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GROWTH AND NUTRIENTS UPTAKE OF RICE AT EARLY SEEDLING STAGE AS INOCULATED WITH *BACILLUS* SPP.

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Abstract

Utilization of plant growth promoting rhizo-bacteria (PGPR) potentially elevates crop productivity. Concurrently, our study was focused on isolating efficient PGPR strains from the rhizosphere of rice plants. This in order to assess its properties under both laboratory and glass house conditions accordingly. Five (5) bacterial isolates were screened out based on sets of qualitative tests. Among the bacterial isolates, UPMRB9 were selected due to higher production of plant growth promoting traits:-1) nitrogen fixation, 2) phosphate and potassium solubilization, 3) indole-3 acetic acid production as well, 4) siderophore and hydrolyzing enzyme production. The isolates were later identified based on 16S rRNA sequence by which known to be *Bacillus tequilensis*. Subsequently, this potential isolates were inoculated to rice seedlings under control environment (glasshouse conditions). The results amply that seedling biomass was 38.2% (BRR1 dhan67), 38.5% (Putra-1) and 33.9% (MR297) higher in compare to uninoculated control. Similar goes for seedling height at 22.5%, 20.2% and 20.0% higher whilst total chlorophyll content was 22.5%, 20.2%, 20.0% higher respectively. The relative increase of nitrogen for the rice variety BRRI dhan67, Putra-1 and MR297 were 34.38%, 99.65% and 52.22% accordingly; phosphorous was 442.7%, 104.9%, 140.7% accordingly and potassium was 41.5%, 45.8%, 153.7% accordingly. Therefore, the isolates of UPMRB9 could be considered as a potential bio-fertilizer source; yet further evaluations are necessary particularly on its performances under field environment.

Key words : PGPR, Rice, Indole acetic acid, Seedling biomass, Biofertilizer.

Introduction

Globally, the crop production system mostly depends on chemical fertilizers to supply essential plant nutrients. However, potential environmental degradation, high cost incurred and accessibility of chemical fertilizers limits its usage under modern agriculture purview (Majed *et al.*, 2015).

Among the agricultural crops; rice is a staple diet for approximately 3 billion people with ever-existing demands (FAO, 2004). It is projected that the rice production need to be increased by 20% to fulfill the demand of upcoming 4.6 billion peoples by 2025 (Tan *et al.*, 2014). Thus, essential nutrients like NPK fertilizers are the pre-requisite to increase the rice production per unit area. Nonetheless, sole dependencies of chemical fertilizers unable to ensure sustainable returns in a long run. Therefore, alternative tactics which is imperative that can ensure economical rice production, environmental safety control whist maintain long- term ecological balance in agro-ecosystem should be pursued.

On recent note, adaptation of plant growth promoting rhizo-bacteria (PGPR) is becoming more widely accepted in practice of intensive agriculture system at different parts of the world (Majeed *et al.*, 2015). The PGPR are said important in bio-geochemical cycles for carbon, nitrogen, sulphur and phosphorous. Previously, 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; Habib *et al.*, 2016). The main mechanism of PGPR is mostly explained by the release of metabolites for direct stimulation of plant growth. Several other mechanisms of PGPR have been assumed to elucidate the benefits of host plant upon inclusions. These include plant growth regulators or phytohormones such as indole acetic acid (IAA),

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cytokinins, and gibberellins (Glick, 1995; Marques*etal*., 2010). It also able to enhance N_2fixation (Khan, 2005); solubilize inorganic phosphate and mineralize organic phosphate (Glick, 1995; Jeon *et al.*, 2003); production of siderophores, synthesis of antibiotics, enzymes and/or fungicidal compounds, and competition with detrimental microorganisms (Dey *et al.*, 2004; Lucy *et al.*, 2004).

