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Full Length Article

Molecular Characterization of Stress Tolerant Plant Growth Promoting Rhizobacteria (PGPR) for Growth Enhancement of Rice

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Abstract

The study was undertaken to characterize plant growth promoting rhizobacteria (PGPR) for growth enhancement and stress tolerant traits and their efficacy on early establishment of rice seedling. *In vitro* growth promoting traits revealed that out of 30 PGPR isolates, 18 fixed nitrogen, 17 solubilized tri-calcium phosphate, 29 and 17 produced IAA with or without addition of L-tryptophane. In case of stress tolerant activities, PGPR isolates tolerated pH ranging from 5 to 10, Nacl from 1 to 6% and polyethylene glycol (PEG) from 10 to 40%, respectively. They showed antagonistic activity against *Pyricularia oryzae* with PIRG values ranging from 7−68%. After two-stage of screening, isolates UPMR7 and UPMR17 were identified based on 16s rRNA gene sequences and matched to the genus *Bacillus* and *Citrobacter* with 97−98% similarity. UPMR 7 and UPMR 17 were further evaluated on early growth promotion of rice variety MR219. Results revealed that PGPR inoculation had significant effects on plant growth compared to non-inoculated plants. Thus, it could be suggested that the isolates UPMR7 and UPMR17 have the potential to be used as biofertilizer and bioenhancer in sustainable rice cultivation.

Keywords: *Oryza sativa*; PGPR; Stress tolerant; Growth promotion

Introduction

Rice is the second most widely grown cereal crop and a primary source of income for millions of Asian. Rice consumption has been increasing over the years due to ever increasing population. Unfortunately, rice productivity is not increasing at the pace required to keep up with the demand due to decreasing soil fertility, poor management of soil resources, build-up of pathogens and accumulation of phytotoxic substances (Pinheiro et al., 2006; Mishra et al., 2014; Pereira and Castro, 2014). Farmers usually rely on agrochemicals to maintain the crop productivity (Wang et al., 2010; Zaman et al., 2010). However, excessive use of agrochemicals to crop fields has been reported to increase nitrate, nitrite, ammonium and phosphate and other reactive chemical species in groundwater and surface water bodies, which causes serious environmental and health hazards (Emilsson et al., 2007; Chandna et al., 2011; Rawat et al., 2010, 2012).

Biofertilizers can boost up crop productivity by minimizing the harmful effects of agrochemicals. Plant performance mostly depends on nutrients availability in soil. Nitrogen and phosphorus are the primary nutrient elements in rice cultivation. However, rice plants have limited ability to get these nutrients from soil. Plant growth promoting rhizobacteria (PGPR) are needed in nutrient recycling to help plant uptake and absorb nutrients at optimal concentration, while plant donates waste by-products as food for microbes (El-kholy *et al.*, 2005).

PGPR are important in biogeochemical cycles including carbon, nitrogen, sulphur and phosphorous. Different PGPR strains such as Bacillus, Citrobacter, Pseudomonas, Rhizobium, Klebsiella, Enterobacter and Burkholderia have been reported to colonize the root of rice plants (Naher *et al*., 2009). They enhance plant growth and improve plant health in stress conditions by various direct and indirect mechanisms (Solano *et al*., 2008). Direct mechanisms include the production of stimulatory volatiles, or phytohormone-like indole acetic acid, lowering ethylene level in plants, improvement of plant nutrient status by liberation of phosphates and micronutrients from insoluble sources, production of siderophores, polysaccharides and organic acids. Indirect mechanisms involve stimulation of defense related mechanisms against infection by pathogens, including induced systemic resistance (ISR) and also induced changes in their physiologies resulting in enhanced

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resistance to pathogens (Jetiyanon and Kloepper, 2002). Potential PGPR perhaps play a vital role in sustaining high crop productivity through growth promotion and stress tolerant traits by minimizing the use of agrochemicals. To best of our knowledge, only few attempts have been made to characterize such indigenous microorganisms living in the rhizosphere of rice plants. Hence, the present study was undertaken to isolate and identify PGPR isolates such as nitrogen fixing and phosphate solubilizing bacteria that have important role in multiple growth promotion and stress tolerance traits and evaluate their efficacy on growth enhancement of rice.

