ISOLATION AND CHARACTERIZATION OF RHIZOBIA AND PLANT GROWTH-PROMOTING RHIZOBACTERIA AND THEIR EFFECTS ON GROWTH OF RICE SEEDLINGS

[1]Tan, K.Z., [1]O. Radziah, [2]M.S. Halimi, [3]A.R. Khairuddin, [2]S.H. Habib and [1]Z.H. Shamsuddin

[1]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia
[2]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia
[3]Agrotechnology and Biosciences Division, Malaysian Nuclear Agency, Malaysia

ABSTRACT

Biofertilizer is a relatively safer, environmentally friendly and cost-effective approach as an alternative to reduce chemical fertilizer usage. The selection of bacterial strains with multiple beneficial characteristics are important to maximize the effectiveness on the host plant. Due to aforementioned interest, several Plant Growth-Promoting Rhizobacterial (PGPR) and rhizobial strains were isolated from rice and legume roots, respectively, at four locations in Malaysia namely Universiti Putra Malaysia (UPM), Serdang, Selangor; Besut, Terengganu; Tunjung, Kelantan and Sik, Kedah. Bacterial isolations were undertaken to select the best isolates which exhibit multiple beneficial effects to the rice plant and a total of 205 bacterial strains were isolated and categorized as follows; 94 rhizospheric and 107 endophytic bacteria from rice roots, one rhizobial strain from soybean and three from *Mimosa pudica*. These isolates were screened for their abilities to fix N_2 and solubilize phosphate; 52 were positive for both tests. The selected isolates were then tested for IAA production and other biochemical tests such as potassium solubilization, hydrolyzing enzymes (cellulase and pectinase) and iron siderophore productions. Four isolates, namely UPMB19 (rhizospheric PGPR from Tunjung, Kelantan), UPMB20 (endophytic PGPR from Besut, Terengganu), UPMR30 (rhizobia from soybean) and UPMR31 (rhizobia from *Mimosa*) were selected for subsequent plant inoculation tests with UPMB10, a PGPR isolated from oil palm root, as the reference strain. Based on 16S rDNA gene sequencing, these bacterial strains were identified under several genera: *Lysinibacillus*, *Alcaligenes*, *Bradyrhizobium*, *Rhizobium* and *Bacillus*, respectively. Results of plant inoculation test indicated that UPMB19 significantly enhanced the seedling height at the early growth stage (7 days after transplanting, DAT) which could be attributed to the higher N_2 fixation rate of this strain as compared to the other strains, except UPMB20. UPMB10 and UPMR31 also showed significant effects with increased seedling height and Total Dry Weight (TDW) at the later stage (14 DAT) possibly due to the higher bacterial population and IAA produced as observed in the residual nutrient solution without addition of fertilizer-N. This study has successfully demonstrated the effectiveness of locally isolated PGPR and rhizobial strains with multiple beneficial characteristics on early growth and vigor of rice seedlings under controlled condition. The result proved to be a vital information in the development of a liquid biofertilizer for rice; thus further studies will be done to evaluate the effectiveness of these isolates under glasshouse and field conditions on growth, tillering and yield of rice.

Keywords: PGPR, Rhizobia, N_2-Fixation, Growth, Rice

Corresponding Author: Shamsuddin, Z.H., Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, Malaysia Tel. No.: +603-8947 4964; Fax No.: +603-8947 4919

1. INTRODUCTION

Rice is the major world food crop and it is the most important staple diet for nearly 3 billion people, approximately half of the world population (FAO, 2004). If the consumption trend continues, 4.6 billion people will consume rice by 2025 and production must be increased by 20% to keep pace with this increasing demand. To increase the local rice yield, farmers tend to apply more chemical fertilizers which can be hazardous to the environment when applied excessively. Alternatively, a relatively safer approach is to use the environmentally friendly biofertilizer which is also cost-effective.

Research on Plant Growth-Promoting Rhizobacteria (PGPR) with non-legumes such as rice have shown beneficial effects through biological N_2 fixation (Malik *et al*., 1997), increased root growth (Mia *et al*., 2012) with enhanced nutrient uptake (Yanni *et al*., 1997), phytohormone production (Chabot *et al*., 1996), plant growth enhancement stimulation by other beneficial bacteria and fungi (Saharan and Nehra, 2011) and disease control (Ramamoorthy *et al*., 2001). Peng *et al*. (2002), however, reported that rhizobial inoculation known for their symbiotic relationship with legumes, could also increaserice grain yield, but little is known about the mechanism(s) involved. The beneficial effects of the selected rhizobialisolates could be due to their plant growth-promoting abilities namely biological N_2 fixation, phosphate solubilization and plant growth-regulator/phytohormone and siderophore production, similar to the known beneficial effects of PGPR (Boddey *et al*., 1997; Verma *et al*., 2001; Araujo *et al*., 2013; Kloepper *et al*., 1980). Yanni *et al.* (1997) have also shown beneficial interactions of rhizobial isolateson growth of rice, which was believed to be due to increased root efficiency in water and nutrient uptake.

The reference strain UPMB10 (*Bacillus* sp.), a local isolate from rhizosphere of oil palm roots, has been studied in detail for the past 10 years. This rhizobacterial isolate has been shown to exhibit several beneficial characteristics namely biological N_2 fixation (Mia *et al*., 2010a), increased accumulation of N, P and K in the plant tissue (Amir *et al*., 2005), increased root and shoot growth (Mia *et al*., 2010b) and overall plant growth and yield (Farzana *et al*., 2007) on various crops such as oil palm, sweetpotato, banana and rice (Amir *et al*., 1999; Farzana *et al*., 2009; Mia *et al*., 2009; 2012). [15]N dilution technique and acetylene reduction assay revealed that the BNF rate values to be 28 and 63%, respectively

(Amir *et al*., 2003; Zakry *et al*., 2012). The large variation of nitrogen derived from atmosphere (Ndfa) values are due to the solid substrate used in the later study as compared to the liquid inoculum used in the earlier report. The solid substrate was reported to have several advantages over liquid inoculum as it provides a substantial amount of carbon source, incubated for 2 weeks prior to application and organic substrate in the carrier which provide 'shelter' for bacterial cells from direct sunlight and act as a buffer zone (Zakry *et al*., 2012).

Thus, it is essential to isolate native rhizobia and PGPR from rice grown fields for multiple beneficial effects on the crops and for potential biofertilizer development. In the present study, isolation and screening of local rhizobia and PGPR with multiple plant growth-promoting abilities were conducted, followed by *in vitro* plant inoculation test.

2. MATERIALS AND METHODS

2.1. Sampling of Rice and Legume Roots for Bacterial Isolation

At every location, eight sampling points, each point approximately 50 m apart, were randomly selected. One healthy rice plant per point was chosen for bacterial isolation. The roots were washed twice with clean water to remove the adhering soil particles. Three fresh and whitish root tips, each approximately 2.5 cm long, were cut and placed into a test tube containing 9 mL sterile distilled water. The test tubes were shaken manually to remove soil particles and other debris on the roots and the water was replaced with new sterile distilled water. The samples were taken to the laboratory in an ice box, the icereplaced regularly every 6 h.

Volunteer leguminous plants (*Glycine max, Mimosa pudica*) in therice field were collected randomly and thenodules from each plant were removed and placed in McCartney bottles containing 15 mL sterile distilled water. The bottles were stored in ice box and taken to the laboratory.

2.2. Isolation of Rhizobia/PGPR

The rice root samples were transferred into McCartney bottles containing 15 mL sterile distilled water using sterile forceps. The bottles were shaken vigorously with a vortex mixer for 10 sec. The solutions were used to isolate rhizospheric bacteria. The roots were taken out and surface sterilized by soaking in 95% ethanol for 10 sec, followed by 3% sodium hypochlorite for 1 min. 6 times washed with sterile distilled water. Then, the roots were cut

longitudinally using sterile blade, followed by vertical sections into 5 pieces. These dissected root pieces were transferred into another McCartney bottle containing 15 mL sterile distilled water and the bottle shaken vigorously with a vortex mixer for 10 sec. These solutionswere used to isolate for endophytic bacteria.

The above procedures were repeated for all 8 rice plants from each sampling location. 0.1 mL of the solution from each bottle wastransferred onto Tryptic Soy Agar (TSA) plates and spread evenly using a hockey stick. The plates were covered with parafilm, inverted and placed in the incubator for 24 h at 33°C.

Similarly, nodules from the leguminous plants were surface sterilized, dissected and crushed using a sterile pestle. It was transferred into a test tube containing 9 mL sterile distilled water and shaken using a vortex mixer. 0.1 mL of the solution from each tube was pipetted into Yeast Mannitol Agar (YMA) plate and spread evenly using a hockey stick. The plates were covered with parafilm, inverted and placed in the incubator for 24 h at 33°C.

After 24 h, all the plates were observed for different types of bacterial growth, based on colony morphology such as color and shape. Different colonies were sub-cultured into new TSA/YMA several times to get the pure, single colony. Each of the isolates was transferred onto agar slant (TSA/YMA) and kept in the refrigerator at -3°C for the next subsequent experiment.

2.3. N_2 Fixation Ability

The N_2 fixation ability was tested by growing the isolates on N-free solid malate medium (Nfb medium) according to the method by Dobereiner and Day (1975). Plates were incubated for 24 h at 33°C. The ability of the isolates to fix N_2 was observed by the change in color of the media from pale green to blue after the incubation period. This is due to the increase in pH attributed to the formation of ammonia and nitrates from the atmospheric N_2 fixation.

The quantitative measurement of N_2 fixation rate of the selected bacterial strains was done using Acetylene Reduction Assay (ARA) method according to Hardy *et al.* (1968) and Somasegaran and Hoben (1985). About 20 µL of the bacterial inoculum (1×10^8 CFU mL^{-1}) was transferred into a 10 mL Vacutainer (Becton, Dickinson and Company, USA) containing 4 mL of N-free semi solid media (Dobereiner and Day, 1975). 10% of the air was replaced with pure acetylene gas (99.8%) using a gas-tight syringe (Hamilton®, USA). The bacterial culture was allowed to incubate for 1 h and 1mL of the air sample was injected into a gas chromatography

(7820A, Agilent Technologies, USA) equipped with a hydrogen Flame Ionization Detector (FID) and stainless steel column with Porapak N media. The concentration of ethylene produced was calculated based on the standard ethylene graph at 2.1 min Retention Time (RT).