In term of rice yield, the PGPR is said able to increase the rice productivity and quality. Glick *et al.,* (2012) indicated that these rhizo-bacteria are the dominant force in the recycling of nutrients and help in improving the soil fertility. Ahmed *et al.*, (2013) reported that inoculation of rice with *Azospirillum brasilense* able to increase the grain yield by 22.7% over control. The PGPR inoculation to rice effectively said to increase the surface area of roots (Richardson, 2001) and root weight (Cakmakci *et al.*, 2007). Similarly, increased in root length, shoot length, aerial biomass and increased nutrient uptake has been reported in response to the PGPR application (Salamone *et al.*, 2012). Mohammadinejhad-Babandeh *et al.,* (2012) confirmed that *Azotobacter, Azorhizobioum* and *Azospirilium* were able to enhance the rice yield components. Significant influence of phosphate solubilizing bacteria (PSB) on grain yield and biological yield of rice was demonstrated by Vahed *et al.,* (2012).

Knowledge of the native bacterial species, their characterization, and identification is required for understanding the distribution and diversity of indigenous bacteria in the rhizosphere of specific crops (Keating *et al.*, 1995; Chahboune *et al.*, 2011). With increasing awareness regarding the efficiency of chemical fertilizers for agricultural practices, it is important to discover the region specific microbial strains that can be used as a growth promoting and enhancing inoculum to achieve desired crop production (Deepa *et al.*, 2010). Therefore, this study was aim to isolate, characterize and identify the PGPR strains from rhizosphere of rice for PGP potentials. Subsequently, the inoculation of the representative isolates was observed on their effects on early seedling growth of rice.

Materials and Methods

Collection of soil sample

The rhizosphere of soil samples were collected from paddy fields located in seven (7) locations.

Isolation of rhizospheric bacteria

The rhizospheric microbes were isolated from roots of rice plants according to method by Quadt-Hallman *et al.,* (1997). Approximately 10g of soil was kept into a 250 mL conical flask containing 95 mL sterilized water. The soil suspension was kept for 30 min with constant agitation. Later, 1 ml of the suspension was transferred into another test tube containing 9 ml of sterilized water, shake and transfer the aliquot to the next test tube. Serial dilution technique was performed up to 10^{-8}. An aliquot of this suspension was spread on tryptic soy agar (TSA) plates and incubated for 24 to 48 h at 28-30°C for observing colonies developed on it. Subsequently, the number of bacterial colony grown in TSA plate was recorded. A fine isolated colonies were picked and streaked on fresh TSA plate for incubation. This process was carried out thrice (3) to get single colony with high purity.

Screening of potential bacterial isolates

Five (5) different isolates were screened out through various qualitative tests. The isolates were UPMRA4, UPMRB9, UPMRE3, UPMRE6 and UPMRG1. These bacterial isolates were further characterized based on plant growth promoting properties.

Characterization of PGPR

The nitrogen fixation ability was tested by growing the isolates on N-free solid malate medium (Nfb medium) according to method by Dobereiner and Day, (1975). For phosphate solubilization test, the isolated cultures were grown on Pikovskaya agar medium according to method by Pikovskaya, (1948). The phytohormone production namely indole acetic acid (IAA) was determined colorimetrically as described by Gordon and Weber (1951). Whilst, for the solublized potassium selected isolates were grown on a modified Aleksandrov agar medium (Hu *et al*., 2006). The siderophore activity was assayed as described by Schwyn and Neilands (1987). The cellulose enzyme production by the selected bacteria was recorded by using Carboxy Methyl Cellulose (CMC) agar plates according to the method by Kasana *et al.* (2008). Yogesh *et al.*, (2009) method was adopted to screen for pectinase enzymes production. Lastly, for Chrome Azurol S (CAS) agar was similar as per described by Schwyn and Neilands (1987).

Bacterial Identification by 16S rRNA gene Sequence

Partial sequencing of the 16S rRNA gene was identified the selected strains. A fragment of 16 SrRNA gene from the total genomic DNA was amplified by polymerase chain reaction (PCR) using universal forward (5′- GAGTTTGATCCTGCTCAG-3′) and reverse (5′GTTACCTTGTTACGACTT-3′) primers (BioSune Biotechnology Co. Ltd., China). The PCR product was purified using Gel/PCR DNA Mini Kit (Real Biotech, Taiwan) and out-sourced for sequencing to First Base

Laboratories Pvt. Ltd., Selangor, Malaysia. Subsequently, the sequence data was aligned and analyzed to identify the bacterium and its closest neighbors by using BLAST technique (NCBI, USA).

