



Full Length Article

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

S.H. Habib^{1,3}, H. Kausar², H.M. Saud^{1*}, M.R. Ismail² and R. Othman^{2,4*}

¹Department of Agricultural Technology, Faculty of Agriculture, Universiti Putra, 43400, Serdang 43400, Selangor, Malaysia

²Laboratory of Food Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

³Oilseed Research Centre, Bangladesh Agricultural Research Institute (BARI), Gazipur-1701, Bangladesh

⁴Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

*For correspondence: halimi@upm.edu.my; radziah@upm.edu.my

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. © 2016 Friends Science Publishers

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

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 4°C 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 $28 \pm 2^\circ\text{C}$ for 24–48 h. Single colony of PGPR was transferred aseptically onto NA media to obtain pure cultures. Pure cultures were kept at 4°C 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, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, NaCl 0.5, $\text{F}_2 \text{SO}_4$ 0.1, Na_2MoO_4 0.005, CaCO_3 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 30°C. 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, $\text{Ca}_3(\text{PO}_4)_2$ 5.0, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 5.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25, KCl 0.2 and $(\text{NH}_2)\text{SO}_4$ 0.1. Using sterile toothpicks, a loop full of each PGPR isolate was placed onto plate and incubated for 2 days at 30°C. 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 ($28^\circ\text{C} \pm 2$) 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 $28 \pm 2^\circ\text{C}$ 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).

$$\begin{aligned} r_1 &= \text{radial growth in control plate} \\ r_2 &= \text{radial growth in treatment plate} \end{aligned}$$

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 28°C 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 CO., Ltd.). The universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA coding region. The 25 μ L PCR reaction mixture comprised of 1 μ L of bacterial DNA, 0.15 μ M of each primer, 1 \times PCR reaction buffer (Fermentas, USA), 0.2 mM of dNTP mix, 2.5 U of Taq polymerase (Fermentas, USA), 25 mM of MgCl₂ and top up with sterile distilled water. The thermal cycling condition was: one cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min followed by an additional cycle of 5 min at 72°C.

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 \pm 2°C. The cultures were harvested by centrifuging at 10,000 rpm for 10 min. Pellets were suspended in NB and bacterial suspension was adjusted to 10⁸ 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% HgCl₂ solution for 3 min followed by rinsing 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 (10⁸ 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 Ca₃(PO₄)₂ in NBRIP medium with various PSI which ranged from 1.1 to 2.3. The significantly (≤ 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 μ g 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 μ g 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 (≤ 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

Table 1: Plant growth promoting activities of bacterial isolates

Isolates	N-fixing ability	P-solubilization index	IAA $\mu\text{g mL}^{-1}$	
			with tryptophan	without tryptophan
UPMR1	+	1.4 ef	12.2 cg	1.01
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	1.01
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	1.01
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

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

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

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

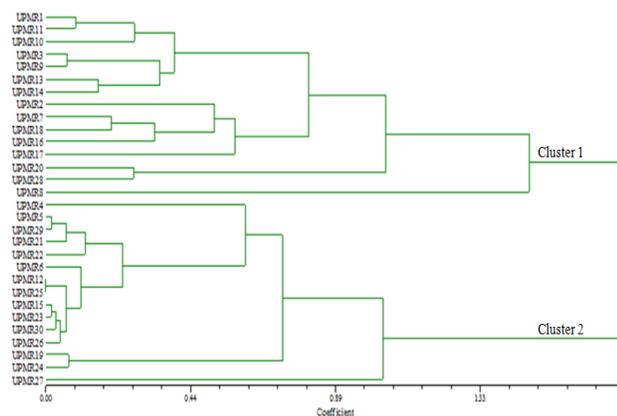


Fig. 1: Dendrogram of 30 bacterial isolates based on their plant growth promoting ability prepared using NTSYS pc 2.02 software

