Application of phosphate solubilising microorganisms to increase the solubilisation of rock phosphates in soil

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Abstract
In this study, phosphate solubilising microorganisms were used to increase the solubility of rock phosphates, a slow release phosphorus (P) fertiliser. A consortium that consists of *Serratia marcens*, *Klebsiella* sp. and *Aspergillus* sp. was mixed with rock phosphate to evaluate the efficiency of rock phosphate solubilisation in soils sampled from various locations with different soil properties and agronomic practices. Total P and soluble P in soils treated with phosphate solubilising microorganisms were significantly higher compared with soils solely applied with rock phosphate. Approximately 70 – 98 % of P was released from rock phosphates in soils. Available P within the range of 13 – 1289 ppm and 832 – 1441 ppm were observed in soils exclusively treated with rock phosphates and soils treated with both rock phosphate and phosphate solubilising microorganisms respectively. Thus, the application of rock phosphates with phosphate solubilising microorganisms should be recommended for a sustainable agriculture practice as it is safe for environment and reduces pollution caused by chemical fertilisers.

Keywords: insoluble phosphate, dissolution, orthophosphate, bio-inoculants

Introduction
Rock phosphates are natural deposits of rocks that contain high phosphate bearing minerals. The phosphate fertiliser industry is very much dependent on rock phosphate as it contains high concentration of total phosphorus pentoxide (P₂O₅). About 90% of the phosphate rock production is used by the fertiliser industry to manufacture phosphate fertilisers. Chemical phosphate fertilisers are produced by a reaction between sulphuric acid with rock phosphates that consumes huge amount of energy and high cost. The remarkable increase in the food production over the years has assured a continuous demand for rock phosphates as it is very essential to sustain food quality and security.

The use of rock phosphates as direct fertiliser has received significant interest in recent years as they are natural, inexpensive and easily available fertilisers. Malaysia, particularly imports most of the phosphate rocks from Christmas Island that have been widely used as direct fertiliser in palm oil and rubber plantations as it is more suitable for perennial crops where the nutrients can be released slowly over time. There are different grades of rock phosphates available in the market which is determined by the percentage of phosphorus (P) in...
the material. However, rock phosphates are generally categorised as slow release fertiliser based on their ability to supply the plant orthophosphate requirement gradually. The solubility of rock phosphates in water is less than 1%. The release of soluble P from rock phosphate highly depends on the weathering. This process contradicts with synthetic fertilisers, whereby a quick and high supply of orthophosphates are available for plant uptake upon application. The main drawback of synthetic fertilisers is the instability of the phosphate anions in soils. The soluble orthophosphates turn into insoluble forms very quickly in the soil via adsorption and precipitation. Fixed P in soils contains phosphate compounds that are resistant to mineralisation due to the crystalline structure. Phosphate ions generally react by adsorbing to calcium, magnesium, aluminium and iron.

In acidic and neutral soils, there are fast and reversible surface based sorption reaction with aluminium and iron oxides, whereas precipitation involves new solid formation of Al-P or Fe-P minerals from highly concentrated P solutions (McLaughlin et al. 2011). These two scenarios have led to the accumulation of insoluble phosphates in the soils. Almost 75 – 80% of the P fertiliser applied to soil had undergone precipitation and fixation (Gyaneshwar et al. 2002; Lin et al. 2006; Zixi et al. 2008). It was previously reported that P accumulated in agricultural soils can sustain for more than 100 years (Gyaneshwar et al. 2002). Accumulation of P in soil eventually will lead to algal bloom in water reservoirs that can cause water pollution (Hong et al. 2006).

It is expected that inoculation of phosphate solubilising microorganisms (PSM) could add value to slow release mineral fertiliser, phosphate rocks by maximising the usage, avoiding wastage and protecting environment. Phosphate rock minerals are too insoluble to provide sufficient P for crop uptake (Goenadi et al. 2000; and Khan et al. 2009). Inoculation of phosphate solubilising bacteria (PSB) has improved P uptake both from native soils and phosphate rocks (Khan et al. 2009). Hamdali et al. (2008) also suggested the use of natural rock phosphates as an alternative source of P fertiliser if a natural non-polluting way could be found to promote its solubilisation. Similarly, rock phosphates and PSB were recommended for a less-expensive fertiliser in view of environmental concerns (Chen et al. 2006).

Thus, in this study, the effectiveness of rock phosphate mixed with PSM of local origin was investigated particularly to increase the available P in soil. Arable soils that were planted with different types of crops and virgin soils were analysed to study the efficiency of PSM in soils of different physical textures that were exposed to various fertilisation and agricultural practices.