Evaluation of PGPR

The potential PGPR isolates were inoculated to three (3) rice variety based on the potential grain yield production. The three rice varieties were BRRI dhan67 (low yield), MR297 (Moderate yield) and Putra-1 (high yield).

Rice seed treatment

Initially, surface sterilization was conducted for tested seed varieties by soaking the seeds in 95% ethanol for 10 seconds. Then the seeds were agitated with 3% sodium hypochloride (chlorox) for 1 min and rinsed 6 times with sterile distilled water before planting.

Inoculums application to rice seedlings

One loop of 24-hour-old bacterial cultures was inoculated to Trypic Soy Broth (TSB) medium and shake for 24 hours. When plumule and radical emerged, seedlings were treated with PGPR suspensions of approximately 10^8 cfu mL^{-1} concentration as well as non-inoculated TSB medium (as control) for 1h at room temperature (±27^{0}C). Seedlings were then transplanted in a plastic pot filled with 2 kg of sterile soil. In inoculated treatments, plants were given second inoculation with 5.0 mL of washed bacterial cells at 14 days after transplanting (DAT).

Data collection

On 30 DAT, the individual plant leaves area were measured by using Leaf area meter (AM-200, ADC Bio Scientific Ltd., England) and total chlorophyll content following the protocol of Lichtenthaler (1987). The seedlings were harvested at 40 DAT. After measuring the root and shoot length, the plants were oven dried at 72^{o}C for 48 hours; then the weight of dry biomass was measured. The oven dried plants were grinded to measure total nitrogen (N), phosphorous (P) and potassium (K) by adopting wet digestion method and quantified using Atomic Absorption Spectrophotometer (AAS).

Statistical Analysis

The collected data were analyzed using analysis of variance (ANOVA) by SAS 9.4 software. The means comparisons were made by Tukey (HSD) test at a probability level of 0.05.

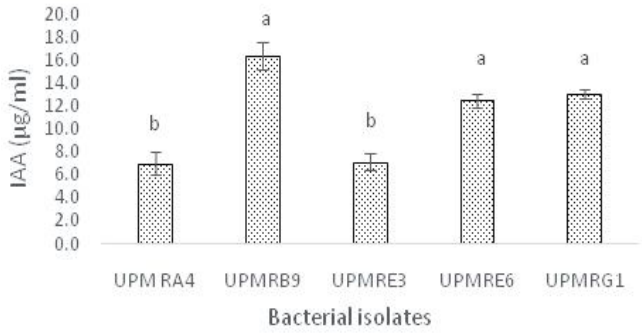
Results and Discussion

Plant growth promoting properties of UPMRB9

The qualitative screening of UPMRB9 reveals that the selected bacterial isolates have the ability to fix atmospheric nitrogen, solubilize phosphate and potassium, producing siderophore as well as hydrolyzing enzyme like cellulase and pectinase table 1.

Indole 3-acetic acid (IAA) production

The IAA production by the bacterial strains was quantified calorimetrically using a spectrophotometer and compared in relative to the IAA standard curve. All of the selected bacterial strains were able to produce IAA with the ranged in between 3.41-16.33 µg mL^{-1}. Among the five (5) isolates highest IAA-producing bacterial strains was UPMRB9 (16.33µg mL^{-1}) followed by UPMRE6 (12.48 µg mL^{-1}) and UPMRG1 (13.06 µg mL^{-1}) accordingly (Fig.1).



Values having same letter in columns do not differ significantly at alpha 0.05 level by tukey (HSD).

Fig. 1: IAA production by the selected bacterial strain.

