Characterization of Effective Microorganism (Phosphate Solubilizing Bacteria) Isolated from Rice- Field Soils

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Abstract. Phosphate solubilizing bacteria (PSB) is included in one of the effective organisms for plant growth-promoting rhizobacteria (PGPR) and considered as promising biofertilizers. This study is focused on identifying with inoculations PSB will give effects to the solubilization of phosphorus in soil and become available for plant uptake. To address this, four treatments are set up which are Merlimau soil (T0), Merlimau soil with Phosphate Solubilizing Bacteria (PSB) (T1), Tanjong Karang soil (T2), and Tanjong Karang soil with PSB (T3). Available P for the treatments and the value of soil pH is being taken as the parameter. Besides, the growth performance of rice plants was also recorded with the measurement of plant height, the number of tillers, and the number of leaves on weekly for five weeks. The soil analysis for available P and the soil pH was found to have a significant increase when treated with PSB as shown for the results of T1 and T3. The plant analysis for the growth performance of rice plants also shows that better growth of plants for the soils that have been treated with PSB. Thus, the application of PSB to the rice-field soil can increase P availability and reduce the acidity of the soil. In addition, it gives effect to the performance of the rice growth where this study shows results in increasing plant height, higher development of tillers, and plant leaves compared to the soils that do not treated with PSB.

Keywords: Soil Analysis; Available P; pH Value; Plant Analysis

1. Introduction

Rice belongs to genus Oryza. There are a lot of Oryza species but only Oryza sativa L. that has been cultivated as a major food crop [1]. Rice crops will produce grains with husk, where the husk will be removed to be consumed by people as food [2]. Rice is the second most important crop in the world after wheat, with Asia being the largest producer and consumer of this crop [3]. Statistic from Paddy Production Survey Report Malaysia, (2015) stated that the world leading rice producers are china (28%), India (21%), Indonesia (10%), Bangladesh (7%), Vietnam (6%), Thailand (4%), Myanmar (4%), and others (20%) produce 744.9 million metric tonnes of rice for supplying world demand. In Malaysia, the total rice production is 2.4 million tonnes which are categorized in the 25th place in the world [4].

Rice is most widely grown as a cereal crop and staple food for more than half the world’s population, which includes 89 countries [5]. People depend on rice for food calories and protein [6]. There are many efforts in increasing rice production. One of the essential management practices is by making proper fertilizer application [7]. Thus, the appropriate fertilizer input is not only for getting a high yield of grain but also focus on attaining maximum profitability [8].
Nutrients in the soil are one of the essential factors that influence soil fertility [9]. Phosphorus is one of the most nutrients needed for plant uptake [10]. Even though a large reservoir of phosphorus contained in soil, the amount of available forms for the plant uptakes is generally low because of some factors such as leaching and acidity of the soil [11]. It has become a concern especially when considering the soil condition that has a high amount of Fe, Al, or Ca, this acidity and oxidic mineralogy will form inorganic phosphates that cannot be taken by plants [12]. The majority of soil P is found in insoluble forms, while the plants mostly can absorb in two soluble forms from the soil solution as orthophosphate ion either HPO₄²⁻ or H₂PO₄⁻ [13]. Researchers have led to the search for an ecologically safe and economically reasonable option to improve crop production in low phosphorus soils. The aim is also to reduce the chemical fertilizer uses as it will harm the environment. In this context, PSB, which is organisms that often helps with phosphate solubilizing activity may provide the available forms of phosphorus to the plants and become an effective alternative to reduce chemical fertilizers [14].

Phosphate solubilizing bacteria (PSB) is included in one of the effective organisms for plant growth promoting rhizobacteria (PGPR) and considered as promising biofertilizers [15]. It is a plant probiotic, which enhance P solubilization in soil and applied phosphate fertilizer, thus give results in better P nutrition for plants [16]. Seed or soil that is inoculated with PSB is known to mobilize the precipitated P resulting in higher crop yield [17]. The benefits of PSB are not only supplying phosphorus but also produce other biological compounds like hormones such as auxin and gibberellic acid as well as vitamins [18]. Besides, it also helps in stimulating the efficiency of biological nitrogen fixation (BNF) [19]. Although PSB already occurs in soil, their numbers are not high enough to compete with bacteria commonly established in the rhizosphere [20]. Therefore, crop yield improvement requires inoculation of target microorganism at a much higher concentration than those found in the soil [21]. According to Kumar (2016), the diversity and richness of established PSB vary from soil to soil depending upon the physicochemical properties [22].