2.4. Phosphate Solubilizing ability

The isolated culture was grown on Pikovskaya medium to determine the ability to solubilize phosphate according to the method by Pikovskaya (1948). The ability to solubilize phosphate was observed by the formation of a halo zone around the colony (10 µL inoculum, 1×10^8 CFU mL^{-1}) after 24 h of incubation. A halo zone will be formed when the bacterial isolates produce organic acid which will solubilize tri-calcium phosphate in the medium.

The quantification of the solubilized phosphate was undertaken by using phosphate solubilizing index, according to the method by Sitepu *et al.* (2007). Phosphate solubilization index was derived by dividing the total diameter of the halo zone (colony+halo zone) with diameter of the colony. These were undertaken in triplicates.

The phosphate solubilization quantification was repeated using vanadomolybdophosphoric acid method (Kumar *et al.*, 2009; Ribeiro and Cardoso, 2012) to measure soluble phosphate concentration in the culture supernatants of NBRIP broth (Nautiyal, 1999). The standard curve was prepared by a stock solution of KH_2PO_4, mixed with 5mL of concentrated H_2SO_4 and diluted to 1 L with distilled water. The bacterial isolates were grown on TSA culture medium for 24 h. Thereafter, 10 µL of bacterial suspension (containing approximately 10^8 CFU mL^{-1}) was inoculated into each NBRIP broth. The control consisted of flasks containing 100 mL of NBRIP culture medium without inoculum. The flasks were incubated at 28°C for 12 days under constant agitation at 100 rpm and the pH of the culture medium in each treatment was checked. After 6 days of incubation, 25 mL of each culture medium was added to 50 mL centrifuge tubes, which were centrifuged at 8000 g for 20 min. 2.5 mL of the supernatant was transferred into a 50 mL beaker and 20 mL of distilled water was added. 2.5 mL of Barton's reagent (Barton, 1948) was added quickly to create a mixing action and color development was allowed for 10 min. The absorbance value was determined colorimetrically using spectrophotometer at 430 nm. The same procedures were repeated after 12 days of incubation. The long incubation periods (6 and 12 DAI) were chosen as the preliminary study revealed that these bacterial strains have slow rate of phosphate solubilization ability.

2.5. Phytohormone Production

Fully grown bacterial cultures were inoculated in 100 mL Tryptic Soy Broth (TSB) and shaken on an orbital shaker for 24 h. 1mL of the bacterial culture was transferred into a new 100 mL TSB with the addition of 5 mL L-Tryptophan as the precursor of Indole-3-acetic Acid. TSB without bacterial inoculation served as the control. 1.5 mL of the bacterial culture were transferred into sterile Appendorf tube and centrifuged at 7000 rpm for 7 min. The supernatant (1mL)wasmixedwith 2mL of Salkowsky reagent (2% of 0.5 M $FeCl_3$ in 35% perchloric acid) according to the method by Gordon and Weber (1951). The solution was allowed to settle for 25 min and development of a pink color would indicate IAA production. The absorbance values were determined using a spectrophotometer at 535 nm and compared to the standard curve to determine the IAA concentration. The IAA standard curve was prepared using pure IAA at 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 μg mL^{-1} of IAA. Supernatants of uninoculated test tubes were used as control, where no visible color was observed.

2.6. Potassium Solubilization Ability

The selected isolates were grown on a modified Aleksandrov agar medium to determine the ability to solubilize potassium (Hu *et al.*, 2006). About 10 μL of bacterial suspension (containing approximately 10^8 CFUmL^{-1}) were streaked onto the media, incubated at 33°C and observed at 5 and 10 Days After Incubation (DAI). The ability to solubilize muscovite mica as a source of insoluble form of potassium was derived by the formation of clear halo zone around the colony.

The quantitative estimation of potassium solubilization rate was examined based on the bacterial abilities to release K from media supplemented with muscovite mica as an insoluble source of potassium. One mL of overnight culture of each isolate was inoculated to 100 mL of Aleksandrov broth and the amount of K released in the broth was estimated at 5, 10 and 15 DAI from triplicate flasks, in comparison with a set of uninoculated control. The broth cultures were centrifuged at 10,000 rpm for 10 min to separate the supernatant from the bacterial cells and insoluble potassium. The available K content in the supernatant was determined by Atomic Absorption Spectrometer (AAS) (Perkin Elmer, AAnalyst 400 AAS). One mL of the supernatant was taken in a 50 mL volumetric flask and the volume was made to 50 mL with distilled water and mixed thoroughly. Then, the solution was fed to AAS for K content determination.

2.7. Iron Sequestration (Siderophore Production)

Chrome Azurol S (CAS) agar similar to that described by Schwyn and Neilands (1987) was prepared to detect the siderophore production by the bacteria.

CAS agar plates were spot inoculated with each of the bacterial strain and incubated for 48 h at 33°C. Development of an orange halo zone around the colony (10 μL inoculum, 1×10^8 CFU mL^{-1}) indicates a positive result as the siderophore removes Fe from the Fe-CAS dye complex and gives the medium its characteristic blue color.

2.8. Cellulase Enzyme Production

Carboxy Methyl Cellulose (CMC) agar plates were prepared by screening for cellulose enzyme production according to the method by Kasana *et al.* (2008).

A sterile paper disc was dipped into microbial culture and transferred onto the CMC agar plates. The plates were incubated overnight at 33°C. After incubation, the plates were flooded with Congo red solution for 15 min, followed by de-staining with the salt solution for 15 min. Unstained areas indicate where the CMC has been degradeddue to production of cellulose by the bacterial strain.

2.9. Pectinase Enzyme Production

Pectinase agar plates were prepared to screen for pectinase enzymes production, according to the method by Yogesh *et al.* (2009).

A sterile paper disc was dipped into microbial culture and transferred onto the pectinase agar plates. The plates were incubated overnight at 33°C. After incubation, the plates were flooded with 50 mM iodine solution for 15 min to observe the halo zonewhich indicates the ability of the strain to produce pectinase enzyme.

2.10. Bacterial Identification using 16S rDNA Gene Sequence

The selected strains were identified by partial sequencing of the 16S rDNA gene. Genomic DNA was isolated from the bacterial culture by using Genomic DNA Mini Kit (Geneaid). 16S rDNA gene was amplified using universal forward (5´-AGAGTTTGATCMTGGCTCAG-3´) and reverse (5´-GGTTACCTTGTTACGACTT-3´) primers (Integrated DNA Technologies, USA). 50 μL reaction mixture was prepared containing 5 μL of DNA template, 5μL 10X

PCR Reaction Buffer, 0.75 µL 10 µM of each primers, 1 µL 10 µM dNTPs mix, 0.5 µL 5µ/µL Taq polymerase, 3µL 25 µM $MgCl_2$ and 34µL ultra-pure water. PCR reactions were carried out in a thermal cycler (MJ Mini Personal Thermal Cycler, Bio-Rad) using cycles as follows: 5 min at 94°C, 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C and final extension for 5 min at 72°C. The amplified 16S rDNA gene was purified with a Gel/PCR DNA Fragments Extraction Kit (Geneaid) and out-sourced for sequencing (First Base Laboratories Pvt. Ltd., Selangor, Malaysia). The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors by using BLAST (NCBI, USA).

2.11. Plant Inoculation Test in Plant Growth Chamber

Bacterial cultures were prepared by growing them in TSB/YMB for 24 h, followed by centrifuging at 4000 g for 40 min. The supernatants were then removed aseptically and replaced with 20 mL 0.85% sterile phosphate buffer saline.

Table 1. Treatment design and description of the plant inoculation test (Control/PGPR and rhizobia)

Treatment	Description
T1: N_0 – PGPR	Control 1 (without N)
T2: N_{33} – PGPR	Control 2 (with 33% of N)
T3: N_0 + PGPR 1	UPMB19
T4: N_0 + PGPR 2	UPMB20
T5: N_0 + PGPR 3	UPMR30
T6: N_0 + PGPR 4	UPMR31
T7: N_0 + PGPR 5	UPMB10
T8: N_{33} + PGPR 1	UPMB19
T9: N_{33} + PGPR 2	UPMB20
T10: N_{33} + PGPR 3	UPMR30
T11: N_{33} + PGPR 4	UPMR31
T12: N_{33} + PGPR 5	UPMB10

Note: [All treatments were laid in Completely Randomized Design (CRD) with three replicates and harvested after 7 and 14 Days of Transplanting (DAT); N0: Treatment without inorganic fertilizer-N, N33: Treatment with 33% of inorganic fertilizer-N recommended rate]

Table 2. Rhizobial and PGPR strains used for inoculation of rice variety MR219

Strains	Species	Isolated plant species
UPMB10	*Bacillus sp.*	*Elaeis guineensis Jacq.*
UPMB19	Rhizospheric PGPR strain isolated from rice roots	*Oryza sativa L.*
UPMB20	Endophytic PGPR strain isolated from rice roots	*Oryza sativa L.*
UPMR30	Rhizobial strain	*Glycine max*
UPMR31	Rhizobial strain	*Mimosa pudica*

The solution was mixed thoroughly using vortex mixer for 30 sec. 1mL of the bacterial suspension from each solution was transferred into a series of test tubes at 10-fold dilutions each and streaked on TSA/YMA to get the Colony Forming Unit (CFU) by total plate count technique, according to method by Somasegaran and Hoben (1985). The optical densitiesof the bacterial cultures were also undertaken by transferring 1.5 mL of the bacterial suspension into 2 mL cuvette and read using a spectrophotometer at 600 nm absorbance.

Sets of sterile 500 mL measuring cylinders and stainless steel sieves were prepared for growth of the rice seedlings according to the method by Egener *et al.* (1999). About 50 mL sterile N-free nutrient solutions were added to each cylinder followed by 5 mL of the respective phosphate buffer saline-washed bacterial inoculum, according to the treatments (approximately 10^{7-8} CFU mL^{-1}, 1 mL per seedling) (**Table 1 and 2**). Five 5-days old rice seedlings were aseptically transferred onto the sieve in the cylinder and adjusted so that only the roots were in contact with the nutrient solution. The lids of the cylinders were covered with aluminium foil and incubated in a plant growth chamber at 30°C for 14 days with alternate 12 h day and night cycle.

2.12. Statistical Analysis

Data on phosphate solubilization index, phytohormone production and plant inoculation test in theplant inoculation experiment were subjected to Analysis of Variance (ANOVA) using SAS software. Means were compared by Least Significant Difference (LSD) Test at a probability level of 0.05.

