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Mohibul A. Khan

Eff Haque

Narayan C. Paul

Abdul Khaleque

Saleh M S Al-Garni

See next page for additional authors

Authors

Mohibul A. Khan, Eff Haque, Narayan C. Paul, Abdul Khaleque, Saleh M S Al-Garni, Mahfuzur Rahman, and Tofazzal Islam

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Enhancement of Growth and Grain Yield of Rice in Nutrient Deficient Soils by Rice Probiotic Bacteria

Md Mohibul Alam KHAN[1, 2], Effi HAQUE[1], Narayan Chandra PAUL[3], Md Abdul KHALEQUE[2], Saleh M. S. AL-GARNI[4], Mahfuzur RAHMAN[5], Md Tofazzal ISLAM[1]

([1]*Department of Biotechnology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh;* [2]*Department of Biochemistry & Microbiology, North South University, Dhaka 1229, Bangladesh;* [3]*Department of Rural Development Administration, Bioenergy Crop Research Institute, Muan 534833, Republic of Korea;* [4]*Department of Biological Sciences, King Abdulaziz University, Jeddah 2589, Kingdom of Saudi Arabia;* [5]*West Virginia University, Morgantown, West Virginia 26505, USA*)

Abstract: Plant associated bacteria are promising alternatives to chemical fertilizers for plant growth and yield improvement in an eco-friendly manner. In this study, rice associated bacteria were isolated and assessed for mineral phosphate solubilization and indole-3-acetic acid (IAA) production activity *in vitro*. Six promising strains, which were tentatively identified as phylotaxon *Pseudochrobactrum* sp. (BRRh-1), *Burkholderia* sp. (BRRh-2), *Burkholderia* sp. (BRRh-3), *Burkholderia* sp. (BRRh-4), *Pseudomonas aeruginosa* (BRRh-5 and BRRh-6) based on their 16S rRNA gene phylogeny, exhibited significant phosphate solubilizing activity in National Botanical Research Institute phosphate growth medium, and BRRh-4 displayed the highest phosphate solubilizing activity, followed by BRRh-5. The pH of the culture broth declined, resulting in increase of growth rate of bacteria at pH 7, which might be due to organic acid secretion by the strains. In presence of *L*-tryptophan, five isolates synthesized IAA and the maximum IAA was produced by BRRh-2, followed by BRRh-1. Application of two most efficient phosphate solubilizing isolates BRRh-4 and BRRh-5 by root dipping (colonization) of seedling and spraying at the flowering stage significantly enhanced the growth and grain yield of rice variety BRRI dhan-29. Interestingly, application of both strains with 50% of recommended nitrogen, phosphorus and potassium fertilizers produced equivalent or higher grain yield of rice compared to the control grown with full recommended fertilizer doses, which suggests that these strains may have the potential to be used as bioinoculants for sustainable rice production.

Key words: indole-3-acetic acid; mineral phosphate solubilization; rice; plant growth promotion; plant associated bacterium; grain yield; fertilizer

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population, making it the most important cereal crop. With the increase of world's population, the global rise in rice consumption portends an increased pressure on our dwindling agricultural land. It has been reported that to feed the fast growing world's population, annual cereal production will need to rise to about 3.0 billion tons by 2050 (FAO, 2009) from around 2.6 billion tons today (FAO, 2017). To meet this demand, high-yielding varieties are being developed, which require extensive application of fertilizers such as nitrogen (N) and phosphorus (P) (Hazell, 2010). As currently practiced, an additional 40 and 20 million metric tons of chemical N and P fertilizers, respectively, will be required for food production by 2040 (Gregory et al, 2010). The alarming increase in synthetic chemical fertilizer has led to degradation in soil, deterioration in

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Corresponding author: Md Tofazzal ISLAM (tofazzalislam@yahoo.com)

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air and water quality, which threatens the environmental sustainability (Tilman et al, 2001). Clearly, there is an urgent need to develop efficient, sustainable and green crop production systems for future use.

Phosphorus is an essential macro-nutrient for plant growth and development. Despite a high level of total P content in most agricultural soils, only 0.1% of it exists in soluble form for plant uptake (Richardson and Simpson, 2011). Therefore, costly and environment-degrading P fertilizers are applied in modern cropping systems to maintain P balance in soil for plant nutrition. However, almost 75%–90% of applied chemical P fertilizers are rapidly immobilized by forming complex with Al^{3+} or Fe^{3+} in acidic soils or with Ca^{2+} in calcareous soils (Stevenson, 1999; Islam and Hossain, 2012), resulting in shortage of available P for plant nutrition (Merbach et al, 2010). Farmers, therefore, apply several folds excess of P fertilizer. However, rapid depletion of global sources of rock P (Steen, 1998), rapidly increasing costs of inorganic P fertilizers and growing demands for organic foods have led to search for alternatives to environment-degrading synthetic P fertilizers.