Materials and Methods

Sampling and Isolation of Plant Growth Promoting Rhizobacteria (PGPR) samples were collected from rice fields of Universiti Putra Malaysia, Serdang, Selangor and Semerak, Pasir Puteh, Kelantan, Malaysia. Each sample consisted of 5 cores of soil randomly taken from 5.0 cm depth of rice rhizosphere and pooled together into clean plastic bag. Samples were stored at 4ºC until use.

Isolation was carried out by dilution plate method. Serial dilutions from 10^{-3} to 10^{-8} were prepared by sequentially transferring of 1 mL sample into each test tube containing 9 mL sterile distilled water. A 100 µL of sample at selected dilution was transferred onto Nutrient agar (NA, Difco™). All plates were incubated at 28 ± 2ºC for 24−48 h. Single colony of PGPR was transferred aseptically onto NA media to obtain pure cultures. Pure cultures were kept at 4ºC until required for further studies.

In Vitro Screening for Plant Growth Promoting Traits

Nitrogen fixing activity: Nitrogen fixing ability of PGPR isolates was determined using Jensen's N-free NA medium (Gibson, 1980). Jensen's medium contained (g/L) sucrose 20.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5, F_2 SO_4 0.1, Na_2MoO_4 0.005, CaCO3 2.0 and agar 20.0. Using sterile toothpicks, a loop full of each bacterial culture was placed onto the plate and incubated for 2 days at 30ºC. Halo zone around the colonies indicated the nitrogen fixing potential of PGPR isolates.

Phosphate solubilizing Activity: Phosphate solubilizing activity of PGPR isolates was determined following the method described by Nautiyal (1999). Isolates were tested by using NBRIP medium containing (g/L), glucose 10.0, $Ca_3(PO4)_2$ 5.0, $MgCl_2.6H_2O$ 5.0, $MgSO_4.7H_2O$ 0.25, KCl 0.2 and $(NH)_2SO_4$ 0.1. Using sterile toothpicks, a loop full of each PGPR isolate was placed onto plate and incubated for 2 days at 30ºC. Halo zones (solubilizing zone) around bacterial colonies indicated phosphate solubilization. The potential of PGPR isolates to solubilize insoluble phosphate was also studied by determination of Phosphate Solubilization Index (PSI). PSI was calculated using the following formula (Edi-Premono *et al*., 1996).

$$\text{PSI} = \frac{\text{Colony diameter} + \text{Halo diameter}}{\text{Colony diameter}}$$

Indole-3-Acetic Acid (IAA) Production

IAA produced by PGPR isolates was determined in the presence or absence of L-tryptophan by using Nutrient Both (NB, Difco™)) and Salkowski colorimetric assay (Gutierrez *et al.,* 2009). All PGPR isolates were grown NB on an incubator shaker (150 rpm) at room temperature (28ºC ± 2) for 24 h. After 24 h, 1 mL of bacterial culture were inoculated into 100 mL of sterile NB amended with 5 mL L-tryptophan and without L-tryptophan solution for 48 h. To determine the amount of IAA produced from the isolates, 1.5 mL of aliquot was sampled and centrifuged at 12,000 rpm for 5 min. One mL of the supernatant was added to 2 mL of Salkowski's reagent. After 25 min of incubation the color density of the mixtures were read using UV-spectrophotometer (Model UV-3600, Shimadzu) at 530 nm absorbance.

In Vitro Screening for Stress Tolerance

PGPR isolates were tested for pH and NaCl tolerance according to the method described by Hayat *et al*. (2013). The range and optimum pH required for bacterial growth were determined by inoculating each PGPR isolate in NB adjusted to a range of pH 4.0 to 10.0 at an increment of one unit pH. NaCl tolerance test was performed by growing bacterial isolates in PGPR with different concentration of NaCl from 0 to 10% (w/v) at optimum pH. The sensitivity of PGPR isolates to drought was tested by growing PGPR isolates in NB amended with polyethylene glycol (PEG 6000). The culture was then shaken for overnight at 180 rpm and the growth rate was determined (Sandhya *et al*., 2009).