3. RESULTS

A total of 205 isolates were obtained from this study, which came from four distinct locations across Malaysia. The bacterial isolates were categorized as follows; 94 rhizospheric and 107 endophytic bacteria from rice roots, one rhizobial strain from soybean (*Glycine max*) and three from *Mimosa pudica* (**Table 3**). The highest number of PGPR was isolated from Besut, Terengganu, followed by UPM, Selangor, Tunjung, Kelantan and Sik, Kedah with a total number of 93, 45, 41 and 22 isolates, respectively. Results also showed that sampling sites with long history of rice cultivation namely at Tunjung, Kelantan and Besut, Terengganu tend to have higher numbers of endophytes from the total isolates (60-61%), compared to the relatively new rice cultivation sites at Sik, Kedah and UPM, Selangor (27-44%). Fifty two of these bacterial isolates were positive for both the N_2-fixation and phosphate solubilization ability tests using agar plates (**Table 4 and Fig. 1**).

Table 3. Rhizospheric and endophytic classification and distribution of bacterial isolates from four locations

Location	Crop	Classification	Isolates	Percentage
Sik, Kedah	*Oryza sativa*	Rhizospheric	16	73
		Endophytic	6	27
	Total isolates		22	100
Tunjung, Kelantan	*Oryza sativa*	Rhizospheric	16	39
		Endophytic	25	61
	Total isolates		41	100
UPM, Selangor	*Oryza sativa*	Rhizospheric	25	56
		Endophytic	20	44
	Total isolates		45	100
Besut, Terengganu	*Oryza sativa*	Rhizospheric	37	40
		Endophytic	56	60
	Total isolates		93	100
Total PGPR isolates collected		201		-
UPM, Selangor	*Glycine max*	Root nodule (legume)	1	25
	Mimosa pudica		3	75
Total Rhizobial isolates collected			4	100

Table 4. N_2-fixation and phosphate solubilization tests result

Location	Isolates	Positive tests on N_2-fixation and phosphate solubilization
Sik, Kedah	22	4 (18.2%)
Tunjung, Kelantan	41	12 (29.3%)
UPM, Selangor	49	6 (12.2%)
Besut, Terengganu	93	30 (32.3%)
Total	205	52 (25.4%)

Note: [Figures in parenthesis represent the percentage values per location]

N-free agar plate
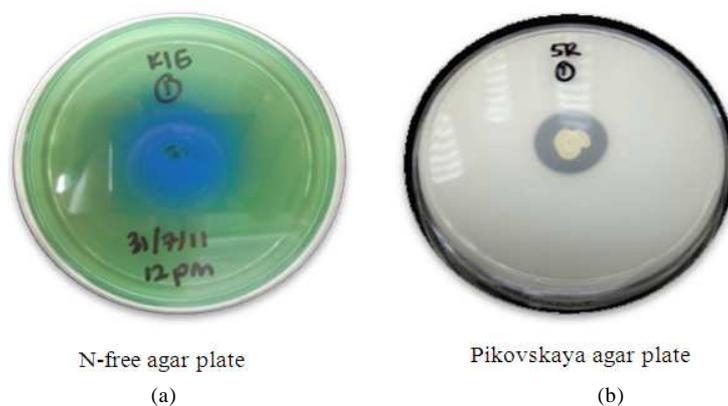(a)

Pikovskaya agar plate
(b)

Fig. 1. N-free(a) and Pikovskaya(b) agar plates after inoculation with selected isolates for 24 h. Positive indications are shown by the change in color from green to blue in (a) and formation of halo zone around the colony in b

A total of 52 isolates (25.4%) from the total of 205 isolates have dual beneficial characteristics namely N_2-fixation and phosphate solubilization.

Based on the IAA production, five isolates were selected, namely UPMB19 (Tunjung rhizospheric PGPR), UPMB20 (Besut endophytic PGPR), UPMR30 (UPM Soybean) and UPMR31 (UPM *Mimosa*) and the reference strain UPMB10, which produced 23.681, 3.327, 13.225, 8.932 and 19.670 µg mL^{-1} of IAA, respectively (**Table 5**).

The five isolates from the current study were then subjected to other biochemical tests such as quantification of N_2 fixation, phosphate and potassium solubilization rates and qualitative observation of siderophore and hydrolyzing enzyme production by using Acetylene Reduction Assay (ARA), phosphate solubilization index and vanadomolybdophosphoric acid method, Aleksandrov broth media, Chrome Azurol S (CAS), cellulase and pectinase agar, respectively.

Table 5. IAA production of the selected PGPR/rhizobial isolates

No	Isolates	IAA (µg/mL)	No.	Isolates	IAA (µg/mL)
1	UPM r 1	1.460	27	Besut e 14	1.212
2	UPM r 2	2.175	28	Besut e 15	7.424
3	UPM r 3	1.303	29	Besut e 16	7.052
4	UPM Mimosa 1 (UPMR31)	8.932	30	Besut e 17	1.929
5	UPM Mimosa 2	1.367	31	Besut e 18	0.994
6	UPM Soybean 1 (UPMR30)	13.225	32	Besut e 19	0.808
7	Besut r 1	4.131	33	Besut e 20	7.207
8	Besut r 2	1.242	34	Besut e 21	0.870
9	Besut r 3	1.895	35	Besut e 22	2.299
10	Besut r 4	2.361	36	Besut e 23	0.870
11	Besut r 5	1.957	37	Tunjung r 1 (UPMB19)	23.681
12	Besut r 6	8.553	38	Tunjung r 2	4.611
13	Besut r 7	8.560	39	Tunjung r 3	3.903
14	Besut e 1	0.932	40	Tunjung e 1	2.442
15	Besut e 2	0.777	41	Tunjung e 2	2.730
16	Besut e 3	1.304	42	Tunjung e 3	1.912
17	Besut e 4	1.336	43	Tunjung e 4	3.040
18	Besut e 5	1.367	44	Tunjung e 5	4.788
19	Besut e 6	2.799	45	Tunjung e 6	3.283
20	Besut e 7 (UPMB20)	23.327	46	Tunjung e 7	5.451
21	Besut e 8	0.683	47	Tunjung e 8	4.633
22	Besut e 9	1.646	48	Tunjung e 9	5.584
23	Besut e 10	3.666	49	Sik r 1	4.013
24	Besut e 11	1.469	50	Sik r 2	4.500
25	Besut e 12	6.151	51	Sik e 1	3.571
26	Besut e 13	7.518	52	Sik e 2	4.080
				Reference strain (UPMB10)	19.670

Note: [Values are based on average of 3 replicates; "r" represent rhizospheric PGPR, "e" endophytic PGPR]

The results of ARA showed a considerable amount of ethylene produced by the selected strains which ranged between 15.60-19.95 nmol C_2H_4/mL/hour (**Fig. 2**). UPMB19 and UPMB20 have significantly higher nitrogenase activities with an increment of 28% and 24%, respectively, compared to the lowest ethylene produced by UPMB10.

Phosphate solubilization index showed that isolate UPMB10 has significantly the highest phosphate solubilization index compared to the other isolates throughout the incubation period of 10 days (**Fig. 3**). The values gradually increased from 2.14 at two days after incubation (D2) to 2.51 at the end of the incubation period (D10). Similar trend was observed for strain UPMB20 with increment of index value from 1.31 to 2.00. Other isolates, UPMB19 and UPMR31, recorded a gradual increase in index value from D2 to D8, followed by a

slight decline at D10. UPMR30 also recorded an increase from D2 to D4, followed by a gradual decline until D10.

The quantification of phosphate solubilization by the bacteria in the current study was repeated using a relatively more accurate technique called vanadomolydophosphoric acid method. The amount of soluble phosphate derived from the solubilization of insoluble form of phosphate [tricalcium phosphate, $Ca_3(PO_4)_2$] was measured. All inoculums media (NBRIP broth) showed a continuous drop in pH upon bacterial inoculation and prolonged incubation period (**Fig. 4**). The pH of the uninoculated broth remained constant at 7.0 throughout the duration of the 12-days experiment. The amount of P solubilized was directly related to the drop in media pH. Inoculation with strain UPMR31 showed the highest drop of pH (25%) at 12 Days After Incubation (DAI), compared to the pH at 6 DAI, followed by the broth inoculated with UPMB10 which

showed a pH drop rate of 9.7%. At 6 DAI, UPMB10 reduced the pH of media to 5.45 which was the lowest among the treatment and this resulted in a significantly high inorganic phosphate solubilization (51.14 µg mL^{-1} P) compared to the other strains **Fig. 5**. However, upon prolonged incubation, UPMR31 reduced the media pH to the lowest value at 4.71 and hence produced the highest phosphate solubilization rate (61.87 µg mL^{-1}). This was also true for inoculation with UPMB10, with the drop in pH to 4.92 and the rate of phosphate solubilization at 56.59 µg mL^{-1}.

Amount of K solubilized by these bacterial strains ranged from 10.70 µg mL^{-1} to 14.15 µg mL^{-1}, with UPMB10 able to significantly solubilize the highest K, followed by UPMB19, UPMB20, UPMR30 and UPMR31 (**Fig. 6**). He selected isolates were also tested for other biochemical tests such as siderophore and hydrolyzing enzyme production (**Table 6 and Fig. 7**). All isolates were able to produce siderophose, with UPMB19 and UPMB20 showing a slightly higher production rates through the halo zone observation. Meanwhile, isolates UPMB10, UPMB19 and UPMB20 were able to secrete cellulase while the ability to produce pectinase only existed for the latter strain.

