Potential of Native Phosphate Solubilizing Bacteria Isolated from the Rhizosphere of Economic Crops and Vermicast in Northeast Thailand to Solubilize Insoluble Phosphates under *in vitro* Conditions

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Abstract Although soils generally contain a large amount of total P, only a small proportion is immediately available for plant uptake making it a major constraint on crop production in many tropical countries. Free-living bacteria and fungi can mobilize orthophosphate from either organic or inorganic P sources such as Phosphate Rock (PR). These phosphate-solubilizing microorganisms (PSM) are characterized by their capacity to solubilize precipitated forms of P, the main P ingredient in PR and could be good bio-fertilizers for improving phosphorus plant nutrition. The present study examined phosphate solubilising bacteria (PSB) isolated from cassava, groundnut, rubber tree, sunchoke, rice, rice-soybean, rice-soybean-corn and rice-chili fields and vermicast of earthworm varieties in Northeast of Thailand where soils are mainly sandy and P-deficient. PSB isolates were tested by using different P sources [Tri-calcium Phosphate ($Ca_3(PO_4)_2$), Ferric Phosphate ($FePO_4$) and Aluminum Phosphate ($AlPO_4$)] on specific culture media (National Botanical Research Institute Phosphate Growth Medium, NBRIP). Our results showed that five of the PSB isolates from economic crops and vermicast of earthworm varieties solubilised a significantly ($P \leq 0.01$) higher amount of $AlPO_4$ and $FePO_4$ over the uninoculated control. The highest activity of solubilization was achieved for $AlPO_4$ followed by $FePO_4$ which are the main forms of insoluble phosphates in acidic sandy soils. We found that PSB isolated from vermicast of earthworm varieties *Pheretima posthuma* and *Eudrilus eugeniae* were able to solubilize both $AlPO_4$ and $FePO_4$ at relatively high rates (up to 1,918.49 mgP/l), in contrast, PSB isolated from cassava, rice-soybean and groundnut field soil tended to have lower solubilisation rates for $FePO_4$. None of the isolates tested were able to solubilize $CaPO_4$. Finally, IAA production was observed only in PSB isolated of *E. eugeniae* vermicast and rice-soybean field soil. These results highlight variability of specific PSB isolates from different rhizospheres and vermicast of earthworm varieties and provide essential information for the management of soil fertility.

Keywords crop productions, phosphate solubilization, phosphate-solubilizing bacteria, vermicast

INTRODUCTION

Phosphorus (P) is an essential element in plant development. Photosynthesis, changing sugar to starch, genetic inheritance, nitrogen fixation, flowering, fruiting seed production (Mehrvarz *et. al.*, 2008) and resistance to plant diseases are the attributes associated with phosphorus nutrition (Khan *et al*., 2009). Although soils generally contain a large amount of total P, only a small proportion is immediately available for plant uptake. Phosphorus deficiency is the main problem to plant growth in tropical and subtropical areas (Bonser *et al*., 1996). Especially, in the Northeast Thailand the soil are acidic with highly reactive of Fe^{3+} and Al^{3+} (Gyaneshwar *et al*., 2002), low phosphorus contents in the parent material and low soil moisture affects the availability of phosphorus (Karmakar *et al.*, 1997; Raychaudhury *et al*., 2003). To meet the crop demand, farmers apply up to 3-4 times the required amount of P to crops, causing a substantial increase in production costs. There are strong evidences showed soil bacteria are capable of transforming soil P to the forms available to plant.

Recent studies reported that plants and microorganisms play a key role in soil P dynamics which catalyze the hydrolysis of organic phosphate esters to orthophosphate anions by using phosphatases. Free-living bacteria and fungi can mobilize orthophosphate (predominantly as HPO_4^{2-} and $H_2PO_4^-$) from either organic or inorganic P sources for improving P availability. Phosphate solubilizing bacteria (PSB) become a source of P to plants upon its release from their cells. The phosphatase efficiency is related to the microbial fauna, the soil temperature and humidity as well as the associated bacteria communities (Zahran, 1999). Other factors that can play a significant role are the physiological state of the plant, the type of rooting system, the age of the plant and the location of ectomycorrhiza on the root (Antibus *et al.*, 1997).

Vermicast, also called worm manure originates from the breakdown of organic matter by earthworms. Its microbial activity is 10 to 20 times higher than in the soil (Chaoui, 2010). Vermicast contains reduced levels of contaminants and a higher saturation of nutrients than organic materials do before vermicomposting (Ndegwa *et al*., 1998). Moreover, vermicast is also believed to contain hormones and enzymes which it acquires during the passage of the organic matter through the earthworm gut. The hormones and enzymes are believed to stimulate plant growth and discourage plant pathogens (Gajalakshmi and Abbasi, 2004). The analysis of the ability of bacterial isolates to solubilize insoluble P form to available P form will enable to create a highly valuable basis for the use of these isolates as bio-inoculums or bio-fertilizer to increase crops yield and for sustaining crop production. The objectives of this research were to isolate phosphate-solubilizing bacteria (PSB) from cassava, groundnut, rubber tree, sunchoke, rice, rice-soybean, rice-soybean-corn and rice-chili fields and vermicast of earthworm varieties in the Northeast of Thailand where soils are mainly sandy and P-deprive.

