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Research Article
Diversity of Phosphate Solubilizing Bacteria under Rubber Intercropping

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Abstract

Background and Objective: Microorganic services like Phosphate Solubilizing Bacteria (PSB) has been shown to enhance the solubilization of insoluble P compounds and produce IAA hormone. There are widely factors effected on phosphatases activities such as the microbial in the soil, soil temperature, soil moisture, the diversity of bacteria associated, the development of the crop, the root system of the plant and the location ectomycorrhiza. The purpose of this study is to address the influence of different intercropping with rubber plantation practices on Phosphate Solubilizing Bacteria (PSB). **Methodology:** The present study was to characterization and estimate the variability of specific PSB isolates from banana, cassava and *Mucuna bracteata* under rubber plantations, in order to make the suggestions regarding fertility management in the poor sandy soils of the region. **Results:** The results of the present study showed that four pure isolates encodes PSB-M-01, PSB-M-02, PSB-B-01 and PSB-C-01 were obtained after intercropping with rubber trees and it was observed that PSB isolate from *Mucuna bracteata* (PSB-M-01) best solubilised $Ca_3(PO_4)_2$ and $AlPO_4$ by releasing inorganic phosphate of approximately 1,050.90 and 561.32 mg P L^{-1}, respectively. Finally, the IAA production was analysed, the results showed that bacterium 5-2 (2013) isolates from root rhizosheres of *M. bracteata* produced a significantly ($p \leq 0.01$) higher amount of IAA hormone (962.53 mg L^{-1}) over the other PSB isolates. **Conclusion:** These results highlight variability of specific PSB isolates from different rhizospheres of intercropping under rubber tree plantation and could be good bio-fertilizers for improving phosphorus plant nutrition.

Key words: Bacteria, intercropping under rubber plantation, phosphate solubilization, IAA production, *Mucuna bracteata*

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In order to get the income before open tapping and improving soil fertility within ecosystem services in rubber plantations, Northeast of Thailand. The agricultural areas in Northeast Thailand are depleting availability with suitable soil (sandy, acidic, low Cation Exchange Capacity (CEC), low nutrient reserves and low water holding capacity)[1]. Furthermore, the average annual rainfall of less than 1,300 mm (with a severe dry season) contrasts the generally favourable biotope usually admitted for rubber tree development (generally about 1,800-2,000 mm of rainfall). The uplands often present severe physical and/or chemical degradations, due to deforestation and the cultivation of both cassava and sugarcane. Microorganic services like Phosphate Solubilizing Bacteria (PSB) has been shown to enhance the solubilization of insoluble P compounds[2] and are therefore, widely used as inoculants to increase P uptake and crop yield[3]. Soil microbes are found around root rhizospheres of agricultural crops[4]. Phosphatases enzymes are released from root exudation process is an organic acid that enhances the solubility of phosphorus in the rhizosphere[5]. The efficiently of phosphatases is associated with factors such as the microbial in the soil, soil temperature, soil moisture and other factors like the diversity of bacteria associated[6], the development of the crop, the root system of the plant and the location ectomycorrhiza[7]. The purpose of this study is to address the knowledge regarding the influence of different intercropping with rubber plantation practices especially microorganic services like Phosphate Solubilizing Bacteria (PSB). The objective of this study was to characterization and estimate the variability of specific PSB isolates from banana, cassava and *M. bracteata* under rubber plantations, in order to make the suggestions regarding fertility management in the poor sandy soils of the region.

MATERIALS AND METHODS

Sampling site and soil sample collection: Intercropping with banana, cassava and *M. bracteata* under rubber tree plantations was conducted at experimental farm, Faculty of Agriculture, Khon Kaen University, Thailand from June, 2012 to August, 2014. Soil samples were collected at a depth of 0-10 cm with four replicates (n = 4) and were preserved in plastic bags at 4°C for chemical and physical analysis and PSB isolation.