Free-living plant-associated bacteria that may directly or indirectly exert beneficial effect on plant growth and development are generally referred as plant probiotic bacteria (Glick, 2012; Islam and Hossain, 2012). They are well-known to enhance plant growth and improve yield by increasing plant nutrient use efficiency by solubilization and mineralization of nutrient components particularly, mineral P (Sarkar et al, 2012), N-fixation (Islam et al, 2016), and synthesis of phytohormones such as indole-3-acetic acid (IAA) (Islam et al, 2016; Khan et al, 2016). Thus, a significant decrease in the use of chemical fertilizers could be achieved by applying plant-associated probiotic bacteria as bio-inoculants, which is an eco-friendly promising alternative to costly and environment-degrading industrial fertilizers (Rodríguez and Fraga, 1999; Islam and Hossain, 2012; Khan et al, 2016). Therefore, the objective of current study was to assess the role of native rice probiotic bacteria in order to reduce the use of chemical fertilizers without compromising with the growth and yield of rice under nutrient poor soil conditions.

MATERIALS AND METHODS

Experimental sites

The plant and soil samples were collected for isolation of bacteria from the experimental farm of Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh (24.75º N and 90.50º E), Bangladesh and the field laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur (24.09º N and 90.25º E), Bangladesh.

Collection of plant materials and isolation of bacteria

Seed samples of rice varieties BINA dhan-4, BINA dhan-5, BINA dhan-6, BINA dhan-7, BINA shail, Iratom-24, Kalijira and root samples along with rhizosphere soils of BINA dhan-7 and Paijam were collected from the experimental sites for bacterial isolation. However, seeds of rice variety BRRI dhan-29 were collected from the field laboratory of BSMRAU to use in pot experiment.

To isolate endophytic bacteria [*Pseudochrobactrum* sp. (BRRh-1), *Burkholderia* sp. (BRRh-2), *Burkholderia* sp. (BRRh-3), *Burkholderia* sp. (BRRh-4), *Pseudomonas aeruginosa* (BRRh-5 and BRRh-6)], 2–5 g seed samples were washed thoroughly with distilled water and rinsed in 70% ethanol for 5 min, followed by 5 times washing with distilled water, and then surface sterilized with 1% NaClO for 1 min followed by 5 times washing with sterilized distilled water, and finally the tissue was rinsed in 100% ethanol for 1 min followed by 5 times washing with sterilized distilled water. The tissue was then aseptically macerated with homogenizers. For rhizoplane bacteria isolation, root samples were washed thoroughly with sterilized distilled water and then homogenized by a vortex mixture for 1 min in 20 mL of sterile distilled water in a sterile test tube. To isolate rhizobacteria, rhizosphere soil samples (1 g) were added to sterile distilled water (10 mL). Serial dilutions (up to 10^{-9}) of each resulting suspension were made, and exactly 100 µL aliquot from each dilution was spread on nutrient agar plates and incubated at 25 ºC for 48 h (Sarkar et al, 2012). Morphologically distinct colonies were purified by repeated streak culture on the same medium. The purified bacterial isolates were then stored in 20% glycerol solution at -20 ºC for further study.

Biochemical characterization of isolated bacteria

The gram reaction was conducted as described by Vincent and Humphrey (1970). A number of biochemical tests were performed to characterize the isolated bacteria following the criteria of Bergey et al (1994). For testing KOH solubility, bacterial isolates

were mixed with 3% KOH solution on a clean slide for 1 min and observed for a thread-like mass formation. Catalase and oxidase tests were done as described by Hayward (1960) and Shekhawat et al (1992), respectively.

DNA extraction, 16S rRNA gene amplification and phylogenetic analysis of isolated bacteria

Bacterial DNA was extracted by lysozyme-SDS-phenol chloroform method using phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol (Maniatis et al, 1982). The extracted DNA was then treated with DNase-free RNase (Sigma Chemical Co., St. Louis, MO, USA) at a final concentration of 0.2 mg/mL at 37 ºC for 15 min. The DNA was further extracted with phenol chloroform-isoamyl alcohol and precipited with isopropanol. Lastly, the DNA pellet was suspended in Tris-EDTA buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0), preserved at -20 ºC, and used in PCR as template DNA for 16S rRNA gene amplification.

The 16S rRNA gene was amplified using universal primer (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach et al, 1992). Amplification was performed in a thermo cycler (Mastercycler® Gradient, Eppendorf, Hamburg, Germany) at 94 ºC for 5 min, followed by 30 cycles of 40 s at 94 ºC, 40 s at 55 ºC and 1 min at 72 ºC with a final extension at 72 ºC for 10 min. A 5 μL aliquot of each PCR amplicon was electrophoresed on 1.5% agarose gel in 0.5× Tris-Borate-EDTA (TBE) buffer at 100 V for 40 min, stained with ethidium bromide for 20 min and visualized under a UV transilluminator (BioDoc-IT system, Japan). Amplified products were purified using Quick PCR purification column (Promega, Madison, WI, USA) and sequenced using the Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Forster City, CA, USA) in an ABI Prism® 310 Genetic Analyzer (Applied Biosystems). The sequences were compared with the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov) using the BLASTN search, and reference sequences were retrieved to perform phylogenetic analyses. Multiple sequence alignment was carried out using the CLUSTALW Multiple Alignment program in BioEdit version 7.2.3 (Hall, 1999), and gaps were edited manually. A phylogenetic tree was constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) in the MEGA version 5.2.2 software package (Tamura et al, 2011). The pair wise evolutionary distances were calculated using the Kimura 2-parameter model (Kimura, 1980). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1 000 replicates.