In Vitro Screening for Antagonistic Activity

A 5 mm diameter of *Pyricularia oryzae* (rice blast disease) agar plugs obtained from the periphery of a seven day old colony were transferred to Potato Dextrose Agar (PDA, Difco™) plates and allowed to grow for 2 days before introducing the PGPR isolate. The respective PGPR isolate was spotted approximately 2 cm apart from the pathogen plug. The plates were incubated at 28 ± 2ºC for 5 days. The inhibition zone was measured when the *P. oryzae* mycelium in control plates had reached the edge of the plate. The antagonistic activities of PGPR isolates were evaluated by using percentage inhibition in radial growth (PIRG) of *P. oryzae*, based on the following formula (Jinantana and Sariah, 1997).

r1= radial growth in control plate
r2= radial growth in treatment plate

Identification of Potential PGPR Isolates

Isolates UPMR7 and UPMR17 in terms of their growth promoting, stress tolerance and antagonistic activity were selected for identification by using 16S rRNA gene sequencing.

PGPR cells were grown in 5 ml of NB at 28ºC for 2−3 days until they reached stationary phase. One mL of culture was transferred to 1.5 mL micro centrifuge tube and centrifuged at 12000 *g* for 5 min using a Eppendorf centrifuge 5810R (Hamburg, Germany). Genomic DNA was isolated by using Genomic DNA mini kit (Yeastern Biotech C0., Ltd.). The universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA coding region. The 25 µL PCR reaction mixture comprised of 1 µL of bacterial DNA, 0.15 µM of each primer, 1 × PCR reaction buffer (Fermentas, USA), 0.2 mM of dNTP mix, 2.5 U of Taq polymerase (Fermentas, USA), 25 mM of $MgCl_2$ and top up with sterile distilled water. The thermal cycling condition was: one cycle at 94ºC for 5 min, 30 cycles at 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1 min followed by an additional cycle of 5 min at 72ºC.

The amplified 16S gene fragments were purified using QIAquick Gel Extraction Kit (QIAGEN Inc. USA) and sent for sequencing. The obtained sequences were analyzed by comparison with sequences in GeneBank database by BLAST program and percent similarity was determined. A phylogenetic tree was constructed by using Mega Version 4 Software (Tamura *et al*., 2007) following neighbor-joining method.

Evaluation of PGPR on Early Establishment of Rice Seedling

Two selected PGPR isolates UPMR7 and UPMR17 were tested on rice to verify their growth promoting traits.

Inoculums Preparation

Isolates UPMR7 and UPMR17 were grown in NB for 48 h at 28 ± 2ºC. The cultures were harvested by centrifuging at 10,000 rpm for 10 min. Pellets were suspended in NB and bacterial suspension was adjusted to 10^8 cfu•mL^{-1} (Kausar *et al*., 2011) for plant inoculation.

Plant Inoculation

Rice seeds variety MR219 were obtained from Seri Merbok Sdn. Bhd., Kangkong, Alor Setar, Kedah, Malaysia. Seeds were surface-sterilized by dipping in 95% ethanol for 1 min and in 0.2% $HgCl_2$ solution for 3 min followed by ringing with sterile distilled water (Zahir *et al*., 2009). The seeds were sown in petri dishes containing double wet filter papers for germination. When plumule and radical were emerged, seedlings were treated with PGPR suspensions (10^8 cfu mL^{-1}) or distilled water (as control) for 1 h at room temperature. Seedlings were transplanted in plastic pot filled with 1.0 kg of sterile soil. In inoculated treatments, each plant was further inoculated with 5.0 mL of bacterial suspension at 14 days after transplanting. On day 30, data on plant height, root length, shoot and root fresh and dry weight, chlorophyll a, chlorophyll b and total chlorophyll of leaves were recorded.