Materials and methods
A thorough screening comprising 72 bacterial and 35 fungal isolates was conducted to select the most effective microorganism to dissolve insoluble aluminium phosphate, ferric phosphate and calcium phosphate in National Botanical Research Institute’s phosphate (NBRIP) medium. The analysis included clear zone formation on insoluble NBRIP phosphate agar medium and the release of soluble P from NBRIP liquid medium supplemented with insoluble ferric phosphate, aluminium phosphate and calcium phosphate. The 35 potential isolates were later tested using Christmas Island rock phosphate (CIRP, mineral fertiliser) using the molybdenum blue assay. Only three potential microorganisms were selected for the study and identified using 16S rDNA molecular method.

The microbial culture was grown in 100 ml of modified NBRIP broth at 28 ºC in orbitary shaker (180 rpm) for 5 days. The calcium phosphate in NBRIP medium was substituted with CIRP. The microbial cultures were centrifuged at 9,000 rpm for 3 min. The supernatant was filtered using
Whatman filter paper no. 42 and 1 ml of the sample was used for the subsequent analysis. The molybdenum blue assay was conducted as described by Olsen and Sommers (1982). This assay is done based on the concentration of soluble inorganic P that correlates with the intensity of blue colour in the sample. The reaction between acid ammonium molybdate with orthophosphate ions forms phosphomolybdenum complexes which are reduced to molybdenum blue by ascorbic acid. The absorbance of sample was read at 882 nm against reagent blank. For standard analysis, a blank sample was prepared and the absorbance of this blank sample was subtracted from the absorbance of the sample. The concentration of the sample was determined from the standard calibration graph.

Soil samples of Serdang series from various cultivated sites and non-cultivated sites in MARDI, Serdang were sampled. The sampling sites, the estimated area for sampling point and soil physical texture were described in Table 1. Soils were sampled from MARDI experimental plots (20 – 47% clay, 10 – 22% silt, 33 – 69% sand) and from secondary forest of approximately 20 years old (9 – 15% clay, 3 – 15% silt, 65 – 81% sand) to evaluate the efficacy of selected PSM in soils of significant different characteristics. The soil samples were collected in triplicates at a random sampling point at the depth of 0 – 20 cm and 20 – 40 cm. Digestion method was used to analyse total P and Bray method was used to analyse available P.

To prepare the inoculum, one loop full of bacterial colony was grown overnight in 40 ml of nutrient broth. The bacterial cells were harvested and mixed with 0.5% glucose to ensure cell activity. The fungal spores were harvested using Tween 80. The microbial population was \(10^7\) cfu/ml for bacteria and fungi. The harvested bacterial and fungal cells were incubated separately with 1 gram of rock phosphate in 50 ml of distilled water for 3 days prior to application. All the three rock phosphate-microbial cultures were mixed together prior to the soil application. About 150 ml of rock phosphate-microbial culture was applied to the soil.

The PSM inoculum was inoculated in the soil samples to determine the ability of dissolving insoluble soil phosphates in soil. Soils were inoculated 2 weeks once to maintain a high population of PSM. After 1 month of treatment, soils were analysed to study the changes in soil nutrient and microbial profile which includes total P, soluble P, nitrogen and potassium content, soil conductivity and soil pH.