Bioassays for plant growth promoting traits

Screening for inorganic phosphate solubilization by isolated bacteria on agar assay

All bacterial isolates were screened for their mineral phosphate solubilizing activity using National Botanical Research Institute's phosphate (NBRIP) growth medium supplemented with 1.5% Bacto-agar (Difco Laboratories, Detroit, MI, USA) (Nautiyal, 1999). Each bacterial isolate was inoculated in triplicate on the NBRIP agar medium and incubated at (25 ± 2) ºC for 72 h. The ability of the bacteria to solubilize insoluble tricalcium phosphate (TCP) was determined in terms of phosphate solubilization index (PSI) [PSI = A / B, where A is the total diameter (colony + halo zone) and B is the colony diameter (Islam and Hussain, 2012).

Quantification of phosphate solubilizing activity in broth

Erlenmeyer flasks (100 mL) containing 40 mL NBRIP broth (pH 7.0) were inoculated with bacterial isolates approximately 1×10^9 cfu/mL. An uninoculated sterile NBRIP medium (pH 7.0) was served as control. After 72 h incubation at (25 ± 2) ºC in a shaker at 160 r/min, cultures were centrifuged at 10 000 r/min for 10 min. The supernatants were decanted and filtered through whatman filter paper (Grade 1) to discard thick polysaccharide-like exudates. The filtrates were assayed for available P, using phospho-molybdate blue complex colorimetric method by measuring the absorbance at a wavelength of 660 nm (Sarkar et al, 2012). The amount of P solubilized was estimated by subtracting the available P of the inoculated sample from the corresponding uninoculated control (Oliveira et al, 2009). Each treatment was replicated three times and data were expressed as the mean value ± standard error.

Bacterial growth and pH value changes of culture broth

Growth of phosphate solubilizing bacterial isolates at different pH, their ability to change the pH value of the culture broth was observed according to Islam et al (2007). Briefly, bacterial isolates were inoculated separately in sterile Erlenmeyer flasks (50 mL) containing 20 mL NBRIP broth medium in a range of pH 3 to 7

and grown in a shaking incubator (100 r/min) for 8 d at (25 ± 2) ºC. At 48 h intervals, the optical density and pH of the culture medium were recorded using a spectrophotometer (ALPO, Germany) (at 595 nm) and a pH meter (Horiba, B-212, Kyoto, Japan), respectively. Each treatment had three replications and data were expressed as the mean value ± standard error.

Determination of IAA production

IAA production by six bacterial isolates was determined according to Gutierrez et al (2009) with little modification. Briefly, the isolated colonies were grown in 50 mL sterilized Jensen's broth (20 g/L sucrose, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 0.1 g/L $FeSO_4$, 0.005 g/L $NaMoO_4$ and 2 g/L $CaCO_3$) (Bric et al, 1991) containing 1 mL of 0.2% *L*-tryptophan and incubated at (25 ± 2) ºC for 72 h with continuous shaking (100 r/min) along with an uninoculated medium as control. The cultures were centrifugated at 12 000 r/min for 10 min and 1 mL clear supernatant was mixed with 2 mL Salkowsky's reagent (50 mL of 35% perchloric acid and 1 mL of 0.05 mmol/L $FeCl_3$ solution) (Gordon and Weber, 1951). The mixture was incubated in the darkness for 30 min at room temperature. Development of visible light to dark pink color indicated the IAA production and absorbance at 530 nm through a spectrophotometer was recorded, and IAA content was calculated from a standard curve of authentic IAA. The data presented were mean value ± standard error.

Effects of probiotic bacteria on growth and yield of rice grown in nutrient deficient soil

In order to investigate plant growth promotion ability of two most efficient phosphate solubilizing isolates BRRh-4 and BRRh-5, a pot experiment was carried out using rice variety BRRI dhan-29 on the roof of Faculty of Graduate Studies at BSMRAU, Salna, Gazipur, Bangladesh, during February to May, 2014. Soil from Salna series in Madhupur tract (AEZ 28), which has been categorized as 'swallow red brown terrace soil' in the Bangladesh soil classification system and falls under the order Inceptisol (Brammer, 1978), was used. The soil is clayey up to 50 cm depth (Islam et al, 2016) and slightly acidic (pH 6.41). Soil contained 1.55% organic matter, 0.08% total nitrogen (N), 9 mg/kg available P and 5.7 mg/kg soil exchangeable potassium (K).