Statistical Analysis

All experiments were conducted using completely randomized design (CRD) with five replications. The data were subjected to analysis of variance (ANOVA) and tested for significance using least significant difference (LSD) by PC-SAS software (SAS Institute, Cary, NC, USA, 2001). To group the PGPR isolates based on their plant growth promoting traits, data were subjected to cluster analysis by using NTSYS pc 2.02 software.

Results

Plant Growth Promoting Activity

Table 1 illustrated the plant growth-promoting traits of all PGPR isolates. The ability to grow in N-free media was positively exhibited by most of the PGPR isolates. Among 30 isolates, 18 were found to form halo zone in N-free media.

With regard to phosphate solubilizing activity, 17 isolates were found to solubilize $Ca_3(PO_4)_2$ in NBRIP medium with various PSI which ranged from 1.1 to 2.3. The significantly ($\leq$0.05) highest PSI activity was found in the isolates UPMR7 and UPMR17 with the value of 2.3 which was closely followed by UPMR18 (2.1) and UPMR16 (1.9), respectively. Among the 17 phosphate solubilizing isolates, UPMR19 showed the least PSI activity (1.1) closely followed by UPMR24 (1.2).

PGPR isolates were able to produce IAA. In the presence of tryptophan, 29 PGPR isolates were found to produce IAA ranging from 1.0 to 43.0 µg ml^{-1}. On the other hand, only 17 isolates produced IAA without addition of tryptophan where the IAA production was ranged from 1.0 to 7.5 µg mL^{-1}.

The plant growth promoting potential of 30 PGPR isolates were combined in a clustering tree by using NTSYS software (Fig. 1). The clustering tree gave two major clusters (cluster-1 and 2) where overall group similarity was above 1.78. Both of the clusters included 15 PGPR isolates. All isolates in cluster-1 produced prominent halo zone in Jensen and NBRIP media as well as higher amount of IAA. Conversely, PGPR isolates in cluster-2 showed the least plant growth promoting potential where 12 isolates did not produce halo zone on N-free media.

Stress Tolerance and Antagonistic Activities

Table 2 shows the abiotic stress tolerant and antagonistic traits of selected PGPR isolates. PGPR isolates UPMR2, UPMR3, UPMR7, UPMR16, UPMR17, and UPMR19 showed the highest potential to grow in wide pH ranging from 5 to 10. On the other hand, UPMR9 appeared as the least pH tolerant PGPR isolate with pH ranging from 7 to 8, whereas the others were of moderate pH tolerant. Isolates UPMR2, UPMR7, UPMR17 and UPMR18 showed the highest NaCl tolerance up to 6%, which was closely followed by the isolate UPMR16 grown on medium amended with NaCl up to 5%. UPMR9 was identified as the least NaCl tolerant isolate.

PGPR isolates UPMR7, UPMR8, UPMR18 and UPMR20 showed the highest potential by growing on 40% PEG amended media. The least PEG tolerant isolate was UPMR9. The remaining 10 isolates showed intermediate PEG tolerant potential where isolates UPMR 2, UPMR10, UPMR11, UPMR16 and UPMR18 grew on 30% PEG amended media and the remaining isolates UPMR1, UPMR3, UPMR13, UPMR14 and UPMR17 grew on 20% PEG amended media.

All 15 tested PGPR isolates showed differential antagonistic activity against fungal pathogen, *P. oryzae*. Isolates UPMR7, UPMR9, UPMR11, UPMR17 and UPMR18 tested were good inhibitors against *P. oryzae* with PIRG values of more than 50%. Isolate UPMR17 achieved significantly ($\leq$ 0.05) higher PIRG (67.8%) which was at par with UPMR7 (63.1%), whilst isolate UPMR3 showed the least antagonistic activity (7.0%).

Identification of PGPR Isolates

PGPR isolates UPMR7 and UPMR17 were identified molecularly. The 16S rDNA gene sequences of PGPR isolates were PCR amplified and approximately 1,400bp were sequenced (Fig. 2). Identification of PGPR isolates based on partial 16S rDNA gene sequences is shown in Table 3. The BLASTX sequence analysis revealed that the isolate UPMR7 matches with the *Bacillus* cereus with 97% similarity. On the other hand, the isolate UPMR17 matches with the *Citrobacter farmeri* strain with 98% similarity. The phylogenetic analysis of PGPR isolates were also done based on neighborhood joining tree method with 100 bootstrap sampling (Fig. 3).