Recently, it has been reported that endophytic diazotroph bacteria such as Acetobacter diazotrophicus, Herbaspirillum spp., Azorarcus spp., Pseudomonas spp., Proteus mirabilis and Azospirillum spp., which colonised the root cortex of sugarcane, rice, maize and palm trees had enhanced plant growth. This has led to a considerable interest in exploiting these plant – microbe interactions [23]. These bacteria have the potential to fix nitrogen, are able to produce IAA (indole-3-acetic acid), and showed some P-solubilising activity [24]. Shen et al, 2016 have reported that, introduction of diazotrophic rhizobacteria to the plant tissues during in vitro propagation process would maintain the beneficial organism within tissues of the host plant. Therefore, the application of plant growth enhancer or microbial fertilizer can be considered as one of the potential alternatives to mineral fertilizer [25].

Plant growth promoting rhizobacteria have been used as biofertilizer for over a century in the agricultural systems. Research has successfully shown that these bacteria have affected the growth of the host plant significantly [26]. Results from different experiments showed that up to 50 – 70 % yield increase was reported due to inoculation of plant growth promoting rhizobacteria [27]. Other reports on the influence of plant growth promoting rhizobacteria for enhancing crops yields were also recorded for sugar beet, sugarcane, wheat, oil palm, maize, pineapple, kallar grass and rice [28].

The recommended rate of fertilizer suggested for mature paddy is an annual application of 50 kg urea kg/ha year⁻¹, 100 kg mixed fertilizer NPK ha/year [29]. This high nutrient demand and high cost of mineral fertilizers for paddy industry has encouraged the growers to find cheaper alternatives that may contribute to efficient nutrient and better profit [30]. In addition, the high input of mineral fertilizers could also affect the environment, in terms of air, soil and water pollutions and contribute to health hazards and environmental pollution [31].
2. Methods

2.1 Experimental Site
The area for this study was conducted in the rain shelter (greenhouse), soil laboratory and mycology laboratory in UiTM Melaka Jasin Campus, Faculty of Plantation and Agrotechnology.

2.2 Soil Sampling
The selected area which located in Tanjong Karang, Selangor and Merlimau, Melaka was chosen for the soil sampling. The reason is to make comparisons between the two areas comparing one of the granary areas for rice crop with rice field in Merlimau, Melaka. Simple random sampling has been taken by using auger was the method that is used. To ensure top soil being obtained, the depth of soil taken is 0-15 cm.

2.3 Soil Preparation
The soils for the experimental were being dried under sunlight for about 48 hours to ensure the dry weight of soil without the presence of water. Besides, any foreign materials such as debris and stones are eliminated from the soil. After that, the soil had been crushed to smaller particles before being transferred to pails. In addition, the pails that will be used also being sterilized by using water and alcohol 70% to maintain the samples are free from microbial contamination. After all the steps were done, the soils were weighed 2 kilograms and placed in each pail.

2.4 Rice Seed Preparation
Variety of MR220CL is selected for the experimental purpose which was taken from the Malaysian Agricultural Research and Development Institute (MARDI). It was chosen as plant indicator because this variety of rice has been commercialized by the farmers for cultivation in most of the rice field areas. Thus, this would ensure that the findings of this case study is relevant for the real application in the field and aligned with the real situation for cultivation practices in Malaysia. Rice seeds being planted under the compost medium for the first stage of growth and as the nursery stage for about one week until it reached 5-8 inches then it will be transplanted to the pails. The number of leaves and height of seedlings must be similar for each pail. Besides, they were also free from the use of any input such as fertilizers so that the transplant process will be considered that all seedlings are homogenous. Other requirements such as water are fulfilled by using the manual method of watering, and the soil was kept maintained for about 60% of moisture content.