Isolation of phosphate solubilizing bacteria by enrichment culture: In order to extract the PSB, it transferred 5 g of each soil sample to the National Botanical study Institute's phosphate growth medium (NBRIP)[8]. This growth liquid medium (per liter) contained 10 g glucose, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl and 0.1 g $(NH_4)_2SO_4$. A modified NBRIP media, containing either $AlPO_4$ or $Ca_3(PO_4)_2$ or $FePO_4$ as the sole source of insoluble P was also used in the initial screening step. The pH of the agar medium was then adjusted to 7.0. The sources of insoluble P were autoclaved separately and other sterile ingredients were aseptically mixed in after autoclaving. Erlenmeyer flasks containing 50 mL of the medium with inoculants were incubated for 1 week at 30°C in an incubator shaker at medium speed (150 cycles min^{-1}). During the next week, 5 mL of the incubated medium with inoculants were again transferred into 50 mL Erlenmeyer flasks with new liquid medium at 30°C, in an incubator shaker again at medium speed for 6 weeks. At the end of 6 weeks cycle of the NBRIP growth liquid media, aliquots of each dilution were spread over the NBRIP medium and further incubated at 30°C for 1-2 weeks. The PSB colonies were selected from the plates on the basis of the appearance of a clear halo (Fig. 1) and the clones were further purified on a minimal medium, based on each insoluble phosphate form. Once purified, each isolate was stored as glycerol stock at -20°C.

Mineral phosphate solubilization: Phosphate Solubilizing (PS) activity of each isolate was determined through four replicates by the molybdenum-blue method[9].

Fig. 1: Appearance of a clear halo from PSB isolates

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 days, at 30°C in an incubator shaker at medium speed (150 cycles min^{-1}). The solubilization efficiencies were determined by reactions with an ammonium molybdate for phosphorus compounds, such as ammonium phosphomolybdate and reduced to molybdenum blue with a compound ascorbic acid. Later, the isolates were incubated for 30 min at room temperature for color development. Lastly, the absorption of light within the wavelength range of 880 nm was measured with a Shimadzu UV-120-01 spectrophotometer. The concentration of PS activity was compared to a standard curve of KH_2PO_4 at concentrations ranging from 0-0.9 mL L^{-1}.

Indole Acetic Acid (IAA) production: Selected PSB strains, based on their ability to solubilize P were analyzed for IAA production[10]. The selective bacterial strains were grown in 50 mL of Luria Bertani (LB) medium containing 10 g L^{-1} tryptone, 5 g L^{-1} NaCl and 5 g L^{-1} yeast extract at 30°C for 2 days. Five microliters PSB solutions were tested through the reaction with 1 mL of tris-TMRT reagent, containing 10 g L^{-1} D-mannitol, 0.2 g L^{-1} yeast extract, 0.2 g L^{-1} $CaCl_2.2H_2O$, 0.2 g L^{-1} $MgSO_4.7H_2O$, 1.21 g L^{-1} tris-base and 0.061 g L^{-1} L-tryptophan at 28°C for 10 days. Two milliliters mixtures were synthesized with 0.01 M $FeCl_3$ in 35% $HClO_4$ for 30 min at 25°C in a dark setting. The positively isolated mixtures presented a red color indicating indole acetic acid production.

To determine the volume of IAA production, the PSB postponed centrifugation (14,000 rpm) at which time the suspended supernatant reacted with 0.01 of M $FeCl_3$ in 35% $HClO_4$ for 30 min, at 25°C in dark conditions. The samples were measured the absorbance at 530 nm to calculate the concentration of indole acetic acid produced by the bacteria, compared to a standard curve of IAA at concentrations, ranging[10] from 0-150 mg mL^{-1}.

PCR amplification of 16S rRNA and sequencing: The gene-encoding 16S rRNA was amplified from selected strains by the Polymerase Chain Reaction (PCR) using bacterial universal primers proR2 (5-AGAGTTTGATCMTGGCTCAG-3) and 907R (5-CCGTCAATTCCTTTRAGTTT-3). The PCR mix consisted of 5 µM of each primer, 10X PCR buffer and 5 U of Immolase™ DNA polymerase. A suspension of cells on MilliQ water, coming from a fresh colony grown on nutrient agar was used as target DNA. The following cycle conditions were used: 95°C for 7 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension step at 72°C for 10 min.

The PCR products were purified from agarose gels with the PCR clean-up gel extraction kit (Macherey-Nagel, Germany) and sequenced. The nucleotide sequences are searched for homology against a public database as GenBank using the BlastN program[11] and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups.

Statistical analysis: An analysis of variance was conducted on data obtained from each parameter in each treatment. All analysis were carried out using statistic version 8.0. Least Significant Differences (LSD) were calculated at $p<0.05$. Duncan's multiple-range test was employed to test the significant differences between treatments.