Evaluation of PGPR Isolates on Growth Enhancement of Rice Seedling

Based on the results of *in vitro* plant growth promotion, stress tolerance and antagonistic activities two best selected isolates *Bacillus sp* (UPMR7) and *Citrobacter sp* (UPMR17) were evaluated on early establishment of rice seedlings.

Analytical results of shoot and root length, fresh and dry weight, and total chlorophyll content of 30 days old-rice seedlings treated with PGPR isolates showed significantly ($\leq$0.05) higher over control treatment (Table 4). The significantly ($\leq$0.05) tallest shoot (42.33 cm) was registered in plants treated with UPMR7, which was

Table 1: Plant growth promoting activities of bacterial isolates

Isolates	N-fixing ability	P-solubilization index	IAA µg mL^{-1}	
			with tryptophan	without tryptophan
UPMR1	+	1.4 ef	12.2 cg	1.0 l
UPMR2	+	1.3 fg	23.0 b	4.5d
UPMR3	+	1.4 ef	16.5 cd	3.0h
UPMR4	-	0.0 i	15.0 ce	0.0m
UPMR5	-	0.0 i	7.3 hk	0.0m
UPMR6	-	0.0 i	0.0 n	1.0 l
UPMR7	+	2.3 a	27.0 b	3.3g
UPMR8	+	1.3 fg	43.0 a	4.3e
UPMR9	+	1.3 fg	16.0 cd	3.0h
UPMR10	+	1.3 fg	10.0 eh	2.3j
UPMR11	+	1.4 ef	11.0 eh	1.3k
UPMR12	-	0.0 i	2.8 cg	0.0m
UPMR13	+	1.8 cd	10.8 eh	2.3j
UPMR14	+	1.7 d	12.8 cg	2.8i
UPMR15	-	0.0 i	1.8 ln	0.0m
UPMR16	+	1.9 c	23.0 b	4.5d
UPMR17	+	2.3 a	16.0 cd	4.8c
UPMR18	+	2.1 b	24.0 b	3.5f
UPMR19	-	1.1 h	7.3 hk	0.0m
UPMR20	+	1.8 cd	14.0 cf	7.75a
UPMR21	-	0.0 i	6.0 il	0.0m
UPMR22	+	0.0 i	4.5 jn	0.0m
UPMR23	-	0.0 i	1.5 ln	1.0 l
UPMR24	-	1.2 gh	8.0 gj	0.0m
UPMR25	-	0.0 i	2.8 kn	0.0m
UPMR26	+	0.0 i	1.0 mn	0.0m
UPMR27	+	0.0 i	5.2 jm	0.0m
UPMR28	+	1.5 e	16.0 cd	7.0b
UPMR29	-	0.0 i	7.0 hk	0.0m
UPMR30	-	0.0 i	2.2 ln	0.0m

Values having the same letter(s) in a column do not differ significantly at the 5% level of probability

Table 2: *In-vitro* stress tolerance ability of the bacterial isolates

Isolates	pH range	Nacl tolerance (%)	PEG tolerance (%)	Dual culture (PIGR)
UPMR1	7-9	-	10-20	12.0 f
UPMR2	5-10	0-6	10-30	31.6 d
UPMR3	5-10	0-4	10-20	7.0 g
UPMR7	5-10	0-6	10-40	63.1 a
UPMR8	5-8	0-4	10-40	44.0 c
UPMR9	7-8	0-1	10	52.6 c
UPMR10	6-9	0-3	10-30	16.7 e
UPMR11	6-9	0-4	10-30	57.7 b
UPMR13	6-10	0-3	10-20	19.2 e
UPMR14	5-9	0-3	10-20	14.0 ef
UPMR16	5-10	0-5	10-30	11.1 f
UPMR17	5-10	0-6	10-20	67.8 a
UPMR18	5-9	0-6	10-40	59.1 b
UPMR19	5-10	-	10-30	9.0 fg
UPMR20	5-9	-	10-40	11.0 f