Identification of PGPR Isolates

Bacterial isolate of UPMRB9 were identified molecularly. The 16S rDNA gene sequences of PGPR isolates were amplified using PCR and approximately 1,413bp for UPMRB9 was notified. The BLASTX sequence analysis revealed that the isolate UPMRB9 matches with the *Bacillus tequilensis* with 99% similarity, the phylogenetic analysis of PGPR isolates were also done based on neighbor-joining bootstrap analysis with 1,000 replications. (Fig. 2)

Effect of bacterial inoculation on total dry biomass of three rice varieties

Significant increments of total dry biomass were recorded via inoculation with UPMRB9 to MR297 rice variety. The value is statistically similar with Putra-1 both for non-inoculated and inoculated conditions. Besides that, highest increase of total biomass was recorded by 38.2%, 38.5%, 33.9% for the rice variety BRRI dhan 67, Putra-1 and MR297 accordingly (Fig. 3)

Effect of bacterial inoculation on seedling height of three rice variety

The selected bacterial isolate exerted a significant

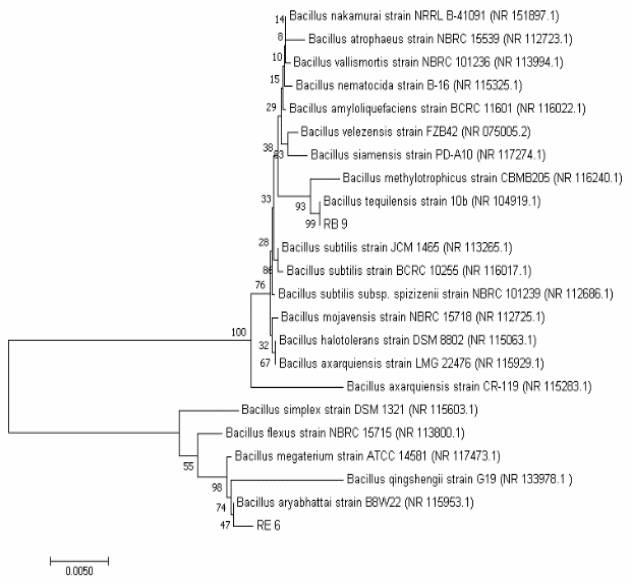
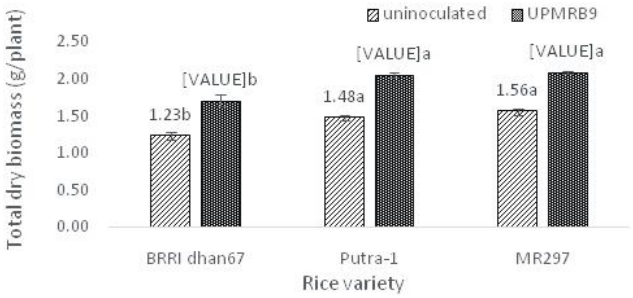
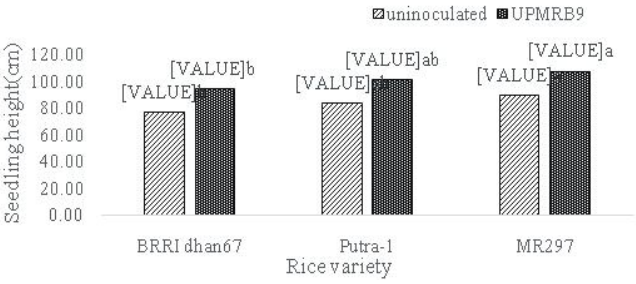


Fig. 2: Neighbor-joining tree based on 16S rRNA gene sequences showing phylogenetic relationship of isolates UPMRB9 to related isolates.



Values having same letter with each variety do not differ significantly at alpha 0.05 level by tukey (HSD).

Fig. 3: Effect of bacterial inoculation on total dry biomass of three rice variety.



Values having same letter with each variety do not differ significantly at alpha 0.05 level by tukey (HSD).

Fig. 4: Effect of bacterial inoculation on seedling height of three-rice variety.

influenced on seedling height of tested rice varieties. The comparisons were made among UPMRB9 inoculated and non-inoculated condition. The rice variety of MR297 was recorded with highest increase of seedling height both in inoculated and non-inoculated condition. The relative increase of seedling height was recorded by 22.5%, 20.2%, 20.1% accordingly (Fig. 4).