2.5 Preparation of NBRIP Media
National Botanical Research Institute’s phosphate growth medium (NBRIP), which is a novel defined microbiological growth medium that is more efficient than PVK, was developed to screen phosphate-solubilizing microorganisms (Mehta and Nautiyal, 2000). NBRIP is the best medium to grow and observed the growth of the PSB. According to Islam et al. (2006), this medium was prepared by mixing 16 gram of agar powder, 10 gram of 1° glucose, 5 gram of magnesium chloride hexahydrate (Mg Cl₂•6H₂O), 5 gram of calcium phosphate (Ca₃(PO₄)₂), 0.2 gram of potassium chloride (KCl), 0.25 gram of magnesium sulfate heptahydrate (MgSO₄•7H₂O) and 0.1 gram of ammonium sulfate (NH₄)₂SO₄ with 1000 ml of distilled water in a scoot bottle. In order to ensure the mixture is dissolved well in the distilled water, the magnetic stirrer is inserted in the scoot bottle and being placed in hot place surface. After that, the solution is then being sterilized by using autoclave at the temperature of 121°C within one (1) hour to ensure fully dissolve of the entire chemical used. Before pouring the agar solution into the petri dish, the solution is being left to be cooled at about 50°C. Then, the petri dishes are being left for a while until the NBRIP agar become solid in the plate and being sealed by using para-film.

2.6 Culture on NBRIP Media
Some of the bacteria in the NA media were subcultured into the NBRIP media to determine whether it is the PSB or not. The method used is by taking the bacteria in the NA media by using sterilized loop and inoculate it into the NBRIP media. To prevent contamination occur, all of the preparation was
carried out in the laminar air flow. Four strains were stabbed in triplicate on a single NBRIP plate by using sterile toothpicks. Both diameters of the halo zones and colony size were measured to the nearest millimetre after 14 d of incubation of the plates at 28 °C (Islam et al., 2006). The ability of bacteria to solubilize phosphate was indicated by the solubilization index which refers to the ratio of the total diameter of both colony and halo zone to the colony diameter (Edi Premono et al., 1996). The positive control used in phosphate solubilizing assay test was Pseudomonas aeruginosa while Escherichia coli was used as the negative control.

2.7 Observation under Microscope
To observe the bacteria under the microscope, gram staining method, which was created by Danish physician Hans Christian Gram in 1844 was commonly used. It is used to identify the nature of the bacteria either it is gram positive or negative (Bacterial Gram Staining, n.d). Gram positive bacteria are bacteria with 90% of cell wall which known as peptidoglycan while gram negative bacteria have a thin layer of peptidoglycan which only 10%. The results from the gram staining method will show gram positive bacteria stains in purple or blue color while gram negative bacteria will stain in red or pink color. The steps were starting with inoculation of bacteria to a slide by using sterilized loop. After being air dried, crystal violet will be added to the slide or it can be changed with methylene blue. Rinse the slide with slow running water and iodine was added. The slide was rinsed again and being decolorized with alcohol before Safranin was added. After that, the slide was rinsed for 5 seconds, being air dried and lastly being observed under the light microscope. If there is change in colour to red for the bacteria strain, the bacteria is gram negative.

2.8 Identification of phosphate solubilising bacteria (PSB)
16S rRNA gene sequencing of PGPB was performed to identify the organisms, 16S rRNA gene from genomic DNA of screened isolate was amplified using universal Forward Primer (27f) : 5’ TACGGYTACCTTGTTACGACTT 3’ and Reverse Primer 1492r 5’ AGAGTTTGATCMTGGCTCAG 3’ by means of polymerase chain reaction. The BLASTn search program (http://www.ezbiocloud.net/eztaxon) was used to look for nucleotide sequence homology. The sequence obtained was then aligned by ClustalW using MEGA 4.0 software [32] and a neighbor-joining (NJ) tree with bootstrap value 500 was generated using the software.

2.9 Test for Phytotoxicity of Selected Isolates
2.9.1 Surface sterilisation of maize seeds
The maize seeds used for this experiment were obtained from a Persatuan Peladang Malaysia. The maize seeds were soaked for overnight in distilled water. The distilled water was then drained out and the seeds were then soaked in 2 % sodium hypochlorite for five minutes. The seeds were then rinsed five times with sterile distilled water prior to use [33].