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

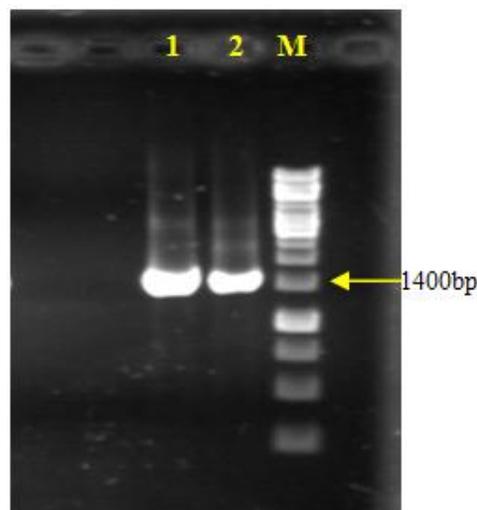


Fig. 2: Agarose gel electrophoresis of the 16S rDNA PCR products of bacterial isolates. Lane M: 1 kb DNA ladder; Lane 1 and 2: bacterial isolates UPMR7 and UPMR17

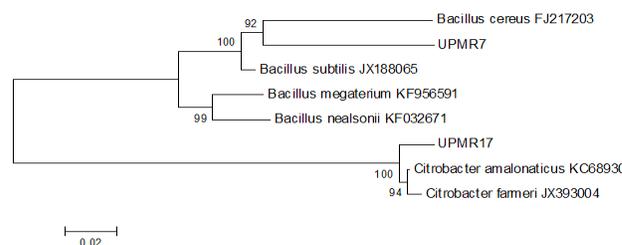


Fig. 3: Neighbor-joining tree based on 16S rDNA gene sequences showing phylogenetic relationship of isolates UPMR7 and UPMR17 to related isolates

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. oryzae* 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 (Klopper *et al.* 2007; Choudhary *et al.* 2007; Jetyanon 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 systemic 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.

Acknowledgements

The authors acknowledge the financial and technical support by Universiti Putra Malaysia and the Ministry of Education Malaysia for Long-Term Research Grant Scheme (LRGS)-Food Security.