Values having the same letter(s) in a column do not differ significantly at the 5% level of probability

Table 3: Molecular identification of Bacterial isolates by 16S rDNA gene sequencing

Isolates	Closest relatives	NCBI accession number	% Similarity
UPMR7	Bacillus cereus strain TBLs6	HQ443236	97
UPMR17	Citrobacter farmeri strain W17-1	JX393004	98

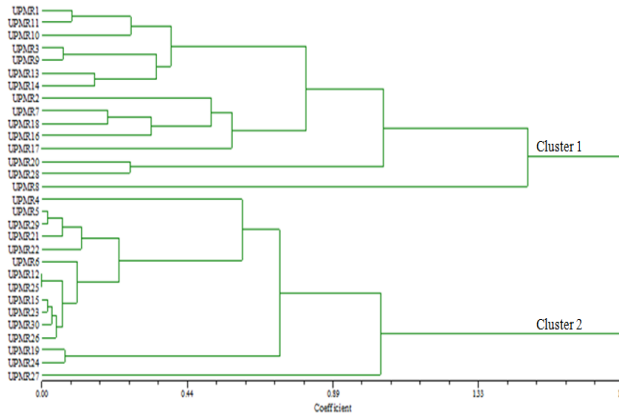

Fig. 1: Dendrogram of 30 bacterial isolates based on their plant growth promoting ability prepared using NTSYS pc 2.02software

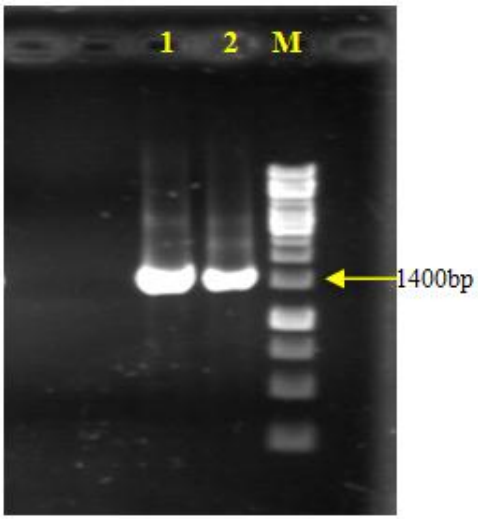

Fig. 2: Agarose gel electrophoresis of the 16S rDNA PCR products of bacterial isolates. Lane M: 1 kb DNA ladder; Lane 1and 2: bacterial isolates UPMR7 and UPMR17

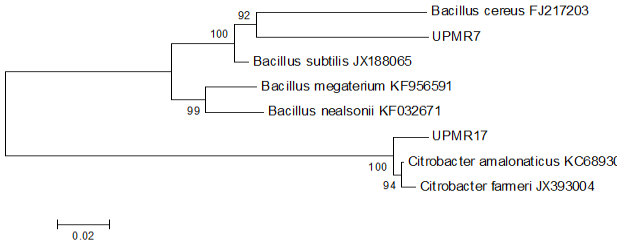

Fig. 3: Neighbor-joining tree based on 16S rDNA gene sequences showing phylogenetic relationship of isolates UPMR7 and UPMR7 to related isolates

approximately 20% more than that of control plants. The significantly (≤0.05) largest root (8.52 cm; 150%), highest shoot fresh weight (2.92 g/plant; 214%), shoot dry weight (0.55 g/plant; 139%), root fresh weight (1.25 g/plant; 220%) and root dry weight (0.25 g/plant; 178%) were recorded in plants inoculated with isolate UPMR17 over control plants. The results also showed that the total chlorophyll content significantly (≤0.05) increased in inoculated plants over control plants. The highest total chlorophyll content (4.5 mg g^{-1} FW) was registered in plants treated with isolate UPMR7 which was 36% higher than that of control plants.