2.9.2 Seed viability test
Prior to the phytotoxicity test, seed viability test was first conducted to test the quality of the seeds. Seed viability test was conducted using the paper towel method [34]. One hundred surface sterilised seeds were placed on a damp paper towel in a petri dish and grown in at room condition (28 °C ± 2 °C). After seven days, the number of seeds the germinated was counted using the formula:

\[
\left( \frac{\text{germinated seed} - \text{non germinated seed}}{100} \right) \times 100
\]

For the phytotoxicity analysis, 30 ml of ISP2 with 2% (w/v) agar was dispensed into plant tissue culture glass jars and autoclaved at 121°C for 15 minutes. Five ml of the bacteria suspension prepared earlier was then added into the cooled molten agar medium. The content was mixed well by gentle shaking and was left to solidify. Five surface sterilized maize seeds were placed onto the solidified agar surface and left to germinate under the light condition for 24 hours per day of fluorescent lighting for 10 days. Agar medium without addition of spore suspension served as the control in this experiment. The root length, plant height, number of leaves and number of secondary roots were
recorded. After 10 days of incubation, the plant height, main root length, number of leaves and number of secondary root branches were recorded. The mean and standard deviation of triplicate for selected isolates from each set were calculated. Data were analyzed using SAS software (windows). Mean separation was accomplished using Duncan’s Multiple Range Test. The statistical significance was determined at P ≤ 0.05.

2.10 Greenhouse experiment

The application of treatment for the inoculation of bacteria to the plant is guided by using McFarland Standard. It is a standard that been widely used by scientist to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension (McFarland Standard, 2002).

The PSB from the NBRIP media was taken by using sterilized loop and been mixed and stirred in 100ml beaker that filled with distilled water. The turbidity of the mixed solution was being compared to the McFarland standard which visually comparable to the concentration of the bacterial suspension. The approximate bacterial suspension was 1.5 x 106 and being treated to the soil at the same rate.

The treated soil was labelled as T1 = Merlimau soil + PSB and T3 = Tanjong Karang Soil + PSB while the other two treatments T0 and T2 was not treated with PSB. The application of treatment is carried out a week after the transplant of the seedlings. It was recorded as week 0 during the application of treatment. To maintain the survivability of microbes in soil, the soil is let to be moist up to 60%.

A total of eight (8) experimental units using pairs of four (4) treatments which are; T0 = Merlimau Soil, T1 = Merlimau soil + PSB, T2 = Tanjong Karang Soil, T3 = Tanjong Karang Soil + PSB with each of it are replicate with two (2) replications are arranged in Randomized Complete Block Design (RCBD).

Alternative to minimize error for the field experiment in the greenhouse, therefore RCBD design was chosen to conduct this project. The experimental error during the experimental process is expected, thus blocking is added to reduce the error. The experimental is applied completely in the greenhouse at UiTM Melaka, Jasin Campus. The diagram visualizes the arrangement of treatments in the greenhouse is showed.

2.11 Parameters Analysis

2.11.1 Determination of available phosphorus

Bray 2 extraction reagent was used to determine Available P from the soil. The preparation of the Bray 2 solution was by using ammonium fluoride and hydrochloric acid as extracting reagent (Bray & Kurtz,1945). 37 g of ammonium fluoride (NH₄F) was weighed and mixed with distilled water in 1000 ml volumetric flask. Hydrochloric acid with 20.4 ml concentrated was diluted with 500 ml of distilled water. The next step was 30 ml of ammonium fluoride (NH₄F) with 200 ml of hydrochloric acid (0.5N HCL) were mixed in a 1000 ml volumetric flask before being added with distilled water.

Plastic vials were used to fill 2 g of each sample soil. Then, 20 ml of Bray 2 solution was being poured into each of the plastic vial and was shaken for 10 minutes at 180 r.p.m. on reciprocating shaker to mix the solution and the soil. After that, the mixed solution was filtered into volumetric flask by using Whatman’s no. 2 filter paper. The extracts were transferred into the plastic vials. The available phosphorus is determined by using Inductively-Coupled Plasma Optical Emission Spectrometer (ICP-OES).