References

- Ali, B., A.N. Sabri, K. Ljung and S. Hasnain, 2009. Quantification of indole-3-acetic acid from plant associated *Bacillus spp.* and their phyto-stimulatory effect on *Vigna radiata* (L.). *World J. Microbiol. Biotechnol.*, 25: 519–526
- Baca, B. and C. Elmerich, 2007. Microbial production of plant hormones. In: *Associative and Endophytic Nitrogen-Fixing Bacteria and Cyanobacterial Associations*, pp: 113–143. Elmerich, C. and W.E. Newton (eds.). Springer, The Netherlands
- Bhoopander, G., G.P. Huong, R. Kumari, R. Prasad and A. Varma, 2005. Microbial diversity in soils. In: *Microorganisms in soils: Roles in Genesis and Functions*, pp: 19–55. Buscot, F. and A. Varma (eds.). Springer, Germany
- Chandna, P., M.L. Khurana, J.K. Ladha, M. Punia, R.S. Mehla and R. Gupta, 2011. Spatial and seasonal distribution of nitrate-N in groundwater beneath the rice-wheat cropping system of India: a geospatial analysis. *Environ. Monit. Assess.*, 178: 545–562
- Choudhary, D.K., A. Prakash and B.N. Johri, 2007. Induced systemic resistance (ISR) in plants: Mechanism of action. *Ind. J. Microbiol.*, 47: 289–297
- Edi-Premono, M., A. Moawad and P.L.G. Merbach, 1996. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonatia J. Crop Sci.*, 11: 13–23
- El-Kholly, M.A., S. El-Ashry and A.M. Gomaa, 2005. Biofertilization of Maize Crop and its Impact on Yield and Grains Nutrient Content under Low rates of Mineral Fertilizers. *J. Appl. Sci. Res.*, 1: 117–121
- Emilsson, T., Berndtsson, J.C., Mattsson, J.E. and K. Rolf, 2007. Effect of using conventional and controlled release fertilizer on nutrient runoff from various vegetated roof systems. *Ecol. Eng.*, 29: 260–271
- Gibson, A.H., 1980. Methods for legumes in glasshouses and controlled environment cabinets. In: *Methods for Evaluating Biological Nitrogen Fixation*, pp: 139–184. Bergersen, F.J. (ed.). John Wiley & Sons, New York, USA
- Gürtler, V. and B.C. Mayall, 2001. Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *Int. J. Syst. Evol. Microbiol.*, 51: 3–16
- Gutierrez, C.K., G.Y. Matsui, D.E. Lincoln and C.R. Lovell, 2009. Production of the phytohormone indole-3-acetic acid by estuarine species of the genus *Vibrio*. *Appl. Environ. Microbiol.*, 75: 2253–2258
- Gyaneshwar, P., G.N. Kumar, L.J. Parekh and P.S. Poole, 2002. Role of soil microorganisms in improving P nutrition of plants. *Plant Soil*, 245: 83–93
- Hayat, R., R. Khalid, M. Ehsan, I. Ahmed, A. Yokota and S. Ali, 2013. Molecular characterization of soil bacteria for improving crop yield in Pakistan. *Pak. J. Bot.*, 45: 1045–1055
- Jetiyanon, K. and J.W. Kloepper, 2002. Mixture of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple diseases. *Biol. Cont.*, 24: 285–291
- Jetiyanon, K. and P. Plianbangchang, 2010. Dose-responses of *Bacillus cereus* RS87 for growth enhancement in various Thai rice cultivars. *Can. J. Microbiol.*, 56: 1011–1019
- Jinantana, J. and M. Sariah, 1997. Antagonist effect of Malaysian isolates of *Trichoderma harzianum* and *Gliocladium virens* on *Sclerotium rolfsii*. *Pertanika. J. Trop. Agric. Sci.*, 20: 35–41
- Kalbe, C., P. Marten and G. Berg, 1996. Strains of genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. *Microbiol. Res.*, 151: 433–439
- Kausar, H., M. Sariah, H.M. Saud, M.Z. Alam and M.R. Ismail, 2011. Isolation and screening of potential actinobacteria for rapid composting of rice straw. *Biodegradation*, 22: 367–375
- Kim, S.H., T. Oikawa, J. Kyoizuka, H.L. Wong, K. Umemura, M. Kishi-Kaboshi, A. Takahashi, Y. Kawano, T. Kawasaki and K. Shimamoto, 2012. The bHLH Rac Immunity1 (RAI1) is activated by OsRac1 via OsMAPK3 and OsMAPK6 in rice immunity. *Plant Cell Physiol.*, 53: 740–754
- Kloepper, J.W., G. Estrad and J.A. McInroy, 2007. Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Can. J. Microbiol.*, 53: 159–167
- Mia, M.A.B., Z.H. Shamsuddin, W. Zakaria and M. Mariah, 2009. The effect of rhizobacterial inoculation on growth and nutrient accumulation of tissue-cultured banana plantlets under low N-fertilizer regime. *Afr. J. Biotechnol.*, 8: 5855–5866
- Mirza, M.S., W. Ahmad, F. Latif, J. Haurat, R. Bally, P. Normand and K.A. Malik, 2001. Isolation, partial characterization and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. *Plant Soil*, 237: 47–54
- Mishra, A. K., K.A. Mottaleb, A.R. Khanal and S. Mohanty, 2014. Abiotic stress and its impact on production efficiency: The case of rice farming in Bangladesh. *Agric. Ecosyst. Environ.*, 199: 146–153
- Mutalib, A.A., O. Radziah, Y. Shukor and U.A. Naher, 2012. Effect of nitrogen fertilizer on hydrolytic enzyme production, root colonisation, N metabolism, leaf physiology and growth of rice inoculated with *Bacillus sp.* (Sb42). *Aust. J. Crop Sci.*, 6: 1383–1389
- Naher, U.A., O. Radziah, Z.H. Shamsuddin, M.S. Halimi and I.M. Razi, 2009. Isolation of diazotrophs from different soils of tanjong karang rice growing area in Malaysia. *Int. J. Agric. Biol.*, 11: 547–552
- Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.*, 170: 265–270
- Ng, L.C., M. Sariah, O. Sariam, O. Radziah, M.A. Zainal Abidin, 2012. Rice seed bacterization for promoting germination and seedling growth under aerobic cultivation system. *Aust. J. Crop Sci.*, 6: 170–175
- Panhwar, Q.A., O. Radziah, M. Sariah and I. Mohd Razi, 2009. Solubilization of phosphate forms by phosphate solubilizing bacteria isolated from aerobic rice. *Int. J. Agric. Biol.*, 11: 667–673
- Patel, K.J., S. Vig, K.G. Naresh and G. Archana, 2010. Effect of transgenic rhizobacteria over expressing *Citrobacter braakii* appA on phytate-P availability to mung bean plants. *J. Microbiol. Biotechnol.*, 20: 1491–1499
- Pereira, S.I.A. and P.M.L. Castro, 2014. Phosphate-solubilizing rhizobacteria enhance *Zea mays* growth in agricultural P-deficient soils. *Ecol. Eng.*, 73: 526–535
- Pinheiro, B.D.S., E.D.M.D. Castro and C.M. Guimaraes, 2006. Sustainability and profitability of aerobic rice production in Brazil. *Field Crop Res.*, 97: 34–42