MATERIALS AND METHODS

Sampling site and soil sample collection

The experiment was conducted within Khon Kaen, Mahasarakham and Roi Et provinces, Northeast Thailand. Northeast Thailand is characterized by a tropical climate with an acid sandy soil. Soil samples (20 points) were collected from cassava, ground nut, rubber tree, sunchoke, rice, rice-soybean, rice-soybean-corn and rice-chili fields while those plants were growing from the soil surface at a depth of 10 cm. Collected soil samples were preserved in plastic bag at 4 °C for phosphate solubilizing bacteria isolation.

Isolation of phosphate solubilizing bacteria by enrichment culture

To extract PSB from soil, 5 g of soil samples and vermicast of earthworm varieties were transferred to the NBRIP growth medium. Per liter, this growth liquid medium contains 10 g glucose with 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl and 0.1 g $(NH_4)_2SO_4$. Additionally,

modified NBRIP media, containing either $FePO_4$ or $AlPO_4$ or $Ca_3(PO_4)_2$ as the sole source of insoluble P, were also used for the initial screening step. The pH of the agar medium was adjusted to 7.0. The sources of insoluble P were autoclaved separately and the other sterile ingredients were aseptically mixed after autoclaving. Erlenmeyer flasks containing 50 ml of the medium with inoculants were incubated for 7 days at 30 °C on incubator shaker at medium speed (150 cycles min^{-1}). For the following week, 5 ml of this incubated medium with inoculants were transferred into 50 ml Erlenmeyer flasks again with new liquid medium for 7 more days at 30 °C on incubator shaker at medium speed (150 cycles min^{-1}). At the end of each week in NBRIP growth liquid media, aliquots of each dilution were spread on NBRIP medium and incubated at 30 °C for 14 days. After PSB isolation for 6 weeks, colonies were selected from the plates on the basis of the appearance of a clear halo; the clones were further purified on minimal medium based on each insoluble phosphate forms. Once purified, each isolate was stored as a glycerol stock at -20 °C.

Mineral phosphate solubilization assays

The phosphate solubilizing (PS) activity of each of the isolates was determined by molybdenum-blue method (Land Development Department, 2005). The isolates were grown in NBRIP liquid medium containing different insoluble forms of phosphate ($AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$) for 3-7 days at 30 °C on incubator shaker at medium speed (150 cycles min^{-1}). The solubilization efficiencies were determined by reaction with ammonium molybdate for phosphorus compounds as ammonium phosphomolybdate and reduced with a compound ascorbic acid to molybdenum blue. Then, the isolates were incubated for 30 min at room temperature for color development. Finally, the absorption of light in the wavelength range 880 nm was measured by Shimadzu UV-120-01 Spectrophotometer.

Indole acetic acid production

Selected PSB strains based on their ability to solubilize P were analyzed for IAA production (Nuntagij, 1997). The selective bacterial strains were grown in 45 ml of Lysogeny Broth medium (LB) contains 10 g/l Tryptone with 5 g/l NaCl and 5 g/l yeast extract at 30 °C for 2 days. The 5 µl PSB solution were determined by reaction with 1 ml of Tris-TMRT reagent contains 10 g/l D-manital, 0.2 g/l yeast extract, 0.2/l $CaCl_2.2H_2O$, 0.2 g/l $MgSO_4.7H_2O$, 1.21 g/l Tris-base and 0.061 g/l L-Tryptophane 28 °C for 10 days. The 2 ml mixture was determined by 0.01 M $FeCl_3$ in 35% $HClO_4$ for 30 min at 25 °C in the dark. The positive isolated were showed red color for indole acetic acid production.

Statistical analysis

An analysis of variance was done on data obtained from each parameter in each treatment. All analyses were carried out using Statistical analysis version 8.0. Least significant differences (L.S.D.) were calculated at $p < 0.05$ and Duncan's multiple-range test was used to test significant differences between treatments. Standard deviation was also calculated for the variance.

RESULTS AND DISCUSSION

Isolation of PSB from the Rhizosphere and vermicast of earthworm varieties

The screening strategy employed during this research enabled the identification of PSB colonies on NBRIP medium containing different insoluble forms of phosphate ($AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$) as sole P source. No colonies exhibiting a clear halo were observed on agar plates supplemented with $AlPO_4$, $Ca_3(PO_4)_2$ and $FePO_4$ for rubber and cassava from Roi Et, rubber tree, cassava and rice from Mahasarakham, sonchoke, rice-soybean-corn and rice-chili from Khon Kaen provinces and from vermicast of *P. peguana* and *E. foetida* (not shown). Five bacterial isolates

from cassava and rice-soybean field at Khon Kaen province, ground nut field from Mahasarakham province and from vermicast of earthworm varieties showed clear halos of $AlPO_4$ and $FePO_4$ solubilization (Table 1 and Fig. 1). Some obvious differences in the size of the halos of different isolates were observed (not shown). This preliminary observation suggests the existence of bacterial isolates exhibiting different degrees of PS efficiencies in the soil samples and vermicast of earthworm varieties collected. This preliminary observation suggested the existence of bacterial isolates exhibiting different degrees of PS efficiencies in the soil samples collected (not shown). Yahya and Azawi (1998) reported that in general high abundances of PSB are found in agricultural and rangeland soils. Furthermore, Kim *et al*. (1989) showed that the cultural activities and different soil properties such as soil physical and chemical properties, organic matter and soil phosphorus content all play role in determining the abundance of PSB in soils.

