported in literature for their phosphate solubilization potential (Alori et al., 2017). The two most efficient PSB, *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7, were isolated from rice plants grown in Kala Shah Kaku district Sheikhupura of rice belt. This region is particularly important for exportable aromatic basmati rice production. Native PSB isolation having multifaceted attribute could be used to foster the yield and for P biofortification of rice. Out of the 11 PSB tested in the present study, *Enterobacter* sp. MR4, *Acinetobacter* sp. MR5, *Pseudomonas* sp. MR7 and *Klebsiella* sp. MR13 additionally released P *in vitro* from phytate, which represents a complementary P-solubilizing trait of these strains for application in soils rich in organic P (Singh et al., 2014b; Hanif et al., 2015).

Organic acid production by rhizosphere bacteria is a well-documented mechanism for mineral phosphate solubilization. Among the detected organic acids, acetic acid and gluconic acid were predominantly produced by the studied PSB strains. Tahir et al. (2013) also reported significant production of acetic acid by PSB isolated from wheat, but these strains did not show gluconic acid production. However, in our study, two most efficient P-solubilizing strains, *Pseudomonas* sp. MR7 and *Acinetobacter* sp. MR5, predominantly produced gluconic acid. Suleman et al., 2018 supported our finding that most prominently detected acid is gluconic acid (102 μg mL^{-1}) by *Pseudomonas* strain isolated from wheat while rice rhizosphere associated PSB reported in the current study, also produced significant amount of gluconic acid (130 μg mL^{-1}) detected during P solubilization.

Otieno et al. (2015) recently described the genetic systems in *Pseudomonas* which is thought to be responsible for gluconic acid mediated phosphate solubilization solubilizing phosphate. It has been reported that glucose dehydrogenase along with the pyrroloquinoline quinone (PQQ) cofactor are required for the conversion of glucose to gluconic acid (Sashidhar and Podile, 2010). In literature, most of the *pqq* operon and *gcd* primers were reported for *Pseudomonas* spp. *Gcd* gene of *Pseudomonas putida* MR7 was amplified using already reported primers. *PqqE* and *pqqC* were not amplified using reported primers, therefore, new primers were designed for *pqqE* and *pqqC* genes against *Pseudomonas putida*. *PqqE* gene was successfully amplified in MR7. But *pqqC* gene was not amplified in repeated PCR experiments even with newly designed primers. This indicates further need for optimization of *pqqC* primers and/or PCR conditions.

No reports are yet available for gene specific primers i.e. *gcd*, *pqqE* and *pqqC* from *Acinetobacter soli*, for detection of glucose dehydrogenase activity. *In silico* PCR analysis as well as no amplification using reported primers in *Acinetobacter soli* MR5 (that produced significant amount of gluconic acid) showed that there is difference in its gene sequence of *gcd*, *pqqE* and *pqqC*. It is worth mentioning that we successfully targeted the *pqqE, pqqC* and *gcd* gene sequences by using newly designed gene specific primers for *Acinetobacter soli* strain MR5. This study expands our knowledge about the genetics of gluconic acid production by *Acinetobacter soli.*

Besides P solubilization, other plant growth promoting attributes suggested that the isolated bacteria not only promote rice growth directly by facilitating nutrient acquisition through the production of organic acids and siderophores, but also by fixing nitrogen and modulating phytohormone levels (Acuña et al., 2011; Ribeiro et al., 2018).

Previously no detailed root morpho-parameters of rice were studied in response to PSB inoculation. *In vitro* plate assay for rhizoscanning of inoculated rice seedling was carried out to study the effect of PSB on root architecture. *Pseudomonas* sp. MR7 showed maximum seed germination rate (100%) and the corresponding inoculated seedlings exhibited shorter primary roots compared to the uninoculated control. Similar characteristics were observed for MR5 and MR13, suggesting that the high P-solubilizing activities of the inoculated PSB may be accompanied by microbe-induced changes in rice root system. An increased root diameter may promote plant growth and development, since thicker roots penetrate more easily into soil, anchor plants more effectively, and yield a higher capacity for water and nutrient transport (Naureen et al., 2017). All root parameters are important for P acquisition especially root length, root diameter, root volume and projection area (Dobbelaere et al., 1999; Contesto et al., 2008). Increase in root hairs was key parameter observed in response to PSB inoculation. One major difference was observed in root length depending upon type of inoculated bacteria like in *Pseudomonas* root length was higher as compared to other strains, while in *Acinetobacter,* small and thick roots were observed.