Table 1. Soil sampling sites and soil physical texture

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Clay (%)</th>
<th>Silt (%)</th>
<th>Sand (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic plot 1</td>
<td>20.55 ± 0.21</td>
<td>10.45 ± 0.21</td>
<td>63.20 ± 0.57</td>
</tr>
<tr>
<td>Pineapple plot</td>
<td>20.20 ± 0.99</td>
<td>14.40 ± 1.98</td>
<td>59.20 ± 0.14</td>
</tr>
<tr>
<td>Organic plot 2</td>
<td>29.10 ± 1.84</td>
<td>10.85 ± 0.21</td>
<td>52.55 ± 4.88</td>
</tr>
<tr>
<td>Maize plot 1</td>
<td>47.50 ± 0.14</td>
<td>13.40 ± 1.84</td>
<td>33.40 ± 0.14</td>
</tr>
<tr>
<td>Mangosteen plot</td>
<td>42.50 ± 6.65</td>
<td>11.15 ± 0.21</td>
<td>38.05 ± 6.57</td>
</tr>
<tr>
<td>Banana plot</td>
<td>25.00 ± 1.41</td>
<td>22.40 ± 0.57</td>
<td>47.45 ± 2.05</td>
</tr>
<tr>
<td>Maize plot 2</td>
<td>39.30 ± 3.11</td>
<td>18.65 ± 1.91</td>
<td>35.60 ± 0.57</td>
</tr>
<tr>
<td>Herbal plant plot</td>
<td>44.80 ± 0.42</td>
<td>13.05 ± 0.78</td>
<td>35.45 ± 0.07</td>
</tr>
<tr>
<td>Rambutan plot</td>
<td>24.60 ± 6.22</td>
<td>12.00 ± 1.84</td>
<td>62.95 ± 9.40</td>
</tr>
<tr>
<td>Papaya plot</td>
<td>44.55 ± 0.07</td>
<td>18.80 ± 0.71</td>
<td>33.80 ± 0.14</td>
</tr>
<tr>
<td>Kenaf plot</td>
<td>23.85 ± 1.49</td>
<td>18.05 ± 0.92</td>
<td>51.10 ± 0.14</td>
</tr>
<tr>
<td>Guava plot</td>
<td>38.95 ± 7.99</td>
<td>17.55 ± 2.01</td>
<td>38.75 ± 6.00</td>
</tr>
<tr>
<td>Virgin soil 1</td>
<td>12.35 ± 4.74</td>
<td>5.95 ± 4.17</td>
<td>75.65 ± 7.85</td>
</tr>
<tr>
<td>Virgin soil 2</td>
<td>13.15 ± 3.47</td>
<td>8.00 ± 0.00</td>
<td>72.95 ± 3.60</td>
</tr>
<tr>
<td>Virgin soil 3</td>
<td>11.75 ± 3.04</td>
<td>13.10 ± 3.11</td>
<td>69.05 ± 5.30</td>
</tr>
</tbody>
</table>
**Statistical analyses**

All data were analysed using one-way ANOVA. The values were means of at least three replications. The differences with \( p < 0.05 \) were considered significant.

**Results**

**Identification of PSB**

Three microorganisms were identified as *Serratia marces*, *Klebsiella* sp. and *Aspergillus* sp. using polymerase chain reaction amplification of the 16S rDNA gene using universal primers. These microorganisms were regarded as good phosphate solubilisers based on clear zone formation on NBRIP medium (*Plate 1*) and molybdenum blue assay method (*Plate 2*). They could release 14.2, 7.4 and 14.9 mg/litre orthophosphates respectively from CIRP medium.

**Soil nutrient analysis**

Total P in soils was very low (0.01 – 0.06%) before treatment. Inoculation of PSM has increased the concentration of P in all soils (0.28 – 0.59%) significantly as described in *Figure 1*. Soluble P content in each soil sample was plotted in *Figure 2*. The range of the soluble P in inoculated soils is from 832 – 1441 ppm, whereas soluble P content in non-inoculated soils is in the range of 13 – 1289 ppm. These values were highly significant. Although P content of soils were highly significant after the addition of PSM, total nitrogen (N) and exchangeable potassium (K) content in all soil samples with and without inoculum did not show significant increase (*Figure 3*). This could be due to the nutrient content of rock phosphate that solely consists of P. Soil conductivity of soils inoculated and non-inoculated with PSM was illustrated in *Figure 4*. The values were significantly different between treatments.

Conductivity of soil represents the concentration of cations and anions present in soil. The greater the concentration of ions in soil solution, the greater the conductivity is. Conductivity can be related to the availability of nutrients for plant uptake. The inoculation of PSM has increased the conductivity of soil solution significantly. The pH range of non-inoculated soils is from 3.95 – 6.85, whereas pH range of inoculated soils is from 4.93 – 6.87 (*Figure 5*). Generally, the pH of all 15 samples has increased after the addition of PSM. The reduction in soil acidity could have released more phosphate from the complex structure.

**Discussion**

Soils were collected from 12 locations of arable soils and three of virgin soils. Various agricultural practices and fertilisation rates were applied to arable soils that were planted with various types of crops and fruit trees. Thus, the nutrient availability in soils would vary according to agricultural management and soil characteristics.
Soil sampling sites

Figure 1. Total phosphorus in soils before and after treatment. Values are means of three independent readings. Bar indicates standard error

Soil sampling sites

Figure 2. Soluble phosphorus concentration in soils treated and untreated with PSM. Values are means of three independent readings. Bar indicates standard error

Soil sampling sites

Figure 3. Total NPK in inoculated and non-inoculated soils. Values are means of 15 independent readings. Bar indicates standard error
The agricultural practice in each plot is not discussed in this study. However, the nutrient status of soils in each sampling plot is the consequence of soil nutrient management and impact of soil physical texture. The variation in soil nutrient content especially in the total and available P is due to leaching; erosion; surface water run-off from rainfall and irrigation; soil property; rate, form, timing and method of P application and formation of insoluble complex structures as the result of over-fertilisation. On the other hand, virgin soils are expected to be naturally fertile and rich with beneficial microbes as no agricultural activity had been taken place.