Preparation of soils and fertilization of the pots

Chemical fertilizers (2.10, 0.86, 0.46, 0.78 and 0.01 g of urea, gypsum and zinc sulphate per 10 kg soil, respectively) were applied as recommended by the fertilizer recommendation guide (FRG) for rice variety BRRI dhan-29. The full doses of triple superphosphate (TSP) and muriate of potash (MoP) were applied as basal dose during pot filling, while urea was applied in three equal installments as top dressing at 20 d after transplanting, during panicles initiation, and at the flowering stage. Cultural operations like irrigation and weeding were done when required.

Seedling raising and transplanting

Seeds of BRRI dhan-29 were collected from the Bangladesh Agricultural Research Institute. Germinated seeds were sown directly in the nursery bed at BSMRAU farm. The seedbed was lightly irrigated regularly to ensure proper growth and development of the seedling. Forty-five-day-old seedlings (3/4-leaf stage) were transplanted to treat and were prepared experimental pots at the rate of two seedlings per pot (20 cm × 20 cm × 30 cm) and were treated with appropriate bacterial suspension.

Design and treatment of pot experiment

Pot experiment was conducted in a complete randomized design with three replications for each treatment. The treatments of the experiment included untreated control (I); treatment with BRRh-4 (II), BRRh-5 with zero (III), half and full doses of recommended N, P and K fertilizers. Plant growth parameters such as root and shoot length (cm), root and shoot dry weight (g), SPAD value of flag leaf at the panicle initiation stage, number of tillers and effective tillers per plant, and total grain weight (g) per pot were recorded.

Bacterial inocula preparation and application to rice

Two most efficient P solubilizer strains, BRRh-4 and BRRh-5, were cultured separately in 250 mL conical flasks containing 200 mL nutrient broth on an orbital shaker with 120 r/min at (28 ± 2) ºC for 72 h, and cells were collected after centrifugation at 15 000 r/min for 1 min at 4 ºC, followed wash with sterilized distilled water by two times. For root colonization, seedlings were uprooted and washed thoroughly, followed by overnight dipping the seedling roots into appropriate bacterial suspension in sterilized distilled water (1×10^9 cfu/mL). Freshly harvested bacterial cells were suspended in sterilized distilled water and then sprayed on rice plants at the tillering and flowering stages.

Table 1. Biochemical and molecular characterization of rice probiotic bacteria isolated from different sources.

Strain	Source of isolation	Biochemical analysis				Molecular analysis		
		KOH test	Gram reaction	Catalase test	Oxidase test	Accession No.	Closest strain from GenBank	Sequence similarity (%)
BRRh-1	Surface sterilized seeds of Kalijira	+	–	+	+	KF937789	*P. saccharolyticum* RD_AZIDI_13	99
BRRh-2	Rhizosphere soil of Paijam	+	–	+	+	KF921289	*Burkholderia* sp. MSh1	99
BRRh-3	Rhizoplane of BINA dhan-7	+	–	+	+	KF974366	*Burkholderia* sp. SAP27_1	100
BRRh-4	Rhizosphere soil of BINA dhan-7	+	–	+	+	KF921290	*Burkholderia* sp. SAP53_1	99
BRRh-5	Rhizosphere soil of Paijam	+	–	+	+	KF937787	*P. aeruginosa* 147	99
BRRh-6	Rhizoplane of BINA dhan-7	+	–	+	+	KF937788	*P. aeruginosa* 147	99

'+' indicates positive response; '–' indicates negative response.

Statistical analysis

The data were statistically analysed using SPSS version 17.0 and Statistix version 8.1. Data obtained under various treatments were compared via ANOVA using the least significant difference (LSD) test at the 5% significance level.

RESULTS

Isolation, biochemical and molecular characterization of bacteria

Eighty bacterial strains were isolated from rice seeds, roots and rhizosphere soils, and purified by repeated streak culture on nutrient agar medium. Six isolates viz. BRRh-1, BRRh-2, BRRh-3, BRRh-4, BRRh-5 and BRRh-6 were selected based on their inorganic P solubilization ability in a preliminary screening. All the six isolates were negative to Gram, but positive to the KOH, catalase and oxidase tests (Table 1).

Based on phylogenetic tree constructed from 16S rRNA sequences showed that the selected strains were members of the genera *Burkholderia*, *Pseudomonas*, and *Pseudochrobactrum* (Fig. 1). After the BLASTN search at GenBank database of NCBI, and the sequence of BRRh-1 was deposited in the GenBank under the accession number KF937789 showed 99%