Discussion

A total of 30 PGPR evaluated in this study varied in all measured traits. Among them isolates UPMR7 and UPMR17 appeared as the most potential bioresources for growth enhancement of rice variety MR219. Based on past research experience, a variety of commonly cited traits including nitrogen fixation, phosphate solubilization, IAA-synthesis, salinity tolerant and antagonistic activity (biochemical); root and shoot length, fresh and dry weight, and leaf chlorophyll content (agronomic) of rice plant were included to evaluate the PGPR isolates. Significant variations among different PGPR isolates were found indicating that selection of these traits was logical.

Nitrogen fixation ability is an important criterion for selection of potential PGPR. In this study most of the PGPR isolates grew well and formed halo zones on N-free NA media confirmed their potential of fixing atmospheric nitrogen on such media. Our result was supported by the findings of Naher *et al.* (2009) who characterized a few N-fixing bacteria by acetylene reduction assay (ARA) at Tanjong Karang Rice Irrigation Project, Malaysia.

Phosphorus is one of the important macronutrient for biological growth and development. Application of phosphate solubilizing rhizobacteria is essential for increasing phosphorus uptake in plant. Seventeen out of 30 PGPR isolates were found to solubilize insoluble phosphate in NBRIP media. Our result was in line with the findings of Bhoopander *et al.* (2005) who found *Bacillus* and *Pseudomonas* were involved in solubilization of inorganic phosphate. Phosphate solubilizing bacteria reduces pH of rhizosphere soils by releasing organic acids which dissolve phosphate mineral through anion exchange or chelation of Fe and Al ions associated with phosphate. This process increases the availability of phosphorus for plant uptake (Gyaneshwar *et al.*, 2002).

Table 4: Effect of inoculation of seven bacterial isolates on different growth parameters of rice seedlings after 30 days of planting in pot experiment

Treatments	SL (cm)	RL (cm)	SFW (g/plant)	SDW (g/plant)	RFW (g/plant)	RDW (g/plant)	Chlorophyll (mg g^{-1} FW)		
							a	b	a+b
UPMR7	42.3 a	7.5 a	1.7 b	0.4 b	0.8 b	0.1 b	3.0 a	1.5 a	4.5 a
UPMR17	39.9 a	8.5 a	2.9 a	0.6 a	1.3 a	0.3 a	2.7 a	1.2 b	3.9 b
Control	35.3 b	3.4 b	0.9 c	0.2 c	0.4 c	0.1 a	2.0 b	0.9 c	2.9 c

Values having the same letter(s) in a column do not differ significantly at the 5% level of probability
SL: Shoot length; RL: root length; SFW: shoot fresh weight; SDW: shoot dry weight; RFW: root fresh weight; RDW: root dry weight

IAA is the most physiologically active auxin in plants that influences root and shoot elongation by cell wall extension (Subba Rao, 1999). Most of the PGPR isolates were found to produce IAA with the addition of tryptophan where synthesis of IAA varied among the tested isolates. In line of our results, Mirza *et al.* (2001) and Ali *et al.* (2009) reported that IAA synthesis of rhizobacteria was tryptophan-dependent. They also observed variations among different species and strains as well as the effects of culture conditions and growth stages. However, some isolates produced IAA without the addition of tryptophan. PGPR also have been reported to synthesis of tryptophan-independent IAA (Baca and Elmerich, 2007; Spaepen *et al*., 2007).

In vitro growth promoting traits of 30 PGPR isolates were combined in a dendrogram derived from NTSYS software to facilitate their comparison. The diverse coefficient was high (1.78) indicating a good clustering structure which provides two clusters of PGPR based on their ability of nitrogen fixation, phosphate solubilization and IAA production. Cluster-1 included 15 PGPR with higher plant growth promoting traits. Based on mean separation by $LSD_{0.05}$ (Table 1) and cluster analysis 15 isolates from cluster-1 were selected for subsequent study.