Effect of bacterial inoculation on total chlorophyll content of three-rice variety

The chlorophyll content in non-inoculated seedlings was remarkably decreased in contrast to UPMRB9 inoculated seedlings. The chlorophyll content was notably higher in MR297 rice seedlings for both conditions. The relative increase of chlorophyll was recorded at 22.5%, 20.2%, 20.1% for the rice variety BRRI dhan67, Putra-1 and MR297 respectively compared to the uninoculated control (Fig. 5).

Effect of bacterial inoculation on leaf area production of three-rice variety

The influence of UPMRB9 on leaf area production of three rice variety were evaluated both in inoculated and uninoculated condition. Significant enhancement of leaf area was recorded for MR297 variety. The comparative value was measured as 54.3%, 28.9% and 20.2% over control for the rice variety BRRI dhan67, Putra-1 and MR297 accordingly (Fig. 6).

Nutrient uptake of three rice varieties by inoculation of UPMRB9

Effect of bacterial inoculation to rice seedlings showed satisfactory nutrients uptake for nitrogen,

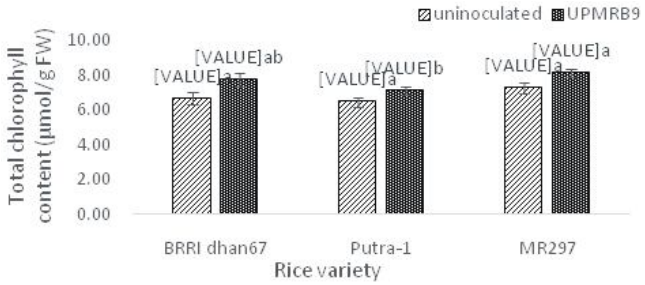


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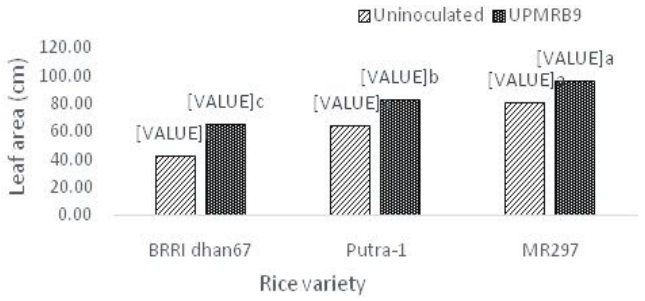
Fig. 5: Effect of bacterial inoculation on total chlorophyll content of three rice varieties.



Values having same letter with each variety do not differ significantly at alpha 0.05 level by tukey (HSD).

Fig. 6: Effect of bacterial inoculation on leaf area production of three-rice variety.

Table 1: Qualitative screening of five bacterial isolates for plant growth promoting traits.

PGP traits	UPMRA4	UPMRB9	UPMRE3	UPMRE6	UPMRG1
Nitrogen fixation	-	++	+	++	+
Phosphate solubilization	+	++	+	+	++
Potassium solubilization	-	+	++	++	+
Siderophore production	-	++	+	+	++
Cellulase production	+	++	-	+	+
Pectinase production	++	++	+	++	++

Note: '+' indicates low ability; '++' indicates high ability.

Table 2: Effect of UPMRB9 inoculation on nutrient uptake of three rice varieties.

	Uninoculated			UPMRB9		
Variety	N(%)	P(g/plant)	K(g/plant)	N(%)	P (g/plant)	K(g/plant)
BRRI dha67	3.17a	1.03c	13.97a	4.26c	5.59a	33.73a
Putra-1	2.82b	2.62a	13.29a	5.63a	5.37a	19.38b
MR297	3.16a	1.72b	5.61b	4.81b	4.14b	14.23c

Values having same letter in a column do not differ significantly at alpha 0.05 level by tukey (HSD).

phosphorous and potassium. Under non-inoculated condition the N uptake were not varied significantly. Besides that, a significant variation was found under inoculated condition and Putra-1 was recorded for highest uptake of nitrogen. Phosphorous uptake was significantly varied both under inoculated and uninoculated condition. Even though Putra-1 was recorded for highest uptake of phosphorous under uninoculated condition, insignificant variation was recorded for both BRRI dhan67 and Putra-1 under inoculated condition. Furthermore, potassium uptake was significantly higher in BRRI dhan67 rice variety under inoculated condition. The relative increase of nitrogen, phosphorous and potassium for the rice variety BRRI dhan67, Putra-1 and MR297 were 34.38%, 99.65% and 52.22% respectively; phosphorous was 442.7%, 104.9%, 140.7% respectively and potassium was 41.5%, 45.8%, 153.7% respectively table2.