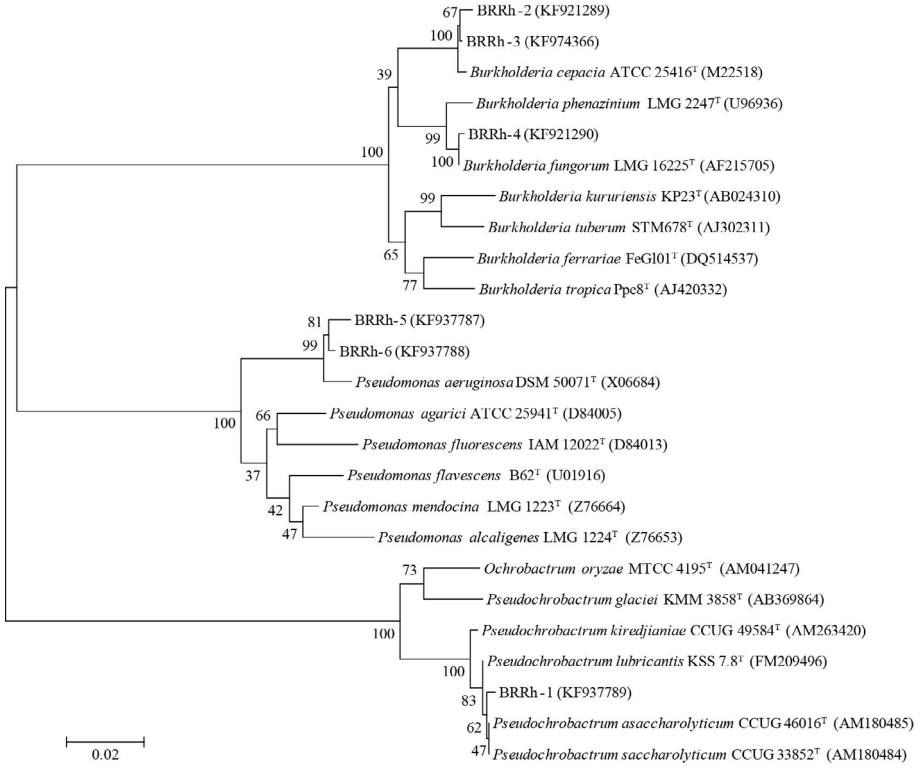

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence.

The significance of each branch is indicated by a bootstrap value based on 1 000 replications.

T indicates type strain.

sequence homology with *Pseudochrobactrum saccharolyticum* RD_AZIDI_13 (Table 1). The sequences of the isolated strains BRRh-2, BRRh-3 and BRRh-4 were submitted to GenBank under the accession numbers KF921289, KF974366, and KF921290, respectively, and had 99%, 100% and 99% similarities with *Burkholderia* sp. MSh1, *Burkholderia* sp. SAP27_1, and *Burkholderia* sp. SAP53_1 (Table 1). However, the isolates BRRh-5 and BRRh-6 were deposited in the GenBank with accession numbers KF937787 and KF937788, respectively, and exhibited 99% sequence homology with *P. aeruginosa* 147 (Table 1).

Characterization for plant growth promoting traits of the isolated bacteria

Screening and quantitative estimation of phosphate solubilization

Out of eighty isolates, only five exhibited halozone on NBRIP agar medium. PSI of five isolates was BRRh-3, BRRh-4 and BRRh-5 (Table 2 and Fig. 2). Among them, BRRh-2 (Fig. 2-A) and BRRh-4 (Fig. 2-C) displayed the lowest and the highest P solubilizing activity, respectively. However, six bacteria exhibited P solubilization ability ranged from 74 to 92 μg/mL in broth culture, and the maximum P (92 μg/mL) was solubilized by BRRh-4 followed by BRRh-5 (87 μg/mL) (Table 2). Although five isolates solubilized P in both agar and broth assays, interestingly, BRRh-1, which did not solubilize P in agar medium and solubilized significant amount of P (74 μg/mL) in broth culture (Table 2).

Growth of bacteria and pH changes of culture broth

All the six isolates were grown in NBRIP broth at pH 3 to 7 for 8 d to observe their growth (optical density) and the changes of pH value in culture medium (Fig. 3). All the isolates could grow well at pH 5 and 7 and displayed optimal growth at pH 7, but insignificantly at pH 3 (Fig. 3-A). As time goes on, the culture broth of the bacteria at pH 7 decreased significantly, but change was nominal when grown at pH 5 (Fig. 3-B).

Table 2. Plant growth-promoting traits of rice probiotic bacteria (Mean ± SE, *n* = 3). μg/mL

Isolate	Phosphate solubilization		IAA production
	PSI in agar assay	PSI in broth assay	
BRRh-1	–	74.0 ± 1.0	25.0 ± 0.1
BRRh-2	3.0 ± 0.1	83.0 ± 2.0	34.0 ± 0.1
BRRh-3	4.0 ± 0.0	86.0 ± 1.0	23.0 ± 1.0
BRRh-4	5.0 ± 0.1	92.0 ± 2.0	9.0 ± 1.0
BRRh-5	4.0 ± 0.1	87.0 ± 1.0	8.0 ± 1.0
BRRh-6	4.0 ± 0.1	85.0 ± 2.0	–

PSI, Phosphate solubilization index; IAA, Indole-3-acetic acid.
PSI = (Halo zone + Colony diameter) / Colony diameter.