2.11.2 Determination of pH value

To determine the acidity of the soil, it is needed to determine the pH value. The method of analysis the pH value is based on Jones in 2001. The steps are starting with 10 g of soil is filled into three 100 ml plastic vials by using an analytical balance. Then, 25 ml of distilled water is poured into a beaker. The distilled water is transferred into the analytical balance with 10 g soil. After that, the plastic vial is shaken by using mechanical shaker for 10 minutes, with 180 r.p.m. speed. The soil mixture and distilled water is being left to suspend within 5 minutes. Lastly, a glass electrode was immersed into
the vial and the pH value is measured by using a calibrated pH meter. The pH value is recorded. Steps 1 – 6 are repeated by using 1M potassium chloride (KCl) and 0.01M Calcium Chloride (CaCl₂).

2.11.3 Plant height measurement

Height of rice seedling is measured weekly, which is from week 1 after the treatment applied until week 5 by using metre rule. Number of tiller and plant leaves was counted manually from week 1 after the treatment applied until week 5

2.12 Data Analysis

This research experiment was using One-way analysis of variance (ANOVA) by using comparisons of Tukey’s test. This method is used for statistical analysis which is used to determine significant difference where the mean of treatment is compared with the p-value (p ≤ 0.05).

3. Results and Discussion

3.1 Pre Analysis of PSB, Available P and pH Value for Two Sample Soils

The availability of P and the soil pH value in both soils show that Tanjong Karang has higher value compared to Merlimau. This would happen because of the Tanjong Karang soil is more fertile and less acidity. The results from the serial dilution and isolation of bacteria from both soils also show that there are more beneficial bacteria (PSB) in the Tanjong Karang rice-field soil compared to the Merlimau rice-field soil. This is due to the halo zones that appeared for T (Tanjong Karang) labelled is more compared to M (Merlimau) labelled. According to Tarah (2017), soil acidity which in low pH value gives impact on low productivity of beneficial microorganism in the rhizosphere area.

3.2 Analysis of Available P

Available of P in soil is analysed fully in three weeks for all treatments respectively. The average results are presented in Figure 1.

Figure 1. Graph of available P versus weeks

Based on Figure 1, a significant decrease is found out occurring throughout the weeks. There are some factors that relate with the declining graph which is the surrounding of the soil environment was disturb and affect the process of solubilizing P by the PSB. There is also possible reason as example low in production of bacterial population or disturbed by the abiotic factor including soil temperature and humidity. It may also decrease because of plant already uptake the available P. According to Sundara et al. (2002), it was assumed that the activity of microorganism is always varied. Thus this factor may lead to the differences of concentration of nutrients being mobilized.
Based on Figure 2, a significant decrease is found out occurring throughout the weeks. There are some factors that relate with the declining graph which is the surrounding of the soil environment was disturb and affect the process of solubilizing P by the PSB. There is also possible reason as example low in production of bacterial population or disturbed by the abiotic factor including soil temperature and humidity. It may also decrease because of plant already uptake the available P. ANOVA shows that there are significant difference between availability of P with all treatments (p=0.003) except for the treatment T0 and T1. Smaller p-value gives meaning that different in application of treatments do related to changes in available P. It also was assumed that the activity of microorganisms in the soils always varied. Thus, this factor would lead to the differences of concentration of nutrients being mobilized in different areas. The of microorganisms’ effectiveness are really depending on the interactions among themselves which also affected by temperature, moisture and other factors. As result shown in the previous figures, it was remarked that the application of PSB with the rice field soil can increase the availability of P.