Fig. 2. Acetylene reduction assay of the selected isolates Note: Different letters within each column are significantly different at 0.05 level
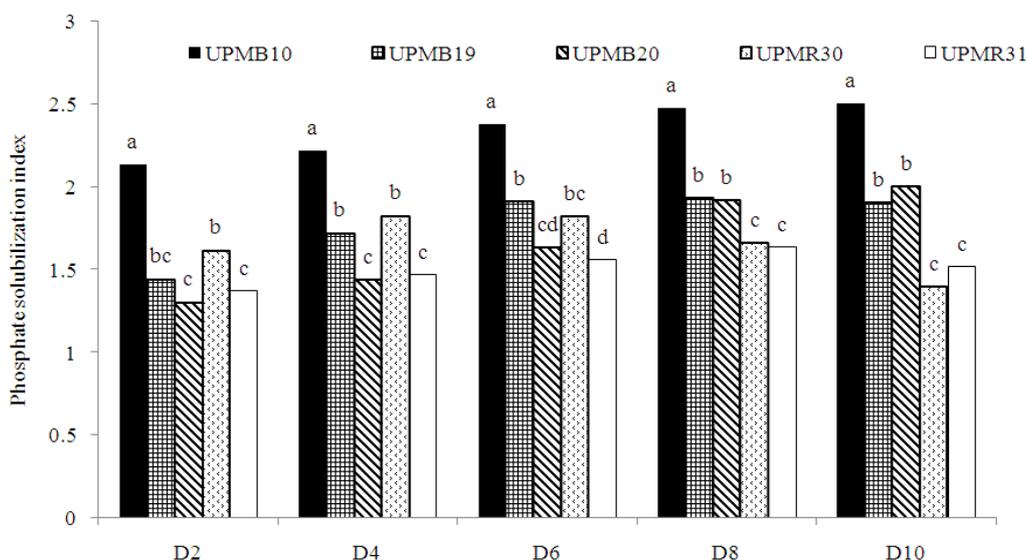

Fig. 3. Phosphate solubilization index of the selected isolates Note: Different letters within each column are significantly different at 0.05 level
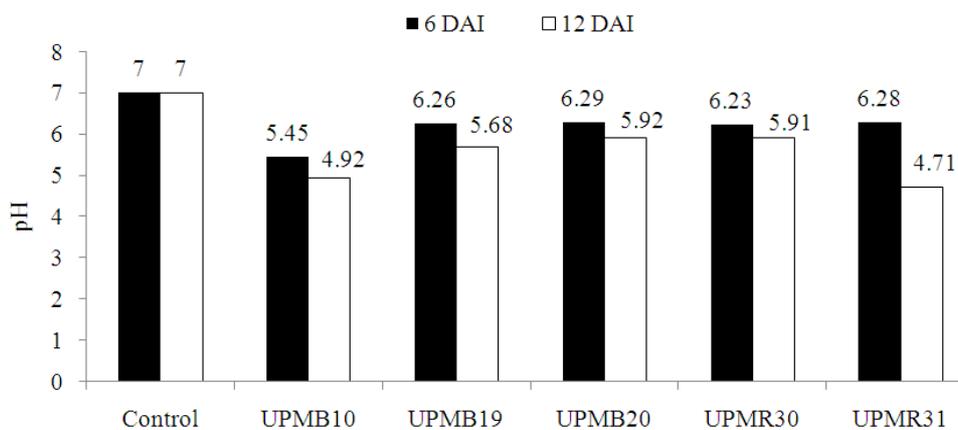
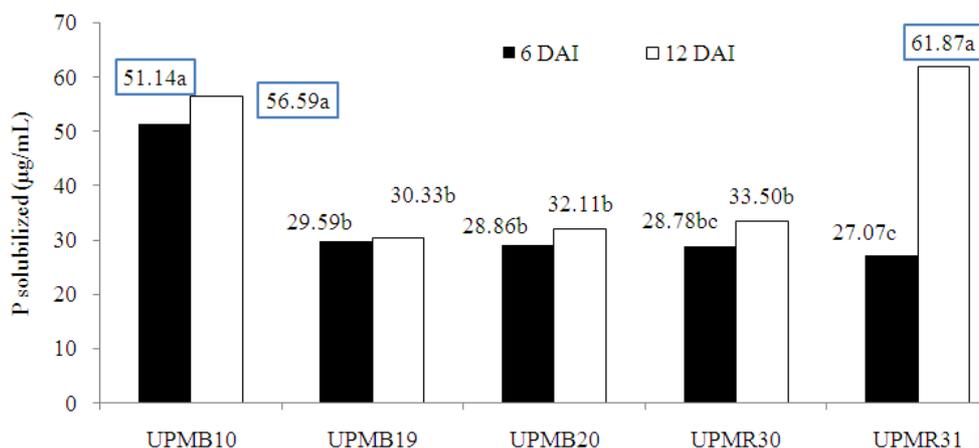**Fig. 4.** Changes in pH of the medium by bacterial inoculations

Fig. 5. Phosphate solubilization rate of the selected isolates Note: Different letters within each column are significantly different at 0.05 level
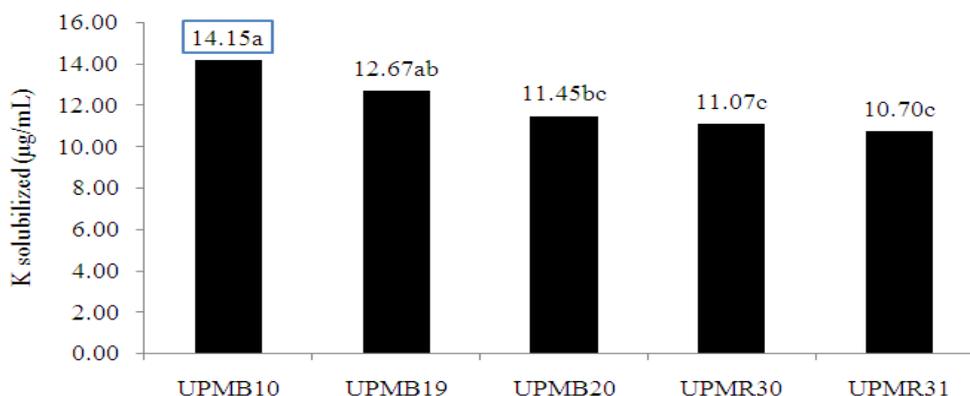

Fig. 6. Potassium solubilization rate of the selected isolates at 5 DAI Note: Different letters within each column are significantly different at 0.05 level
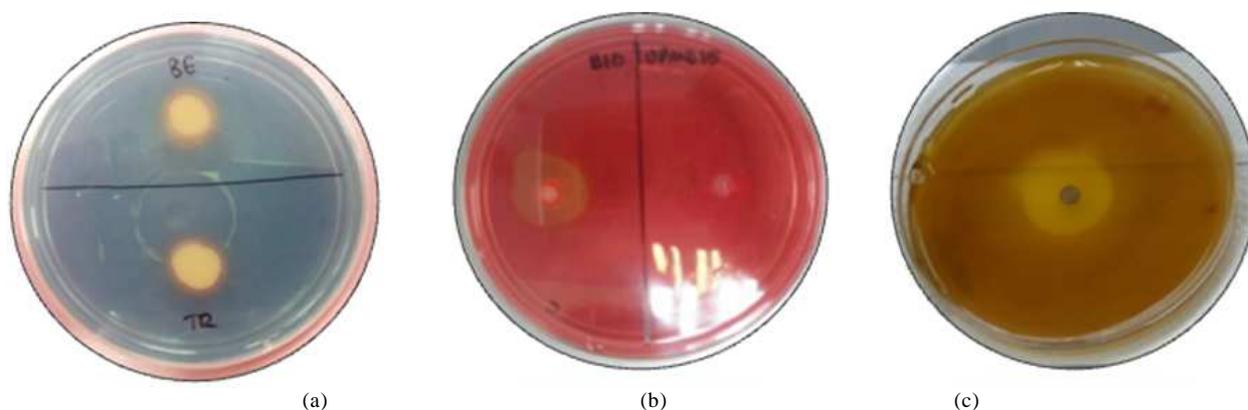
(a)	(b)	(c)

Fig. 7. Chrome Azurol S (a) cellulose (b) pectinase (c) agar plates after inoculation with selected isolates **Note:** Positive results areindicated by the formation of halo zonearound the colonies

Table 6. Assessment of siderophore and hydrolyzing enzyme production of selected isolates

Strain	Siderophore production	Cellulase production	Pectinase production
UPMB19	++	++	-
UPMB20	++	+	-
UPMR30	+	-	-
UPMR31	+	-	-
UPMB10	+	+++	+++

Note: + = Positive; - = Negative

Table 7. Bacterial identification using 16S rDNA gene amplification

Isolates	Molecular Identification			
	16S rDNA fragment length (bp)	Closest relatives in NCBI	NCBI accession number	Similarity (%)
UPMB10	1451	*Bacillus subtilis* strain Ljb-4	KF649246.1	97
UPMB19	1441	*Lysinibacillus xylanilyticus* strain GDLY1	JN999887.1	94
UPMB20	1443	*Alcaligenes faecalis* strain B17	KF641851.1	97
UPMR30	1486	*Bradyrhizobium japonicum* strain HHB-02	DQ517954.1	99
UPMR31	1429	*Rhizobium etli bv. mimosae* strain Mim-1	DQ648573.1	99

The selected bacterial isolates were identified via sequencing of its 16s rDNA gene. As shown in **Table** 7, BLAST searches against the NCBI nucleotide database revealed close relationship to known plant-associated bacteria, including genera *Bacillus*, *Lysinibacillus*, *Alcaligenes*, *Bradyrhizobium* and *Rhizobium*.

These five selected isolates were then used in plant inoculation studies in plant growth chambers under sterile condition. The rice seedlings were inoculated during transplanting at the rate of 2 mL/seedling. At 14 Days After Transplanting (DAT), plants were harvested and various parameters including plant height and Total Dry Weight (TDW) and bacterial population and IAA production in the residual nutrient solution were taken.

Inoculation with UPMB10 and UPMR31 produced significantly taller rice seedlings at 17.15 cm and 17.5 cm and heavier total dry weight at 74.7 mg and 74.65 mg, respectively, at 14 DAT (**Fig. 8 and 9**). All other inocula also resulted in significantly taller rice seedlings at 7 and 14 DAT and total dry weight

without fertilizer-N application. UPMB19 treatment produced the tallest riceseedlings at 7 DAT but not towards the end of the experiment.

Results on survival of bacteria in residual plant nutrient solution showed that UPMB10 and UPMR31 isolates survived better in the residual nutrient solution by maintaining their population at 5.9×10^8 and 6.6×10^8 CFU mL^{-1}, respectively (**Fig. 10**). In the current study, the addition of 33% fertilizer-N lowered the bacterial

population in all treatments except for UPMB19 while UPMB10 maintained the significantly high population at 2.6×10^8 CFU mL^{-1}.