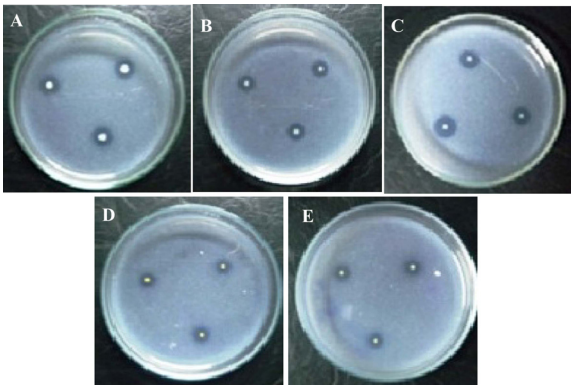

Fig. 2. Phosphate solubilization assay of BRRh-2 (A), BRRh-3 (B), BRRh-4 (C), BRRh-5 (D) and BRRh-6 (E) on National Botanical Research Institute's phosphate agar plate containing tricalcium phosphate (TCP).

Halozone indicates inorganic phosphate solubilization ability of the isolates.

Production of IAA

All the six strains, except for BRRh-6, produced 8–34 μg/mL IAA in presence of *L*-tryptophan. IAA production was optimum for BRRh-2 (34 μg/mL), followed by BRRh-1 (25 μg/mL), and BRRh-5 synthesized the lowest amount of IAA (8 μg/mL) (Table 2).

Promotion of growth and yield of rice variety BRRI dhan-29

Two most efficient P solubilizers BRRh-4 and BRRh-5 were tested for their ability to improve growth and grain yield of rice variety BRRI Dhan-29 in pot culture, and both of them improved all measured plant growth parameters than the untreated controls (Fig. 4 and Supplemental Fig. 1). Application of full dose of fertilizer to the plants treated with BRRh-4 produced the highest shoot and root lengths (85 and 46 cm, respectively), followed by BRRh-5 (80 and 43 cm, respectively), which were significantly higher than the un-inoculated control (77 and 39 cm, respectively) grown with the same doses of fertilizers (Fig. 4-A and -B). Interestingly, about 13% and 5% increases of root length were observed in BRRh-4 (44 cm) and BRRh-5 (41 cm) treated plants with half fertilizer doses, respectively, compared to the untreated control which received full fertilizers (Fig. 4-B). However, shoot dry weight increased more in BRRh-4 treated plants grown with full fertilizer (26 g) over similarly grown untreated control (25 g) (Fig. 4-C). Similarly, treatments with BRRh-4 and BRRh-5 along with full fertilizers significantly enhanced root