This study is aimed to look into the differences in the efficiency of P solubilisation in soils with various nutrient content and physical texture. As referred
to the results, it is obvious that microbial inoculant could increase available P, soil pH and conductivity when applied with rock phosphates. Chen et al. (2006) and Duarah et al. (2011) indicated the potentiality of PSM as an efficient bio-fertiliser for improving P-nutrition of crops. Bacillus sp. and Aspergillus sp. were known as efficient phosphate solubilisers for black pepper as described by Usha and Padmavathi (2012). Aspergillus niger (Kang et al. 2008), Serratia sp. (Chen et al. 2006), were also reported as efficient rock phosphate and tricalcium phosphate solubilisers.

It has been revealed through studies that fungi have greater ability of phosphate solubilisation than bacterial strains (Deepa et al. 2010). The reported results of soil P (Figure 2 and Figure 3) are in agreement with all the aforementioned findings. Therefore, in this study a mixed culture of bacteria and fungi was used to increase the solubility of rock phosphates. Serratia sp. and Aspergillus sp. were equally good in solubilising rock phosphates. A combination of bacteria and fungi showed better solubilisation activity as compared with single type of microorganism (results were not shown). Bacterial cells were harvested using glucose to increase the activity of the cells in liquid culture.

The P content in most soils is about 0.05% of which only 0.1% is plant-available (Vassileva et al. 2000). The initial amount of total P in soils tested in this study was in the range of 0.01 – 0.06%. The low concentration of total P indicates that although these soils were actively fertilised, the P could not be retained in the soils. The P could have been lost via leaching or surface runoff. Rock phosphate application alone and rock phosphate inoculated with PSM were the two treatments introduced to soils collected from various locations. It was observed that untreated soil and non-inoculated soil (treated with rock phosphate) did not show much difference in the concentration of total P. Interestingly, soils inoculated with PSM had shown a significant hike in the concentration of total P and soluble P. The results revealed that PSM could have released more inorganic P compounds present both in soil and CIRP. Total P is the combination of both organic and inorganic P, whereas soluble P measures mainly inorganic P. Thus, the increase in total P of the inoculated soils could have come from the microbial cells itself. The dead microbial cells add in the organic P content in soils.

Chemical fertiliser applied to soil is immobilised rapidly and becomes unavailable to plants. The excessive use of chemical fertilisers causes residue toxicity and environmental pollution due to the low use efficiency by plants and leaching (Duarah et al. 2011). If the total P is high and P fertilisers are applied regularly, the P rapidly fixed to unavailable forms and causes low P use efficiency (Nautiyal et al. 2000). In this study, it was proven that PSM that inoculated with CIRP was able to increase the total P and soluble P in soil significantly.

Scientists have found that pH influences the P dissolution. P becomes insoluble due to precipitation and fixation processes in soils of low pH. Aluminium and ferric ions in acidic soils bind with phosphates to form insoluble compounds that are not available for plants. Thus, the solubility of P is very low in acidic soils (Whitelaw et al. 1999; Igual et al. 2001; Fankem et al. 2006; Naik et al. 2008; Song et al. 2008). Phosphate solubilisation is regarded as the result of combined effect of pH decrease and organic acid production (Fankem et al. 2006). The solubility may increase with the decrease in pH of the surroundings by the secretion of organic acids (Park et al. 2011).

In this study, it was observed that PSM could increase the pH of all soils and subsequently was able to increase the solubility of P as well. P in the form of $H_2PO_4^-$ is available to the optimum level at a pH range of 6 – 7. Secretion of organic acids and pH reduction were
regarded as the major mechanism of P solubilisation, but not the sole reason (Vassilev et al. 2006). This study has confirmed that P solubilisation may also occur without extreme pH reduction of the surroundings. P solubilisation mechanism is a complex process as Hamdali et al. (2008) has reported that calcium chelator facilitated rock phosphate solubilisation, whereas production of exopolysachharide is also found to have a stronger ability in P solubilisation (Yanmei et al. 2008).