In planta evaluation of PSB in pots showed a positive effect with an increase in grain weight ranged from 40 to 55% in plants inoculated with *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7, respectively (Table S3) even though the amount of recommended fertilizer dose was reduced by 20%. *Acinetobacter calcoaceticus and Acinetobacter Baumannii* have been used for pearl millet and rice (Zamin et al., 2011) but *Acinetobacter soli* has not been reported as P solubilizer for rice crop. Furthermore, significant increase in plant P contents (seed and straw of MR5 and MR7 treated plants) was observed compared to the uninoculated control. P content of seeds was maximum as compared to that of the straw at the time of harvesting. It may be due to translocation of P from shoot to seed as previously reported (Hedley et al., 1994). In our present study, P content at the harvesting stage in rice shoot and seed was 0.7 g kg^{-1} and 2.2 g kg^{-1}, respectively. These findings also supported by previous reports that P contents in the rice shoot was 3.5 g kg^{-1} after 19 days of sowing and decreased to 0.7 g kg^{-1} at harvest. The P contents in the grain of rice was about 2 g kg^{-1} (Fageria et al., 2013). Inoculation with *Acinetobacter* sp. has also documented for significant increase in wheat P content by 27% at 15 days after emergence (Ogut et al., 2010). Significant increases of plant P uptake after PSB inoculation were also reported for chickpea (Singh et al., 2014a), Aloe vera (Gupta et al., 2011), sorghum (Jisha and Alagawadi,1996), walnut seedlings (Yu et al., 2011), mung bean (Walpola and Yoon, 2013). Our results contribute to this increasing number of reports on PSB-improved plant P nutrition.

Available P in soil was quantified to estimate the amount of P, which can become available for plant uptake (Liebisch, 2011), and an increase of this P fraction after PSB inoculation, particularly for MR5 and MR7, was observed. Whether these changes were related to direct PSB activity, or/and indirectly by an increase in lateral root growth. Secondly, due to their phytase activity these three strains may also convert organic P complexes in the soil into available P form for plant uptake.

The survival and colonization of rice roots by the two most promising strains *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 were confirmed by FISH, viable count and BOX-PCR (Basheer et al., 2016; Yasmin et al., 2017). After successful re-isolation of these strains from the roots, by using colony counts, a significant increase in bacterial population was observed in the rice rhizosphere. Moreover, viable count, plant growth promoting traits and specific banding pattern of re-isolated bacteria obtained with BOX-PCR in comparison with those of the pure culture of inoculated strain confirmed the survival and viability of PSB. These traits of PSB enhanced their scope to be used as inoculant for rice.

After detailed *in vitro* and *in vivo* characterization of potential PSB strains, there is a need to test the biosafety aspects before field application. Based on preliminary tests, MR5 and MR7 did not show hemolytic activity on blood agar plates (unpublished data) but these strains will be subjected to other toxicity tests to confirm the biosaftey aspect of the potential bioinoculant before commercial release for P deficient fields.

Our result provide evidence that *Acinetobacter soli* and *Pseudomonas putida* improved the plant P uptake may be due to *glucose dehydrogenase* gene, in particular *gcd, pqqE and pqqC*. To validate this evidence in the future, *pqq* and *gcd* mutants of *Acinetobacter* and *Pseudomonas* will be explored for plant growth promoting effects of these mutants as compared to wild type strain treated plants. "*Acinetobacter soli* reported first time in the present study for rice to solubilize phosphate, supports the possibility of using *Acinetobacter* for agricultural purposes especially in P deficit areas".

5. Conclusions

The study is of prime significance because of global economic constraint of P and soil available P is deficient in most of the agricultural fields. We need to develop an efficient cost effective, environmentally safe bioinoculant for plants to get maximum output with minimum input of chemicals. To the best of our knowledge, this study is for the first-time reporting *Acinetobacter soli* as potent P solubilizer for Basmati rice. Glucose dehydrogenase (*gcd*) gene containing phosphobacteria *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 were identified as promising strains for P biofortication and enhanced Basmati rice production. Colonization studies and the presence of other multifaceated PGPR traits also strongly authenticated *Acinetobacter* sp. MR5 and *Pseudomonas* sp. MR7 as potential candidate for this smart approach to increase P content in soil and plant. This study has laid a solid and significant foundation for further research to study the genetics/ P-solubilization mechanism of PSB and offers a basis for the application and development of eco-friendly, high-yield agriculture.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.03.004.

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