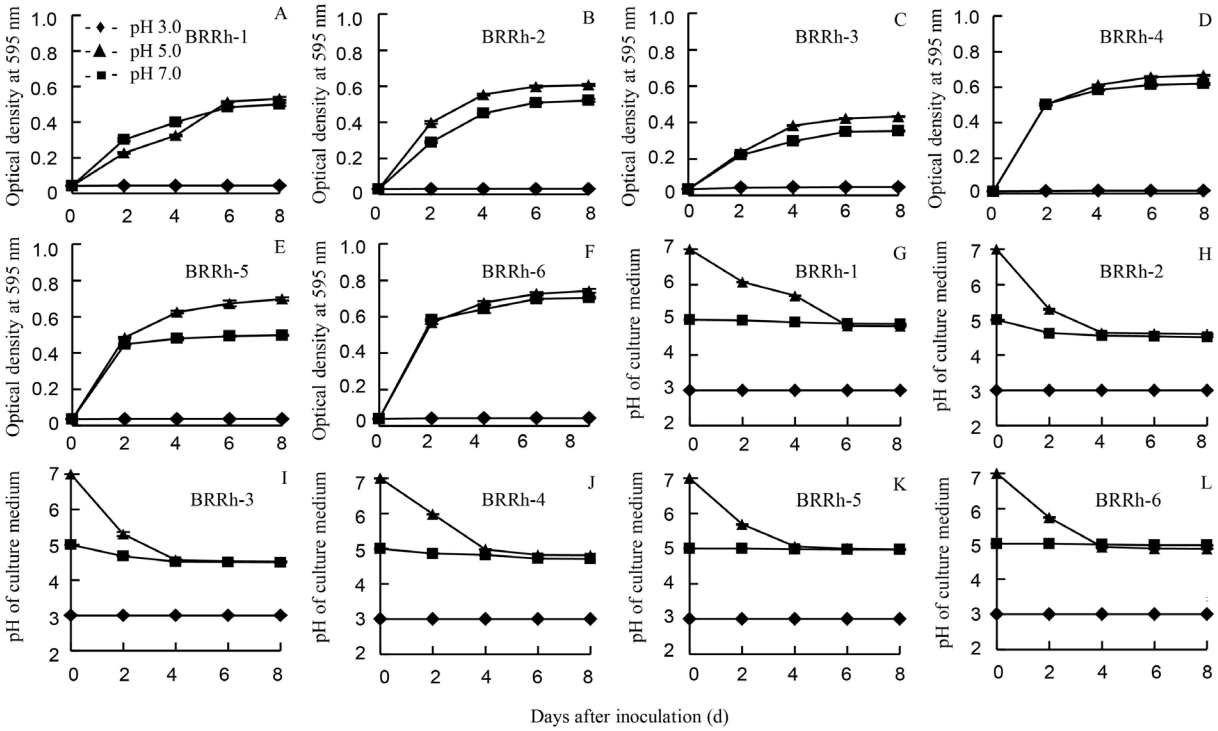

Fig. 3. Changes of the bacterial growth represented by optical density at 595 nm (A–F) and pH value of culture medium (G–L) with time of inoculation in National Botanical Research Institute's phosphate growth medium (Mean ± SE, *n* = 3).

dry weight (20 and 19 g, respectively), compared to un-inoculated control cultivated with similar fertilizer doses (16 g) (Fig. 4-D).

Pronounced improvement in SPAD value, tiller number per pot, effective tiller number per pot and grain yield per pot were also recorded from the bacteria treated plants. The maximum SPAD value (50) was observed for BRRh-5, followed by BRRh-4 (47) treatments, which were significant higher over untreated control (37) when they were cultured with

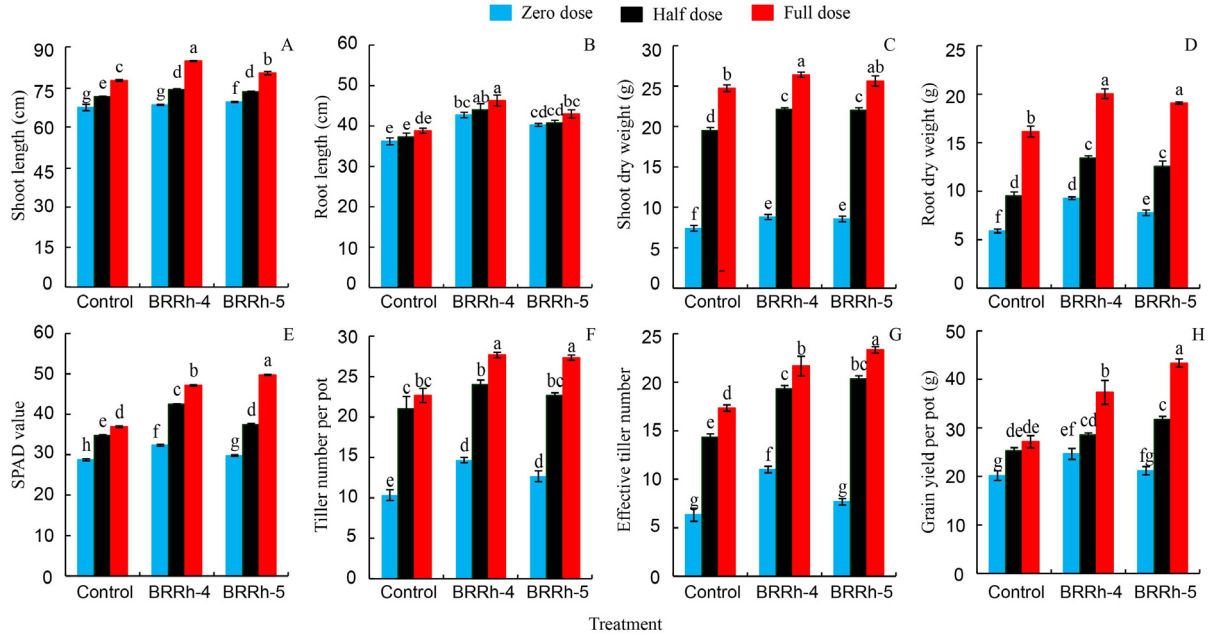

Fig. 4. Effects of BRRh-4 and BRRh-5 along with different fertilizer levels on various growth parameters of BRRI dhan-29.
Values (Mean ± SE, *n* = 3) followed by the same letter(s) in the same graph did not differ significantly at the 0.05 level by the LSD test.

full fertilizer doses (Fig. 4-E). About 15% and 2% higher SPAD values were also noted by BRRh-4 (42) and BRRh-5 (37) along with half fertilizer treatments, respectively compared to the un-inoculated control that received full fertilizer (Fig. 4-E). In addition, application of full fertilizer to BRRh-4 and BRRh-5 treated plants remarkably increased total tillers per pot (28 and 27, respectively), and effective tillers per pot (22 and 23, respectively) over untreated control that were grown with equal fertilizer dose (Fig. 4-F and -G). Interestingly, more than 5% and 11% increases of total tillers per pot (24) and effective tillers per pot (19), respectively, were recorded for BRRh-4 along with half N, P and K fertilizer treated plants, and equal number of total tillers per pot (23) and more than 17% increase of effective tillers per pot (20) were recorded for BRRh-4 and half fertilizer treated plants compared to un-inoculated control that received full fertilizer (Fig. 4-F and -G).