Table 1 Phosphate solubilizing effectiveness of tested bacteria, 10 days after inoculation.

Isolate	Solubilized Phosphate (mgP/l) from		
	$Ca_3(PO_4)_2$	$FePO_4$	$AlPO_4$
Cassava (Khon Kaen)	0	959.53b	1,860.00 a
Rice-Soybean (Khon Kaen)	0	682.13b	1,789.71a
Groundnut (Mahasarakham)	0	741.17b	1,605.09 a
Eudrilus eugeniae	0	1,582.46a	1,834.96 a
Pheretima posthuma	0	1,405.00a	1,918.49 a

Efficiency of phosphate solubilization by NBRIP

To confirm this observation, the 5 purified isolates were tested following the protocol of Land Development Department (2005), a method previously shown to be a reliable and qualitative indicator of the PS activity of different bacterial isolates. Table 1 shows the OD880 nm shift of the culture supernatants of each of the 5 PSB isolates after a 3-7 day cultivation period in NBRIP

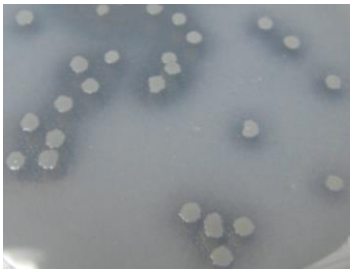

Fig. 1 The appearance of a clear halo from PSB isolates.

medium. Indeed, some isolates did not show any significant change in the absorbance of the supernatant while others exhibited OD880 nm changes in the absorbance. After evaluating their P solubilization capacity, the results showed that all of the 5 isolates can solubilize $AlPO_4$ and $FePO_4$ better than $Ca_3(PO4)_2$ especially the PSB isolates from the vermicast of earthworm varieties (Table 1). Moreover, PSB isolate from the cassava and rice-soybean at Khon Kaen province and groundnut at Mahasarakham province look interesting for solubilizing $FePO_4$ and $AlPO_4$, which are the main forms of insoluble phosphates in acid sandy soils.

Indole acetic acid production of PSB isolates

The plant growth promoting effect of selected PSB was evaluated by analyzing IAA production (Table 2). The IAA production was observed from the isolates of rice-Soybean field and vermicast of *E. eugeniae* (Fig. 2), indicating that these strains could utilize l-tryptophan as a precursor for growth. Phosphate solubilizing bacteria isolated from rice-Soybean field and vermicast of *E. eugeniae* showed the highest IAA production (111.30-111.34 mg/L). Generally,

bacteria can enhance plant growth directly or indirectly by increasing available P, fixing nitrogen, sequestering iron by siderophores, producing antibiotics and plant hormones (Glick *et al*., 1998 and Mantelin and Touraine, 2004). This group of bacteria is known as plant growth promoting bacteria (PGPB). Moreover, PSB have been widely used as inoculants to increase P uptake and crop yield (Chen *et al*., 2008). IAA production contributes to phytostabilization by increasing root and shoots biomass (Liphadzi *et al*., 2006). In the context of sustainable agriculture with bio-fertilizers (phosphate solubilizing bacteria) to supply phosphorus at a favorable level for enhance plant growth by providing nutrients in a readily absorbable form. The application of inoculants provided from these microorganisms seem to increase an abundant population of active and effective microorganisms to the root activity zone which increases plant ability to uptake more nutrients.

Table 2 Plant growth promoting properties of isolates.

Isolate	Phosphate solubilization	IAA production
Cassava (Khon Kaen)	+	-
Rice-Soybean (Khon Kaen)	+	+
Groundnut (Mahasarakham)	+	-
Eudrilus eugeniae	+	+
Pheretima posthuma	+	-

Fig. 2 IAA production from PSB isolates.

CONCLUSION

The native PSB isolated from cassava, rice-soybean and groundnut field soil, vermicast of *P. posthuma* and *E. eugeniae* seems to have the capacity to solubilize insoluble forms of $AlPO_4$ and $FePO_4$ which are the main forms of insoluble phosphates in acid sandy soils. The PSB isolates from vermicast of earthworm varieties *P. posthuma* and *E. eugeniae* show the highest P solubilization capacity in vitro conditions follow by the PSB isolated from cassava and rice-soybean field soil from Khon Kaen province and groundnut field soil from Mahasarakham province. However, the PSB isolates from vermicast of earthworm varietie *E. eugeniae* and rice-soybean field soil from Khon Kaen province look interested for IAA production. This work provides a first step towards by using bio-fertilizers (phosphate solubilizing bacteria) for improving plant nutrient uptake, soil fertility and sustainable crop production in nutrient poor systems.

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