- Hongrattipun, P., S. Youpensuk and B. Rerkasem, 2014. Screening of Nitrogen Fixing Endophytic Bacteria in *Oryza sativa* L. *J. Agric. Sci.*, 6: 66–74
- Rawat, S.K., R.K. Singh and R.P. Singh, 2010. Seasonal variation of nitrate level in ground and surface waters of Lucknow and its remediation using certain aquatic macrophytes. *Int. J. Lake Riv.*, 3: 25–35
- Rawat, S.K., R.K. Singh and R.P. Singh, 2012. Remediation of nitrite in ground and surface waters using aquatic macrophytes. *J. Environ. Biol.*, 33: 51–56
- Sandhya, V., S.K.Z. Ali, G. Minakshi, G. Reddy and B. Venkateswarlu, 2009. Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-P45. *Biol. Fert. Soil*, 46: 17–26
- Singh, S., R. Chandra, D.K. Patel and V. Rai, 2007. Isolation and characterization of novel *Serratia marcescens* (AY927692) for pentachlorophenol degradation from pulp and paper mill waste. *World J. Microbiol. Biotechnol.*, 23: 1747–1754
- Solano, B.R., J.B. Maicas and F.J.G. Mañero, 2008. Physiological and Molecular mechanisms of plant growth promoting rhizobacteria (PGPR). In: *Plant-Bacteria Interactions: Strategies and Techniques to Promote Plant Growth*, pp: 41-54. Ahmad, I., J. Pichtel and S Hayat (eds.). Wiley-Vch Verlag GmbH and Co. KGaA, Weinheim. Germany
- Spaepen, S., J. Vanderleyden and R. Remans, 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol. Rev.*, 31: 425–448
- Subba Rao, N.S., 1999. The rhizosphere and the phyllosphere, In: *Soil Microbiology*, 4th edition, p: 85. Science Publishers, Inc., Enfield, New Hampshire, UK
- Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4. *Mol. Biol. Evol.*, 24: 1596–1599
- Wang, Q., F. Li, L. Zhao, E. Zhang, S. Shi, W. Zhao, W. Song and M.M. Vance, 2010. Effect of irrigation and nitrogen application rates on nitrate nitrogen distribution and fertilizer nitrogen loss, wheat yield and nitrogen uptake on a recently reclaimed sandy farmland. *Plant Soil*, 337: 325–339
- Wang, E.T., Z.Y. Tan, X.W. Guo, R. Rodríguez-Duran, G. Boll and E. Martínez-Romero, 2006. Diverse endophytic bacteria isolated from a leguminous tree *Conzattia multiXora* grown in Mexico. *Arch. Microbiol.*, 186: 251–259
- Yadav, J., J.P. Verma, S.K. Yadav, K.N. Tiwari, 2011. Plant growth promoting activities of fungi and their effect on chickpea plant growth. *Asian J. Biol. Sci.*, 4: 291–299
- Zahir, Z.A., U. Ghani, M. Naveed, S.M. Nadeem and H.N. Asghar, 2009. Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. *Arch. Microbiol.*, 191: 415-424
- Zaman, M., M.L. Nguyen and J.D. Blennerhassett, 2010. The effect of different rates of urea with or without urease inhibitor (NBPT) on wheat yield and quality. *Agric. J.*, 5: 309–312

(Received 14 July 2014; Accepted 05 January 2015)