Concomitant to growth parameters, grain yield of rice was also significantly increased in BRRh-5 treated plants, followed by BRRh-4 treated plants compared with untreated control (Fig. 4-H). Interestingly, rice grain yields in BRRh-4 and BRRh-5 treated plants with half reduced fertilizers were 5% and 17%, respectively, higher than the plants treated with full recommended doses of N, P and K fertilizers, which indicated that application of these two bacteria can reduce 50% of major fertilizers in rice productions (Fig. 4-H).

DISCUSSION

Out of 80 bacterial isolates, 6 potential plant probiotic bacteria were identified as *Pseudochrobactrum* sp. (BRRh-1), *Burkholderia* sp. (BRRh-2), *Burkholderia* sp. (BRRh-3), *Burkholderia* sp. (BRRh-4), *Pseudomonas aeruginosa* (BRRh-5 and BRRh-6) on the basis of their 16S rRNA sequences and phylogenetic relationship (Table 1 and Fig. 1).

It was found BRRh-1 that did not show any phosphate solubilizing activity on agar plate, but solubilized a significant amount of tricalcium phosphate (TCP) in broth culture (Table 2). This is consistent with Nautial (1999), who reported that many isolates did not solubilize phosphate on plate but would mineralize phosphate in broth assay. Other, five bacteria consistently solubilize TCP in both agar and broth cultures (Table 2). A number of earlier studies also reported similar results of phosphate solubilization by the bacteria (Nautial, 1999; Islam et al, 2007; Sarkar et al, 2012). Among the isolates, *Burkholderia* sp. (BRRh-4) and *P. aeruginosa* (BRRh-5) were the most efficient TCP solubilizers (Table 2). The abilities of mineral phosphate solubilization by some strains of *Burkholderia* sp. (Oliveira et al, 2009), *B. fungorum* (de Oliveira-Longatti et al, 2014) and *P. aeruginosa* (Young et al, 2013) have also been reported.

All the isolates grew well in slightly acidic conditions (pH 5–7) (Fig. 3-A) would probably be due to the fact that they were isolated from rice grown in upland soils with pH 6.3–6.5 (Islam et al, 2007). Significant decline in pH of the culture broth (pH 7.0) with bacterial growth might be due to secretion of organic acids into the medium (Fig. 3-B). Decrease in medium pH by phosphate solubilizing *Burkholderia* and *Pseudomonas* species has also been observed by several researchers (Jha et al, 2009; Walpola and Yoon, 2013). In fact, secretion of organic acids such as gluconic, 2-ketogluconic, lactic, oxalic, succinic, isovaleric, caproic, isobutyric, acetic, citric acid as a principal mechanism for inorganic phosphate solubilization by plant-associated bacteria has been well documented (Chen et al, 2006; Islam and Hossain, 2012).

Some rice probiotics produced a good amount of IAA in the presence of *L*-tryptophan (Table 2). *In vitro* production of IAA by some phosphate solubilizing strains of *Burkholderia cepacia* (Singh et al, 2013), *B. fungorum* (de Oliveira-Longatti et al, 2014) and *P. aeruginosa* (Oves et al, 2013) have been documented.

Treatment of rice (BRRI dhan-29) seedlings before transplanting and subsequent foliar spray of BRRh-4 and BRRh-5 at the tillering and flowering stages along with varying doses of N-P-K fertilizers significantly promoted growth and grain yield compared to the untreated control (Fig. 4). Interestingly, application of BRRh-4 and BRRh-5 in combination with half doses of the recommended fertilizers displayed equal or higher root length, SPAD value, numbers of tiller per pot and effective tiller per pot, and grain yield of rice compared to the untreated control plants with full doses of N-P-K fertilizers (Fig. 4). These findings indicate that half reduction of N-P-K fertilizers could be achieved by application of the native two probiotic bacterial strains *Burkholderia* sp. (BRRh-4) and *P. aeruginosa* (BRRh-5) without compromising the growth and yield of rice (Fig. 4). Improved growth and grain yield of treated plants might be associated with the better uptake of plant nutrients (Islam and Hussain, 2012), endogenous changes in phytohormone levels

(Kurepin et al, 2015), and other unknown mechanisms exerted by probiotic bacteria. Inoculation with several strains of *Burkholderia* spp. and *P. aeruginosa* was reported to improve plant growth and yield in several crop plants such as rice (Singh et al, 2013), maize (Young et al, 2013) and chickpea (Oves et al, 2013).

CONCLUSIONS

In conclusion, application of rice-associated *Burkholderia* sp. and *P. aeruginosa* with half N-P-K fertilizers produced equivalent or higher grain in pot culture probably through production of IAA, solubilization of phosphates and other unknown mechanisms which needs detailed study to elucidate the underlying mechanisms and evaluation under field conditions to develop effective formulations for rice production with reduced fertilizer requirement.

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SUPPORTING DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Fig. 1. Effect of BRRh-4 and BRRh-5 along with different fertilizer doses on whole plant of BRRI dhan-29.

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