Discussion

The plant growth promoting rhizo-bacteria isolated from rice rhizosphere was examined and characterized accordingly. A set of five (5) PGPR isolates were evaluated in this study for confirming the PGPR traits. The UPMRB9 rhizobacteria were selected with promising returns based on the measurement of IAA production and the qualitative screening for other PGPR traits. Significant growth enhancement of three (3) rice varieties under glass house condition reveals that the selection of the isolate was suitable. As that, the isolates of UPMRB9 were selected for molecular identification. Subsequently, the 16S rRNA sequence analysis of the genes was carried out. It is revealed that the isolate of UPMRB9 showed 99% similarity with *Bacillus tequilensis*. Previous studies also indicated that the 16SrRNA sequence analysis as a suitable technique for isolating bacterial according to species (Imran *et al.*, 2010; Alam *et al.*, 2011). Similarly, molecular phylogenetic analysis provides the platform for understanding the conventional sequence identification (Sing *et al.*, 2007).

The inoculation of three (3) selected bacterial isolates increased total dry biomass and length of seedlings, which might be the consequence of considerable amounts of IAA production. The bio-chemical test of the selected PGPR observed under laboratory condition showed high IAA production and have the ability for N-fixation as well as phosphate and potassium solubilization. This is in accordance with the findings of Naher *et al*., (2009) in rice who documented that Malaysian tropical soils are pools of varied microorganisms, which able to fix atmospheric-N solubilize inorganic phosphate and produce IAA. At the same time, our selected isolates showed ability for phosphate and potassium solubilizer that might be another reason for higher biomass production. Similar findings were reported by Sharma *et al.*, (2014) who showed that the enhancement rice growth performance and yield in response to P solubilizing PGPR (*P. putida, P. fluorescens, and A. lipoferum*) upon inclusions. Whilst, Vargas *et al.,* (2012) inoculation with diazotrophic nitrogen fixing bacteria (*Azospiril lumbrasilense* sp245, *Bukholderia kururiensis*)in rice seedlings able to increase lateral root development via ethylene signaling. Majeed *et al.*, (2015) indicated that the presence of P- solubilizing microbial population in soils might be considered a positive indicator of utilizing the microbes as bio-fertilizers for higher and sustainable crop production. It is documented that among the soil bacterial communities which described as effective K solubilizers were *B. mucilaginosus, B. edaphicus* and *B. circulanscan* (Meena *et al.*, 2015a; Meena *et al.*, 2014; Meena *et al.*, 2016).

Inoculation of UPMRB9 to three (3) rice varieties resulted in increased in leaf area and chlorophyll content compared to the uninoculated plants since this isolates have diverse plant growth promoting traits. Previous study by Hahm *et al.*, (2017) documented that three PGPR strains *Microbacterium oleivorans* KNUC7074, *Brevibacterium iodinum* KNUC7183, and *Rhizobium massiliae* KNUC7586) inoculation able to elevate total chlorophyll content of pepper leaves. The increased of

nitrogen uptake, phosphorous and potassium were recorded for all tested varieties. This findings was similarly supported by Egamberdieva *et al.*, (2017) who reported that an improved of root growth by bacterial inoculants. These are facilitated towards plants to have better access to soil minerals deposits such as nitrogen (N), phosphorus (P) and potassium (K).

Conclusion

The isolates of UPMRB9 as identified showed a promising prospective growth stimulating properties for early rice seedling establishment. This suggested that the potential use of UPMRB9 could be recommended as a bio-fertilizer with multiple plant growth promoting properties and benefits.

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