At 7 DAT, UPMB10, UPMB20 and UPMR30 significantly increased the IAA production to 0.678, 0.592 and 0.603 µg mL^{-1}, respectively (**Fig. 11**). UPMB10 maintained a significantly higher IAA, 0.774 µg mL^{-1}, at 14 DAT. A similar result was also observed for treatment with UPMR31, 0.773 µg mL^{-1}.
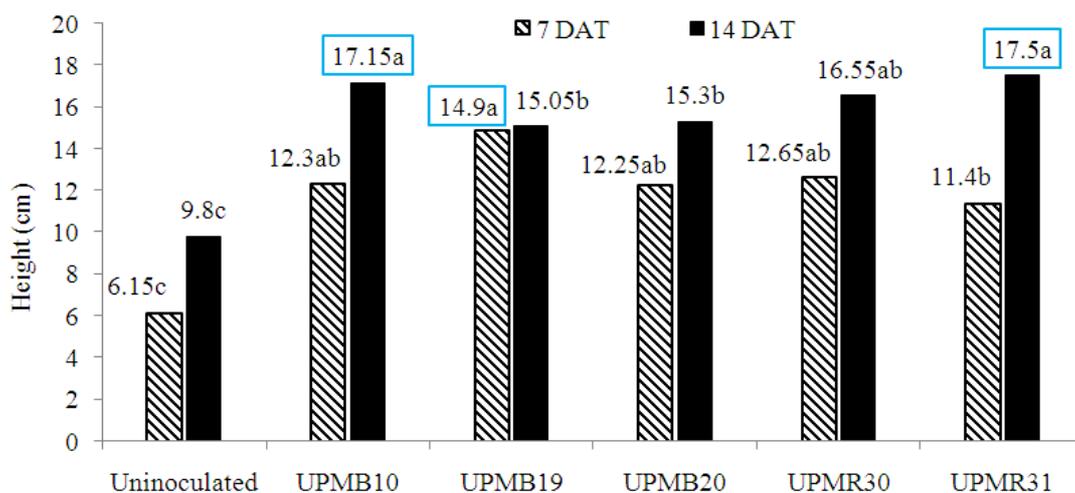

Fig. 8. Effects of rhizobial and PGPR inoculation (with no fertilizer-N) on height of seedlings at 7 and 14 DAT Note: Different letters within each column are significantly different at 0.05 level
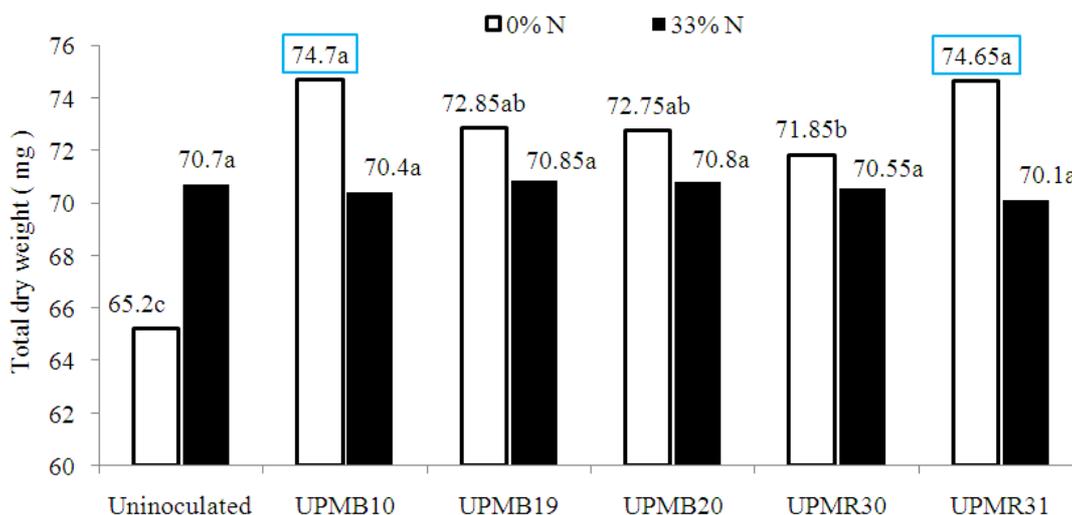

Fig. 9. Effects of rhizobial and PGPR inoculations on TDW of seedlings provided with 0 and 33% fertilizer-N(14 DAT) Note: Different letters within each column are significantly different at 0.05 level
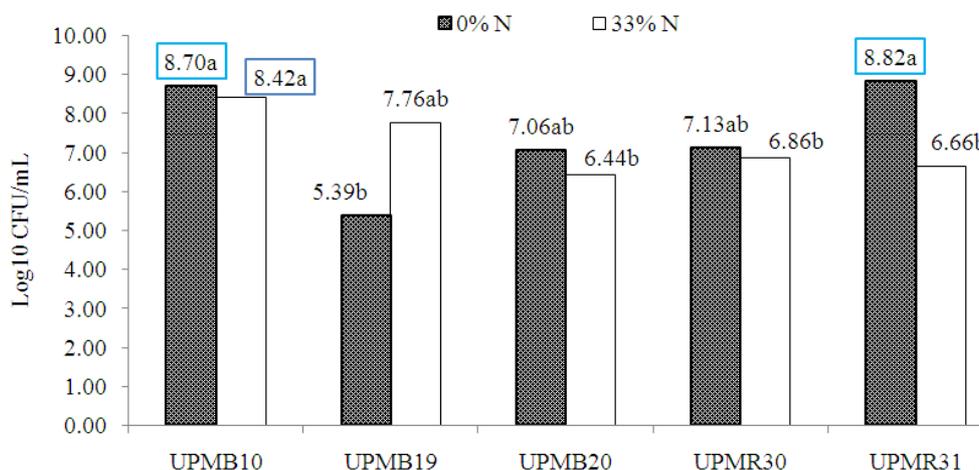
Fig. 10. Total bacterial population in residual plant nutrient solution provided with 0% and 33% fertilizer-N at 7 DAT
   Note: Different letters within each column are significantly different at 0.05 level
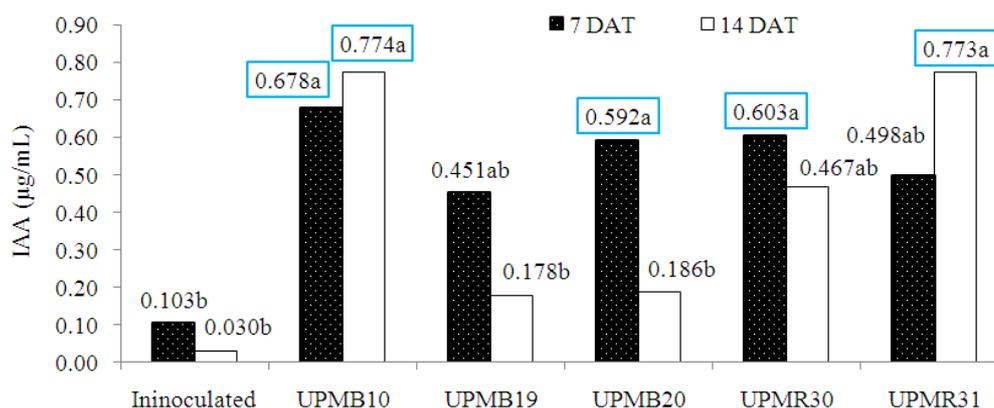

Fig. 11. Effects of different isolates on Indole-3-acetic acid production at 7 and 14 DAT with 0% fertilizer-N addition
   Note: Different letters within each column are significantly different at 0.05 level

4. DISCUSSIONS

Several locations were chosen to observe biodiversity of bacterial isolates due to different cultural agronomic practices and geographical distribution. It is hypothesized that there would be greater chances of beneficial bacterial interactions with plant roots from locations which practice minimal and chemical-free planting systems, namely in Terengganu, Kedah and Kelantan. Isolation at Sik, Kedah represented a major rice growing state located on the north-west coast of Peninsular Malaysia. Rice fields at Tunjung, Kelantan and Sik, Kedah practiced the System of Rice Intensification (SRI) which is a chemical-free system. Bacterial isolation at these locations are vital due to the

higher chances of bacterial interactions with the rice plant roots. PGPR and rhizobia play more important roles especially in supplying nutrients to the plants in a less fertilized soil. Adesemoye and Kloepper (2009) reported an equivalent plant growth, yield and nutrient uptake of tomato when supplied with 75% of recommended fertilizer with addition of microorganism inoculants, compared to fully fertilized plants. ^{15}N studies in 2012 have revealed that UPMB10 (*Bacillus sp.*) fixed 67% of nitrogen in the young oil palm through biological N$_2$ fixation (Zakry *et al.*, 2012). It seems like the PGPR isolates at sites with long history of rice cultivation have developed a survival mechanism against harsh rhizospheric environment by acquiring an ability to penetrate the cell wall and reside within the root tissues

as endophytes. One of the probable mechanism is through production of hydrolyzing enzymes namely cellulase and pectinase, as suggested by Reinhold-Hurek and Hurek (1998). Besut produced the highest number of PGPR isolation and this was anticipated since the rice farmers at Besut practised minimal chemical fertilizer system, which increased bacterial interactions with plant roots. Nutrients were supplied to the plant through conventional system at UPM, thus lesser bacterial population was found. Meanwhile, organic farming areas in Kedah and Kelantan which did not apply chemical fertilizers would not have the starter plant nutrients for the bacteria to interact initially with the plant roots for plant growth promotion. This hypothesis was supported by Anderson and Peterson (1978) who suggested the importance of starter fertilizer in sugar beet when there was limited root growth due to nutrient deficiencies. The soil microbes also required minimal supply of nitrogen as starter-N to grow and establish to produce beneficial effects on oil palm seedlings (Ai'shah *et al.*, 2010). Nevertheless, the numbers of PGPR isolated from each location were almost similar with findings by Jha and Subramaniam (2012), Panhwar *et al.* (2012) and Bhromsiri and Bhromsiri (2010) who had collected 35, 43 and 68 bacterial strains, respectively, from various locations of rice field.

N$_2$-fixation ability was the first screening criteria of the isolates since this characteristic is vital and crucial to the plants as a potential alternative to reduce chemical N-fertilizer application. Bacterial strains with dual beneficial characteristics isolated from this study are slightly higher than isolation percentage of N$_2$-fixing bacteria from Korean rice fields (Islam *et al.*, 2012), 32 out of 165 (19.4%) and lower than isolation of diazotrophs from pepper vine (Zakry *et al.*, 2010, 14 out of 45 (31.1%). Diazotrophs contain nitrogenase enzyme and thus capable of fixing atmospheric nitrogen into ammonium which are subsequently absorbed by plants via Biological N$_2$ Fixation (BNF) process (Boddey *et al.*, 1995). It has been reported by Vargas *et al.* (2012) that rice seedlings can obtain variable contributions from BNF namely increased lateral root development and ethylene signalling responses upon inoculation with diazotrophic bacteria such as *Azospirillum brasilense* sp245 and *Bukholderia kururiensis* M130.