Salt tolerant bacteria are a potential bioresource for saline prone areas (Hayat *et al*., 2013). Our results showed that isolates UPMR2, UPMR7, UPMR17 and UPMR18 tolerated higher NaCl concentration (up to 6%) confirmed their capability of surviving in saline environment. Soil pH is another limiting factor for PGPR as well as rice cultivation (Hayat *et al*., 2013). In our study PGPR tolerance to a wide pH range confirmed their ability to survive both in acidic and alkaline soils. Rice blast caused by *P. oyzae* is a major disease reported to cause yield reduction in rice (Kim *et al*., 2012). In dual culture tests volatile inhibitory compounds suppressive on *P. oryzae* were produced. Most of the PGPR isolates did not exhibit excellent antagonistic effects against *P. oryzae*. Production of inhibitory metabolites is an established mechanism of antagonism (Kalbe *et al*., 1996). Our results showed that PGPR isolates UPMR17 (68%) and UPMR7 (63%) had significantly higher values for PIRG and production of volatile inhibitory compounds against *P. oryzae*.

On the basis of the obtained results, it can be summarized that UPMR7 and UPMR17 were the most potential growth promoting, abiotic and biotic stress tolerant PGPR isolates. Considering the plant growth promoting characteristics and the stress tolerance potential, isolates UPMR7, UPMR17 were selected for molecular identification. Molecular phylogenetic analysis provides the basis for the conventional sequence identification techniques (Singh *et al*., 2007). The 16S rRNA sequence analysis of the genes has been well established as a standard procedure for identification of bacteria at the level of species, genera, and family (Gürtler and Mayall, 2001). The sequence analysis revealed that isolate UPMR7 matched with *Bacillus sp* and UPMR17 matched with *Citrobacter sp* and phylogenetic analysis showed their relationship.

Several studies have showed that *Bacillus sp* enhances the growth, development and yield of various crops under controlled and/or field condition. Bacillus is known as potential elicitor of ISR and exhibits significant reduction in the incidence or severity of various diseases on diverse crops including rice (Kloepper *et al*. 2007; Choudhary *et al*. 2007; Jetiyanon and Plianbangchang, 2010).

Isolate UPMR17, identified as *Citrobacter sp* is recognized as PGPR as well as biocontrol agents (Patel *et al*., 2010) in production of antibiotics, degrading enzymes, competition, parasitism, induced ststemic resistant and a combination of these possibilities (Wang *et al*., 2006).

Rice plants inoculated with *Bacillus sp* (UPMR7) and *Citrobacter sp* (UPMR17) produced higher plant biomass and chlorophyll content proved that PGPR possessed multiple plant growth promoting and stress tolerant traits. Bioassays of plant growth were also reflected during *in vitro* biochemical test of PGPR where the studied isolates showed higher efficiency in N–fixation, phosphate solubilization, IAA production and antagonistic effects against *P. oryzae*. Our results were supported by the findings of Naher *et al*. (2009) who documented that tropical soils of Malaysia harbor diverse microorganisms and most of them are able to fix atmospheric-N, solubilize insoluble phosphate, and synthesize IAA in association of rice plants. Phosphate is an essential nutrient for plant growth and development. Rhizobacteria have been shown to solubilize rock phosphate and enhanced root and shoot growth, yield of rice (Panhwar *et al*., 2009). IAA is an important determinant in shoot and root growth of plant. The significant improvement in root and shoot growth in inoculated plants might be due to the production of IAA by the studied isolates *Bacillus sp* (UPMR7) and *Citrobacter sp* (UPMR17). PGPR produced

phytohormone caused morphological and physiological changes in roots resulted in increased nutrients and water uptake from soil (Mia *et al*., 2009). In line with our findings, *Bacillus spp.* and *Citrobacter* spp. have also been reported to fix atmospheric-N (Mutalib *et al*., 2012; Hongrittipun *et al*., 2014), solubilize phosphate, synthesize of plant growth hormones and increase growth and yield of rice (Yadav *et al*., 2011; Ng *et al*., 2012).

In conclusion, among the 30 PGPR, isolates UPMR7 and UPMR17 appeared as the most potential growth enhancer and stress tolerant bioresource on early establishment of rice. UPMR7 and UPMR17 were identified as *Bacillus sp* and *Citrobacter sp* by using 16s rRNA gene sequences. The results obtained in this study suggested that UPMR7 and UPMR17 have the potential to be used as biofertilizer and biopesticide with multiple plant growth promoting and stress tolerant activities for sustainable rice production.

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