3.3 Analysis of pH Value
The average value of pH for all treatment of soil were measured weekly from week 1 to week 3 after the treatment applied. It is to indicate any changes occurred that related to the changes of pH value. The graph below shows weekly pH value for each treatment applied.
Result from ANOVA statistical method shows that there is significant difference in soil pH related with the treatments applied. The p-value is \( p = 0.000 \). According to the Jabatan Pertanian Negeri Selangor (2010), rice cultivation in Malaysia is mostly suitable to be planted in soil areas that have pH value of 5.5 to 6.5. The observation from the graph can be seen that increasing in soil pH at week 1 and start to decline at week 2 to week 3 for all types of treatments that been applied. This result may cause and relate with the increasing number of phosphorus in soil that also effect in increasing of soil pH for the week 1 to week 2. However, decreasing number of available P on the week 2 to week 3 gives effect to the decreasing of the pH value thus cause the increase in acidity to the soil. As stated by Ghazi (2017), the other expected reason is because of the decomposition by microorganism that produced acid in the soil that will lead to the lower pH value.

3.4 Plant analysis

![Graph growth performance of rice versus treatments](image)

In this study, the parameters for the plant analysis are the plant height, number of leaves and also number of tillers. The experimental plots for all treatments were planted with rice seeds for variety of MR220. This is due to the common and widely used variety that been cultivated by farmers in Malaysia. The bar chart shows growth performance of the rice plant versus treatments that been applied.

The first treatment (T0) which is Merlimau soil shows that all the parameters for the plant analysis are the lowest numbers compared to the other treatments which are T1, T2, and T3. Nevertheless, for the second treatment (T1) which is Merlimau soil that been treated with PSB, it shows that the growth performance of rice plant is higher than T1 and T2 treatment. T3 treatment gives the best result which all the parameter of the plant analysis are the highest compared to others treatment.

Thus, it concludes that PSB do gives effects to the growth performance of rice plant as stated in journal by Joseph et al. in 2015. However, for the statistical analysis by using Minitab software 18 of each of the parameters for the plant analysis gives result in no significantly different between the parameters with the treatments applied. It is because of the p-value for all the analysis gives results in higher value than \( p = 0.05 \). Collection of data from longer term of measurements is needed to get more obvious results that are potentially can produce significant results for this study. The p-value for each parameter is shown as below:

| Parameters of plant analysis | Analysis Treatment versus parameters of plant analysis (P-value) |
|-----------------------------|---------------------------------------------------------------|
|                             |                                                               |

Figure 4. Graph display growth performance of rice versus treatments
3.5 Identification of potential isolates

From the screening result, the selected potential most promising isolates from chosen for 16S rRNA nucleotide sequence analysis. The 16S rRNA nucleotide sequence of two selected strains for each strain; SA37 and SN27 is showed in table below. The nucleotide sequence were identified by the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim, et al. (2012) [35]. All two isolates contained different nucleotide sequences for the 16S rRNA gene, indicating that they were different strains.

Table 2. Blast results of the 16S rRNA gene sequences for all strains showing the closest relatives (type strains) based on nucleotide similarity.

| Strain | Sequence Length (bp) | Closest match               | Pairwise similarity (%) |
|--------|----------------------|------------------------------|-------------------------|
| SN27   | 1401                 | Azospirillum brasilense      | 99.8                    |
| SA37   | 1340                 | Bacillus subtilis            | 99.1                    |

4. Conclusion

In conclusion, the application of beneficial microorganism which is phosphate solubilizing bacteria (PSB) to the rice-field soil can help in increasing P availability for the plant uptake. This study as well shows that PSB do give effect to the pH value where it helps reducing the acidity of the soil when the available P is high. In addition, it also gives effect to the performance of the rice plant growth where this study shows the results in increasing of plant height, higher development of tillers and plant leaves compared to the soils that does not treated with PSB. Many studies had revealed that inoculation of beneficial microorganisms for example the PSB as biofertilizer might be a better approach in assessing absorption of P for plant uptake. Nevertheless, this application is not always being the best choice for nutrient acquirement if mechanistic basis of the microorganisms was not understood well. Besides, there was not strongly proved that there are significant difference between all the treatments relate with growth performance of rice plant. In order to inoculate the PSB or other microorganism in soil under field conditions, it is recommended that we should also concern about the inherent limitations of these inoculation such as temperature and soil moisture. Further research on the multifunctional of effective microorganism (PSB) must be done. Thus, application of PSB as bioamplification and biofertilizer to the soil in the rice cultivation can be practiced widely. There would be needed to take more comprehensive identification and preparation so that successful implementation can be established for the real rice cultivation in Malaysia.

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