The IAA production by bacterial isolates in this study were almost similar with selected isolates of *Azotobacter*, fluorescent *Pseudomonas* and *Bacillus* from rhizospheric soils of different crops namely mustard, barseem, wheat, sugarcane, brinjal, onion, cauliflower, cabbage and root

nodules of chick pea (Ahmad *et al.*, 2008); *Corynebacterium agropyri*, *Enterobacter gergoviae* and *Bacillus amyloliquefaciens* (Ng *et al.*, 2012) and from rice soil diazotrophic isolates (Naher *et al.*, 2009) which ranged from 7.03-22.02, 3.56-24.32 and 32-69 μg mL^{-1} respectively, when using the same concentration of tryptophan as the precursor (1 mL/20 mL broth). IAA production is believed to be crucial for promotion of root elongation and root hair development. Biswas *et al.* (2000) suggested a probable connection between increased tiller production and yield of rice with a change in hormonal balance, particularly IAA, due to rhizobial inoculation. Duangpaeng *et al.* (2012) conducted the same method as in the current study by selecting endophytic bacteria based on their IAA production and showed that several high IAA-producing bacteria increased shoot and root length of rice, while producing the highest number of shoots per plant and shoot height in paper germination on Petri dish and in pot experiments.

The Acetylene Reduction Assay (ARA) is an indirect method to quantify Biological N$_2$ Fixation (BNF) rate since it measures the conversion of acetylene to ethylene by the nitrogenase enzymes, similar to the reduction of N$_2$ to NH$_3$ in BNF by diazotrophs. The ethylene produced by these bacterial strains were much higher than those recorded by Katupitiya *et al.* (1995) with *Azospirillum* (Sp7) inoculation which produced only 2.3 nmol C$_2$H$_4$/plant/hour. This is also true when compared with diazotroph isolates from rice by Naher *et al.* (2009) which ranges from 6.1×10^{-8} to 1.2×10^{-3} nmol C$_2$H$_4$/cfu/hr. The selection of these bacterial strains in the current study was crucial for the next plant inoculation test to minimize inorganic fertilizer-N usage while still benefitting other positive effects of the plant-bacterial interactions.

Meanwhile, phosphate solubilization index value for UPMB10 and UPMB20 showed a gradual increased from D2 to D10 which suggested that these bacterial strains could still solubilize more insoluble phosphate even after 10 days of incubation period. Thus, these two PGPR strains could be more beneficial to plants, since the insoluble phosphate solubilization process occurs in the soil for a longer period. Phosphate solubilization occurs when the microbes release metabolites such as organic acids (Ramachandran *et al.*, 2005; Panhwar *et al.*, 2012) which will reduce the media pH and consequently solubilize the insoluble tricalcium phosphate, Ca$_3$(PO$_4$)$_2$. This criterium is vitally important in the preliminary screening for potential biofertilizer inoculum, especially to minimize the excessive inputs of inorganic phosphate fertilizers in soil environment, as earlier suggested by

Sitepu *et al.* (2007). This criterium was also assessed by Afzal and Asghari (2008) whereby inoculation with Phosphate Solubilizing Bacteria (PSB) along with 30-40% of P fertilizer resulted in improved grain yield of wheat compared to sole application of P fertilizer without PSB. There are reports of contradictory results between halo zone plate detection and P solubilization in liquid culture (Rashid *et al.*, 2004). Nautiyal (1999) has shown that phosphate solubilization test based on NBRIP broth assay has higher efficiency up to 3-fold. Inorganic phosphate solubilization was directly related to the decline in pH (Asea *et al.*, 1988) and this has been reported to be due to the production of organic acids and acid phosphatases by the microbes (Perez *et al.*, 2007; Rodriguez and Fraga, 1999). Similar findings were found in this study which further supports the previous reports. Meanwhile, a group of researchers from China have also shown that P released from insoluble form of phosphate was negatively correlated to the solution pH (Liu *et al.*, 2012). Several strains of *Rhizobium* were also reported to be able to solubilize insoluble phosphate compound, hydroxyapatite and the mechanism involved was the production of 2-ketogluconic acid by the rhizobial strains (Halder and Chakrabartty, 1993).

Potassium solubilization rates for these bacterial strains were higher than *Bacillus mucilaginous* MCRCp1 which was reported to solubilize K at the rate of 4.29 µg mL^{-1} after 4 days of incubation (Sugumaran and Janarthanam, 2007). The bacteria might produce acids, alkalis or chelants which enhance the release of elements from potassium bearing minerals such as muscovite mica.

Siderophore production test using CAS agar plate is useful for rapid screening of potential beneficial bacterial isolates; bacteria with the ability to produce siderophore can enhance plant growth by increasing the availability of iron near the roots for plant uptake (Alexander and Zuberer, 1991). Several reports have claimed that hydrolyzing enzyme production, particularly cellulase and pectinase, could be the mode of entry for the bacteria into the roots and act as endophytes. This is because cell wall is mainly composed of cellulose while the middle lamella between cell walls contains mainly pectin. However, this probability has not been fully studied in diazotrophic endophytes (Reinhold-Hurek and Hurek, 1998), thus the significance of this study. Strain UPMB20 is an endophyte, while strain UPMB19 was isolated from rice root rhizosphere. Previous study using Scanning Electron Microscopy (SEM) has shown that UPMB10 has the ability to act as an endophyte by colonizing the root interiors of rice plant (Premalatha, 2006). Hence, there is a probability that strain UPMB19 could also become endophytic with its cellulase production. Vessey (2003) also had earlier stated in a review that PGPR which have cellulase and pectinase activities could certainly aid in the infection process by gaining entry into the root system.

Based on 16S rDNA gene sequencing, UPMB19, UPMB20, UPMR30, UPMR31 and UPMB10 were identified under several genera: *Lysinibacillus, Alcaligenes, Bradyrhizobium, Rhizobium* and *Bacillus*, respectively. *Bacillus subtilis* has been explored as a great biocontrol agent in various crops by acting as an elicitor of plant induced systemic resistance (ISR) to pests and pathogens (Kloepper *et al.*, 2004; Lugtenberg and Kamilova, 2009; Lucy *et al.*, 2004). *Lysinibacillus xylanilyticus* was isolated by other researchers from a compost of fruit and vegetable waste and shown to be able to solubilize tri-calcium phosphate, produce phytohormone (IAA), carried nif*H* gene for BNF activities and successfully improved growth of wheat (*Triticum aestivum*) under controlled conditions (Hayat *et al.*, 2013). *Alcaligenes faecalis* has been shown to secrete IAA as plant growth-promoter in young lettuce seedling (Barazani and Friedman, 1999), while others have classified this strain as a salt-tolerant bacteria and a potential human pathogen (Egamberdieva *et al.*, 2008). *Bradyrhizobium* and *Rhizobium* are two well-known bacteria in legume symbiosis, with vital roles on nodule formation and BNF (Nguyen *et al.*, 2012; Suzuki *et al.*, 2014; Klonowska *et al.*, 2012).

Results from the plant inoculation test showed that isolates from different crops are able to increase the growth of rice plants (taller and heavier) since UPMB10 was isolated from oil palm and UPMR31 was from *Mimosa pudica*. UPMB19 produced tallest seedlings in the early observation but not towards the latter stage, indicating that this strain has the ability to promote seedling growth as early as 7 DAT, while UPMB10 and UPMR31 performed better at 14 DAT. The beneficial effects of bacterial inoculation were possibly derived through biological N_2 fixation particularly when no fertilizer-N was provided. Treatment with UPMB19 also showed significantly higher N_2 fixation rate compared to the other strains except UPMB20 (**Fig. 1**). A greater rhizobial inoculation effects on plant photosynthesis rate has been reported with zero-N treatment, compared to treatment with 90 kg N Ha^{-1} (Peng *et al.*, 2002). Yanni *et al.* (1997) also stated that two endophytic isolates of *Rhizobium leguminosarum* bv. trifolii

significantly increased shoot and root growth of rice in plant growth chamber experiment. This early growth promotion effects on rice seedlings has also been observed by Vargas *et al.* (2012) upon inoculation with diazotrophic bacteria which promoted lateral root development. Ashrafuzzaman *et al.* (2009) reported a significant increase in plant height, root length and dry matter production of shoot and root of rice seedling in a pot experiment. Isolates UPMB10 and UPMR30 maintained a significantly higher bacterial population possibly due to their abilities to actively interact with the rice roots when provided with zero amount of fertilizer-N. Similarly, Chi *et al.* (2005) and James *et al.* (2002) observed high endophytic rhizobial population in rice which reached 9×10^{10} rhizobia per cm^3 tissues and 10^6 CFU per gram of fresh weight, respectively. The increased population of UPMB19 with increased fertilizer-N supply indicates that this PGPR favours condition with minimal inorganic-N for a population increment of 44% compared to the treatment with zero fertilizer-N. The importance of minimal fertilizer-N as a starter nutrient for plants and bacteria to grow and interact beneficially has been well-demonstrated by other researchers (Becker *et al.*, 1991; Mia *et al.*, 2010a). This could have led to the increase in plant height and total dry weight observed in UPMB10 and UPMR31 inoculations. The significantly higher IAA productions by UPMB10 and UPMR31 were believed to have caused greater root development and consequently increased plant height and TDW (**Fig. 6 and 7**) as also observed by Patten and Glick (2002). In their case, inoculation with bacterial isolates which produced IAA caused longer primary roots of canola and more numerous adventitious roots in mung bean cuttings. PGPR and rhizobia have been suggested to stimulate growth of rice seedlings by producing phytohormones, particularly IAA, which promote seed emergence and other seedling attributes (Mia *et al.*, 2012; Keyeo *et al.*, 2011). Battacharjee *et al.* (2012) also found that rhizobial strain namely *Rhizobium leguminosarum* could secrete phytohormones such as IAA and increase the biomass, root branching and N content of rice plants.