Nitrogen (N) and potassium (K) are the other two major elements important for crops besides P. The N and K content in soils did not show significant increase after the addition of microbial inoculums, but highly significant increase was observed in soil total P after the inoculation. This is due to the content of CIRP which consists of 30% P₂O₅ and 40% CaO. N or K fertilisers were not applied to soils. Therefore, the N and K values were not significant before and after treatment. However, a slight increase was observed in the K content of the soils after the inoculation. This could be caused by the organic acids produced by PSM (Priyanka and Sindhu 2013). P solubilisation is a complex phenomenon that attributes many mechanisms and relies on many factors (Chen et al. 2006). Some studies have reported that organic acids secreted by microorganisms are capable of solubilising insoluble P and K (Priyanka and Sindhu 2013). Soil minerals make up more than 90 – 98% of soil K such as K-bearing minerals and most of it is unavailable for plant uptake (Priyanka and Sindhu 2013). Since organic acid production is considered as one of the major mechanism of P solubilisation (Kil et al. 1998; Rodriguez and Fraga 1999; Perez et al. 2007), it is assumed that insoluble silicate minerals in the soil were also dissolved by the organic acids (Priyanka and Sindhu 2013). Insoluble silicate minerals in soil may release aluminium, iron, silicon and potassium during the solubilisation (Vasanthi et al. 2013). Although PSM and CIRP have solubilised high amount of P, it also has indirectly solubilised silicate minerals in soil that contain aluminium, iron and potassium.

Research conducted in Australia (McLaughlin et al. 2011) has highlighted fertiliser design strategies to make P available in the soil and limiting the rate at which it reacts with soil constituents. It has suggested altering the pH around fertiliser granules, slowing the release rate of P from fertiliser granules or increasing the P solubility to improve P use efficiency. This approach was adapted by the current study, whereby CIRP, a slow release mineral fertiliser was used with microorganisms to increase the solubility of P. Phosphate biofertiliser can help increasing the availability of accumulated phosphates for plant growth by solubilisation or by applying low grade rock phosphate along with microorganisms (Hameeda et al. 2008).

Likely, in the current study, rock phosphate application alone which simulated direct fertilisation of rock phosphates failed to increase the soil soluble P content compared with rock phosphate applied together with PSM. However, rock phosphate that has slow release characteristics was recommended for slow growing perennial species and said to be relatively ineffective when applied to annual cropping systems, where crops have a high P demand over a relatively short time period (McLaughlin et al. 2011). This opinion can change with the finding of the current study that PSM could add the value of CIRP by meeting the P demand of crop and P use efficiency of plants.

**Conclusion**

The solubility of P in acidic soils seems to be a great challenge in agriculture. Rock phosphate that has been used as direct mineral fertilisers in organic and conventional farms is considered as a slow release fertiliser with low effectiveness. In this study, it was reported that inoculation of PSM consortium could not only increase soluble P, but also could accelerate the release of soluble P from rock phosphate.
The use of PSB bio-inoculants can minimise the P-fertilisation, reduce environmental pollution and promote sustainable agriculture practice in soils regardless of its characteristics.

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References


Rock phosphate solubilising microbes


Abstrak

Dalam kajian ini, mikroorganisma pelarut fosfat digunakan untuk meningkatkan kelarutan batuan fosfat, baja forforus (P) perlepasan perlahan. Sebuah konsortium yang terdiri dari Serratia marcens, Klebsiella sp. dan Aspergillus sp. telah dicampur dengan fosfat batuan untuk menilai kecekapan kelarutan batuan fosfat dalam tanah yang disampel dari pelbagai lokasi dengan kandungan tanah dan amalan agronomi yang berbeza. Jumlah P dan P larut dalam tanah yang dirawat dengan mikroorganisma pelarut fosfat adalah lebih tinggi berbanding dengan tanah yang semata-mata menggunakan fosfat batuan sebagai baja langsung. Kira-kira 70 – 98% daripada fosforus diberikan dengan fosfat batuan. Fosfat larut dalam tanah yang dirawat dengan mikroorganisma pelarut fosfat adalah lebih tinggi berbanding dengan tanah yang semata-mata menggunakan fosfat batuan sebagai baja langsung. Oleh itu, penggunaan fosfat batu dengan mikroorganisma pelarut fosfat perlu disyorkan untuk amalan pertanian mampu kerana ia adalah selamat untuk alam sekitar dan mengurangkan pencekalan yang disebabkan oleh baja kimia.