5. CONCLUSION

From the current study, inoculation of rice plant with three Plant Growth-Promoting Rhizobacteria (PGPR) and two indigenous rhizobial isolates from tropical Malaysian soils promoted plant growth which could be directly attributed to the beneficial effects from biological N_2 fixation and phytohormone production and indirectly to phosphate and potassium solubilizations, iron siderophore and hydrolyzing enzyme productions. These strains belong to genera *Bacillus*, *Lysinibacillus*, *Alcaligenes*, *Bradyrhizobium* and *Rhizobium*, respectively. Hence, these findings could be useful and crucial for the preliminary biofertilizer development for rice and other non-legume crops in Malaysia and the tropics. These initial findings are useful as a fundamental information on the effects of local beneficial strains on early plant growth, thus a subsequent glasshouse and field trials need to be undertaken to elucidate the contributions of these PGPR and rhizobia on tillering and yield of rice with reduced fertilizer-N rates.

6. ACKNOWLEDGEMENT

7. REFERENCES

Adesemoye, A.O. and J.W. Kloepper, 2009. Plant-microbes interactions in enhanced fertilizer-use efficiency. Applied Microbiol. Biotechnol., 85: 1-12. DOI: 10.1007/s00248-009-9531-y

Afzal, A. and B. Asghari, 2008. *Rhizobium* and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum* L.). Int. J. Agri. Biol., 10: 1560-8530. Ahmad, F., I. Ahmad and M.S. Khan, 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res., 163: 173-181. DOI: 10.1016/j.micres.2006.04.001

Ai'shah, O.N., H.G. Amir, C.L. Keng and A.R. Othman, 2010. Influence of various combinations of diazotrophs and chemical N fertilizer on plant growth and nitrogen fixation capacity of oil palm seedlings (*Elaeis guineensis* Jacq.). Thai J. Agric. Sci., 43: 139-149.

Alexander, D.B. and D.A. Zuberer, 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol. Fertil. Soil., 12: 39-45. DOI: 10.1007/BF00369386

Amir, H.G., Z.H. Shamsuddin, M.S. Halimi, M.F. Ramlan and M. Marziah, 1999. Potential use of rhizobacteria for sustainable oil palm seedling production. Malaysian J. Soil. Sci., 3: 39-50.

Amir, H.G., Z.H. Shamsuddin, M.S. Halimi, M.F. Ramlan and M. Marziah, 2003. Nitrogen fixation, nutrient accumulation and plant growth promotion by rhizobacteria in association with oil palm seedlings. Pak. J. Biol. Sci., 6: 1269-1272.

Amir, H.G., Z.H. Shamsuddin, M.S. Halimi, M.F. Ramlan and M. Marziah, 2005. Enhancement in nutrient accumulation and growth of oil palm seedlings caused by PGPR under field nursery conditions. Commun. Soil Sci. Plant Anal., 36: 2059-2066. DOI: 10.1080/00103620500194270

Anderson, F.N. and G.A. Peterson, 1978. Optimum starter fertilizer placement for sugarbeet seedlings as determined by uptake of radioactive ^{32}P isotope. J. Am. Society Sugar Beet Technol., 20: 19-24.

Araujo, A.E.D.S., V.L.D. Baldani, P.D.S. Galisa, J.A. Pereira and J.I. Baldani, 2013. Response of traditional upland rice varieties to inoculation with selected diazotrophic bacteria isolated from rice cropped at the Northeast region of Brazil. Applied Soil. Ecol., 64: 49-55. DOI: 10.1016/j.apsoil.2012.10.004

Asea, P.E.A., R.M.N. Kucey and J.W.B. Stewart, 1988. Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. Soil Biol. Biochem., 20: 459-464. DOI: 10.1016/0038-0717(88)90058-2

Ashrafuzzaman, M., F.A. Hossen, M.R. Ismail, M.A. Hoque and M.Z. Islam *et al.*, 2009. Efficiency of Plant Growth-Promoting Rhizobacteria (PGPR) for the enhancement of rice growth. African J. Biotechnol., 8: 1247-1252.

Barazani, O.Z. and J. Friedman, 1999. Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? J. Chemical Ecol., 10: 2397-2406. DOI: 10.1023/A:1020890311499

Barton, C.J., 1948. Photometric analysis of phosphate rock. Anal. Chem., 20: 1068-1073. DOI: 10.1021/ac60023a024

Battacharjee, R.B., P. Jourand, C. Chaintreuil, B. Dreyfus, A. Singh and S.N. Mukhopadhyay, 2012. Indole acetic acid and ACC deaminase -producing *Rhizobium leguminosarum* bv. *trifolii* SN10 promote rice growth and in the process undergo colonization and chemotaxis. Biol. Fert. Soils, 48: 173-182. DOI: 10.1007/s00374-011-0614-9

Becker, M., K.H. Diekmann, J.K. Ladha, S.K.D. Datta and J.C.G. Ottow, 1991. Effect of NPK on growth and nitrogen fixation of *Sesbania rostrata* as a green manure for lowland rice (*Oryza sativa* L.). Plant Soil, 132: 149-158. DOI: 10.1007/BF00011021

Bhromsiri, C. and Bhromsiri, 2010. Isolation, screening of growth-promoting activities and diversity of rhizobacteria from vetiver grass and rice plants. Thai J. Agric. Sci., 43: 217-230.

Biswas, J.C., J.K. Ladha and F.B. Dazzo, 2000. Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci. Society Am. J., 64: 1644-1650. DOI: 10.2136/sssaj2000.6451644x

Boddey, R.M., J.C.D.S. Moraes, B.J.R. Alves and S. Urquiaga, 1997. The contribution of biological nitrogen fixation for sustainable agricultural systems in the tropics. Soil Biol. Biochem., 29: 787-799. DOI: 10.1016/S0038-0717(96)00221-0

Boddey, R.M., O.C.D. Oliveira, S. Urquiga, V.M. Reis and F.L.D. Olivares *et al.*, 1995. Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. Plant Soil, 174: 195-209. DOI: 10.1007/BF00032247

Chabot, R., H. Antoun and M.P. Cescas, 1996. Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum* biovar. *phaseoli.* Plant Soil, 184: 311-321. DOI: 10.1007/BF00010460

Chi, F., S.H. Shen, H.P. Cheng, Y.X. Jing and Y.G. Yanni *et al.*, 2005. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Applied Environ. Microbiol., 71: 7271-7278. DOI: 10.1128/AEM.71.11.7271-7278.2005

Dobereiner, J. and J.M. Day, 1975. Associative symbiosis in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites. Proceedings of the 1st International Symposium on Nitrogen Fixation, (SNF' 75), Washington State University Press, Pullman, pp: 518-538.

Duangpaeng, A., P. Phetcharat, S. Chanthapho, N. Boonkantong and N. Okuda, 2012. The study and development of endophytic bacteria for enhancing organic rice growth. Procedia Eng., 32:172-176. DOI: 10.1016/j.proeng.2012.01.1253

Egamberdieva, D., F. Kamilova, S. Validov, L. Gafurova and Z. Kucharova *et al.*, 2008. High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. Environ. Microbiol., 10: 1-9. DOI: 10.1111/j.1462-2920.2007.01424.x

Egener, T., T. Hurek and B. Reinhold-Hurek, 1999. Endophytic expression of *nif* genes of *Azoarcus* sp. strain BH72 in rice roots. Molecular Plant-Microbe Interact., 12: 813-819. DOI: 10.1094/MPMI.1999.12.9.813

FAO., 2004. Food and Agriculture Organization. Rice is Life. Italy: FAO.

Farzana, Y., O. Radziah, S. Kamaruzaman and S.S. Mohd, 2007. Effect of PGPR inoculation on growth and yield of sweet potato. J. Biol. Sci., 7: 421-424. DOI: 10.3923/jbs.2007.421.424

Farzana, Y., O. Radziah, S. Said and S. Kamaruzaman 2009. Growth and storage root development of sweetpotato inoculated with rhizobacteria under glasshouse conditions. Australian J. Basic Applied Sci., 3: 1461-1466.

Gordon, S.A. and R.P. Weber, 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol., 26: 192-195. DOI: 10.1104/pp.26.1.192

Halder, A.K. and P.K. Chakrabartty, 1993. Solubilization of inorganic phosphate by *Rhizobium*. Folia Microbiol., 38: 325-330. DOI: 10.1007/BF02898602

Hardy, R.W.F., R.D. Holsten, E.K. Jackson and R.C. Burns, 1968. The acetylene-ethylene assay for nitrogen fixation: Laboratory and field evaluation. Plant Physiol., 43: 1185-1207. DOI: 10.1104/pp.43.8.1185

Hayat, R., R.A. Sheirdil, M. Iftikar-ul-Hassan and I. Ahmed, 2013. Characterization and identification of compost bacteria based on 16S rRNA gene sequencing. Ann. Microbiol., 63: 905-912. DOI: 10.1007/s13213-012-0542-4

Hu, X., J. Chen and J. Guo, 2006. Two phosphate- and potassium solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. World J. Microbiol. Biotechnol., 22: 983-990. DOI: 10.1007/s11274-006-9144-2

Islam, M.R., T. Sultana, J. Cho, M.M. Joe and T.M. Sa, 2012. Diversity of free-living nitrogen-fixing bacteria associated with Korean paddy fields. Ann. Microbiol., 62: 1643-1650. DOI: 10.1007/s13213-012-0421-z

James, E.K., P. Gyaneshwar, N. Mathan, W.L. Barraquio and P.M. Reddy *et al.*, 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* z67. Molecular Plant-Microbe Interact., 15: 894-906. DOI: 10.1094/MPMI.2002.15.9.894

Jha, Y. and R.B. Subramaniam, 2012. Isolation of root associated bacteria from the local variety of rice GJ-17. World Res. J. Geo Inform., 1: 21-26.

Kasana, R.C., R. Salwan, H. Dhar, S. Dutt and A. Gulati, 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current Microbiol., 57: 503-507. DOI: 10.1007/s00284-008-9276-8

Katupitiya, S., J. Millet, M. Vesk, L. Viccars and A. Zeman *et al.*, 1995. A mutant of *Azospirillum brasilense* Sp7 impaired in flocculation with a modified colonization pattern and superior nitrogen fixation in association with wheat. Applied Environ. Microbiol., 61: 1987-1995. PMID: 7646034

Kennedy, I.R., A.T.M.A. Choudhury and M.L. Kecskes, 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: Can their potential for plant growth promotion be better exploited? Soil Biol. Biochem., 36: 1229-1244. DOI: 10.1016/j.soilbio.2004.04.006

Keyeo, F., O.N. Ai'shah and H.G. Amir, 2011. The effects of nitrogen fixation activity and phytohormone production of diazotroph in promoting growth of rice seedlings. Biotechnology, 10: 267-273. DOI: 10.3923/biotech.2011.267.273

Kloepper, J.W., C. Ryu and S. Zhang, 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology, 94: 1259-1266. DOI: 10.1094/PHYTO.2004.94.11.1259, PMID: 18944464

Kloepper, J.W., J. Leong, M. Teintze and M.N. Schroth, 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature, 286: 885-886. DOI: 10.1038/286885a0

Klonowska, A., C. Chaintreuil, P. Tisseyre, L. Miche, R. Melkonian and M. Ducousso *et al.*, 2012. Biodiversity of Mimosa pudica rhizobial symbionts (Cupriavidus taiwanensis, Rhizobium mesoamericanum) in New Caledonia and their adaptation to heavy metal-rich soils. Microbiol. Ecol., 81: 618-635. DOI: 10.1111/j.1574-6941.2012.01393.x

Kumar, K.V., S. Srivastava, N. Singh and H.M. Behl, 2009. Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea*. J. Hazardous Materials, 170: 51-57. DOI: 10.1016/j.jhazmat.2009.04.132

Liu, Y., L. Feng, H. Hu, G. Jiang and Z. Cai *et al.*, 2012. Phosphorus release from low-grade phosphate rocks by low molecular weight organic acids. J. Food, Agric. Environ., 10: 1001-1007.

Lucy, M., E. Reed and B.R. Glick, 2004. Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek, 86: 1-25. DOI: 10.1023/B:ANTO.0000024903.10757.6e

Lugtenberg, B. and F. Kamilova, 2009. Plant-growth-promoting rhizobacteria. Annual Rev. Microbiol., 63: 541-556. DOI: 10.1146/annurev.micro.62.081307.162918

Malik, K.A., R. Bilal, S. Mehnaz, G. Rasul and M.S. Mirza *et al.*, 1997. Association of nitrogen-fixing, Plant-Growth-Promoting Rhizobacteria (PGPR) with kallar grass and rice. Plant Soil, 194: 37-44. DOI: 10.1023/A:1004295714181

Mia, M.A.B., Z.H. Shamsuddin and M. Mahmood, 2012. Effects of rhizobia and plant growth promoting bacteria inoculation on germination and seedling vigor of lowland rice. African J. Biotechnol., 11: 3758-3765. DOI: 10.5897/AJB09.1337

Mia, M.A.B., Z.H. Shamsuddin, Z. Wahab and M. Marziah 2010a. Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (*Musa* spp. cv. 'Berangan'). Scientia Horticulturae, 126: 80-87. DOI: 10.1016/j.scienta.2010.06.005

Mia, M.A.B., Z.H. Shamsuddin, Z. Wahab and M. Marziah, 2009. The effect of rhizobacterial inoculation on growth and nutrient accumulation of tissue-cultured banana plantlets under low N-fertilizer regime. African J. Biotechnol., 8: 5855-5866.

Mia, M.A.B., Z.H. Shamsuddin, Z. Wahab and M. Marziah, 2010b. Effect of Plant Growth Promoting Rhizobacterial (PGPR) inoculation on growth and nitrogen incorporation of tissue-cultured *Musa* plantlets under nitrogen-free hydroponics condition. Aus. J. Crop Sci., 4: 85-90.

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. DOI: 10.1111/j.1574-6968.1999.tb13383.x

Ng, L.C., M. Sariah, O. Sariam, O. Radziah and M.A.Z. Abidin, 2012. Rice seed bacterization for promoting germination and seedling growth under aerobic cultivation system. Australian J. Crop Sci., 6: 170-175.

Nguyen, T.H.N., L. Brechenmacher, J.T. Aldrich, T.R. Clauss and M.A. Gritsenko *et al.*, 2012. Quantitative phosphoproteomic analysis of soybean root hairs inoculated with bradyrhizobium japonicum. Molecular Cellular Proteom., 11: 1140-1155. DOI: 10.1074/mcp.M112.018028

Panhwar, Q.A., O. Radziah, A.R. Zaharah, M. Sariah and I.M. Razi, 2012. Isolation and characterization of phosphate-solubilizing bacteria from aerobic rice. African J. Biotech., 11: 2711-2719. DOI: 10.5897/AJB10.2218

Patten, C.L. and B.R. Glick, 2002. Role of *Pseudomonas putida* indoleaccetic acid in development of the host plant root system. Applied Environ. Microbiol., 68: 3795-3801. DOI: 10.1128/AEM.68.8.3795–3801.2002

Peng, S., J.C. Biswas, J.K. Ladha, P. Gyaneshwar and Y. Chen, 2002. Influence of rhizobial inoculation on photosynthesis and grain yield of rice. Agron. J., 94: 925-929. DOI: 10.2134/agronj2002.9250

Perez, E., M. Sulbaran, M.M. Ball and L.A. Yarzabal, 2007. Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biol. Biochem., 39: 2905-2914. DOI: 10.1016/j.soilbio.2007.06.017

Pikovskaya, R.I., 1948. Mobilization of phosphorus in soil in connection with vital activity by some microbial species. Microbiologica, 17: 362-370.

Premalatha, P., 2006. Colonization of oryza sativa roots by plant growth-promoting rhizobacterium, Bacillus sphaericus UPMB10 and subsequent inoculant formulation. PhD Thesis, Universiti Putra Malaysia.

Ramachandran, K., V. Srinivasan, S. Hamza and M. Anandaraj, 2005. Phosphate solubilising bacteria isolated from the rhizosphere soil and its growth promotion on black pepper (*Piper nigrum* L.) cuttings. Proceedings of the 1st International Meeting on Microbial Phosphate Solubilization, (MPS' 03), Springer Netherlands, pp: 325-331. DOI: 10.1007/978-1-4020-5765-6_51

Ramamoorthy, V., R. Viswanathan, T. Raguchander, V. Prakasam and R. Samiyappan, 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protect., 20: 1-11. DOI: 10.1016/S0261-2194(00)00056-9

Rashid, M., S. Khalil, N. Ayub, S. Alam and F. Latif, 2004. Organic acids production and phosphate solubilization By Phosphate-Solubilizing Microorganisms (PSM) under *in vitro* conditions. Pak. J. Biol. Sci., 7: 187-196.

Reinhold-Hurek, B. and T. Hurek 1998. Life in grasses: Diazotrophic endophytes. Trend Microbiol., 6: 139-144. DOI: 10.1016/S0966-842X(98)01229-3

Ribeiro, C.M. and E.J.B.N. Cardoso, 2012. Isolation, selection and characterization of root-associated growth promoting bacteria in Brazil Pine (*Araucaria angustifolia*). Microbiol. Res., 167: 69-78. DOI: 10.1016/j.micres.2011.03.003

Rodriguez, H. and R. Fraga, 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv., 17: 319-339. DOI: 10.1016/S0734-9750(99)00014-2

Saharan, B.S. and V. Nehra, 2011. Plant growth promoting rhizobacteria: A critical review. Life Sci. Med. Res., 21: 1-29.

Schwyn, B. and J.B. Neilands, 1987. Universal chemical assay for the detection and determination of siderophores. Analytical Biochem., 160: 47-56. DOI: 10.1016/0003-2697(87)90612-9

Sitepu, I.R., Y. Hashidoko, E. Santoso and S. Tahara, 2007. Potent phosphate-solubilizing bacteria isolated from dipterocarps grown in peat swamp forest in central Kalimantan and their possible utilization for biorehabilitation of degraded peat swamp forest in Central Kalimantan. Proceedings of the International Symposium and Workshop on Tropical Peatland. Aug. 27-29, Yogyakarta, Indonesia.

Somasegaran, P. and H.J. Hoben, 1985. Methods in Legume-*Rhizobium* Technology. 1st Edn., University of Hawaii NifTAL Project and MIRCEN, pp: 367.

Sugumaran, P. and B. Janarthanam, 2007. Solubilization of potassium containing minerals by bacteria and their effect on plant growth. World J. Agric. Sci., 3: 350-355.

Suzuki, Y., D. Adhikari, K. Itoh and K. Suyama, 2014. Effects of temperature on competition and relative dominance of bradyrhizobium japonicum and bradyrhizobium elkanii in the process of soybean nodulation. Plant Soil, 374: 915-924. DOI: 10.1007/s11104-013-1924-5

Vargas, L., T.L.G.D. Carvalho, P.C.G. Ferreira, V.L.D. Baldani and J.I. Baldani *et al*., 2012. Early responses of rice (*Oryza sativa L.*) seedlings to inoculation with beneficial diazotrophic bacteria are dependent on plant and bacterial genotypes. Plant Soil, 356: 127-137. DOI: 10.1007/s11104-012-1274-8

Verma, S.C., J.K. Ladha and A.K. Tripathi, 2001. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J. Biotechnol., 91: 127-141. DOI: 10.1016/S0168-1656(01)00333-9

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil, 255: 571-586. DOI: 10.1023/A:1026037216893

Yanni, Y.G., R.Y. Rizk, V. Corich, A. Squartini and K. Ninke *et al*., 1997. Natural endophytic association between Rhizobium leguminosarumbv. trifolii and rice roots and assessment of its potential to promote rice growth. Plant Soil, 194: 99-114. DOI: 10.1023/A:1004269902246

Yogesh, K., K.K. Vamsi, B. Amol, G. Nikhil and T. Soham *et al*., 2009. Study of pectinase production in submerged fermentation using different isolates of Aspergillus Niger. Int. J. Microbiol. Res., 1: 13-17.

Zakry, F.A.A., M.S. Halimi, K.B.A. Rahim, H.A. Osumanu and S.K. Wong *et al*., 2010. Isolation and plant growth-promoting properties of rhizobacterial diazotrophs from pepper vine (*Piper nigrum* L.). Malays. Appl. Biol., 39: 41-45.

Zakry, F.A.A., Z.H. Shamsuddin, K.A. Rahim, Z.Z. Zakaria and A.A. Rahim, 2012. Inoculation of Bacillus sphaerichus UPMB-10 to young oil palm and measurement of its uptake of fixed nitrogen using the ^{15}N isotope dilution technique. Microbes Environ., 27: 257-262. DOI: 10.1264/jsme2.ME11309