RESULTS AND DISCUSSION

Soil chemical and physical analysis: The results of chemical properties within the soil in all sites are shown in Table 1. Generally, a pH of 5.4 represents the successful growth of specific microorganisms, like bacteria. Soil pH, soil moisture, soil density, total N, available P, exchangeable K and Ca, Organic Matter (OM) as well as EC were not significant different given the different intercropping of rubber tree. The relevant chemical properties of soil are: (i) Soil reaction

Table 1: Soil properties in different intercropping with rubber tree plantations in Khon Kaen province, Thailand

	Different intercropping		
Soil properties	*Mucuna bracteata*	Banana	Cassava
pH (1:1 H_2O)	4.90	4.65	4.73
Total N (%) (micro-Kjeldahl)	0.06	0.05	0.06
Available P (mg kg^{-1}) (Bray II method)	10.55	13.23	12.68
Exchangeable K (mg kg^{-1})	20.00	22.25	20.00
Exchangeable Ca (ppm)	451.27	311.57	418.99
OM (%)	1.52	1.40	1.50
EC (dS m^{-1})	0.06	0.06	0.07
Moisture (%)	2.04	1.58	2.51
Density (g cm^{-2})	1.39	1.41	1.40

(pH), (ii) Organic matter content and (iii) Fertility status. Microorganisms like bacteria, fungi, actinomycetes, algae and protozoa help in improving soil structure, aeration, water permeability and soil nutrient availability. The optimal pH of soil for rubber cultivation falls within the range of 4.0-6.5. Soil pH may decrease in summer, due to the varied activities of the microorganisms. Acidity or alkalinity of soil influences the biological activity in soil and the availability of certain minerals, such as phosphates. Organic matter after decomposition, attains a relatively stable form call humus, which carries negative electric charges on its surface which attract and hold ions like potassium (K), calcium (Ca) and magnesium (Mg). The humus acts as a cementing agent and assists in granulating clay particles to form stable crumbs. It facilitates movement of water in the soil and improves aeration[12]. The organic matter content of well-drained soil varies (from 0.6-1.0%) and therefore, requires maintenance through the regular application of plant and animal residues in the soil. Soil moisture improves nutrient uptake and its fertility status can be further improved through proper management of the soil, which includes growing a leguminous cover crop and applying fertilizers[12].

PSB isolation: Four PSB isolates were solubilized $AlPO_4$, which are the major elements in tropic soil[13] (Table 1). This preliminary observation suggests the existence of bacterial isolates, exhibiting different degrees of phosphate solubilizing efficiencies in the different root rhizosheres of intercropping under rubber trees soil samples. Similar to the study of Kothandaraman *et al.*[14] by isolating PSB from the rhizosphere of different leguminous cover crops, the results showed that there are the diversity of PSB under the root rhizosphere of *M. bracteata* compared with *P. javanica*, which exhibited 9×10^4 and 5.29×10^4 colony forming unit (CFU g^{-1}) of soil, respectively. Saengsanga[15] reported that Plant Growth Promoting Bacteria (PGPB) found in the agricultural rhizosphere of plants and microorganisms play a key role in soil P dynamics, which catalyze the hydrolysis of organic phosphate esters into orthophosphate anions, through phosphatases. The phosphatase efficiency is related to the microbial fauna, soil temperature, humidity, pH, carbon and nitrogen sources[16], as well as the associated bacterial communities[6]. Other significant factors include the physiological state of the plant, the type of rooting system, the age of the plant and the location of the ectomycorrhiza on the root[7].

Phosphate solubilization efficiency: The isolates were based upon morphological observation and biochemical characterization (Table 2). For the four purified isolates, this results showed that free-living bacteria play a key role in soil P dynamics, which catalyze the hydrolysis of inorganic phosphate into orthophosphate anions, through the use of phosphatases to improve P availability. The four isolates that solubilize $AlPO_4$ and one isolates that solubilize $FePO_4$ are the main forms of insoluble phosphates in acidic sandy soils[17]. After evaluating their P solubilization capacities, the results showed that PSB-M-01 isolated from root rhizosphere of *M. bracteata* can solubilize $Ca_3(PO_4)_2$ better than $AlPO_4$ and the highest solubilize $AlPO_4$ (561.32 mg P L^{-1}) than PSB isolated from root rhizosphere of cassava and banana, respectively.

Table 2: Phosphate solubilizing effectiveness of tested bacteria, 3 days after inoculation

	Solubilized phosphate (mg P L^{-1})		
Isolates	$Ca_3(PO_4)_2$	$FePO_4$	$AlPO_4$
PSB-M-01	1,050.90[a]	-	561.32[a]
PSB-M-02	-	139.63[a]	378.91[b]
PSB-B-01	-	-	65.80[d]
PSB-C-01	-	-	230.02[c]

M: *Mucuna bracteata*, B: Banana and C: Cassava, different letters indicate a significant difference, at $p \leq 0.01$

Table 3: Indole acetic acid production properties of PSB isolates

PSB-Isolates	IAA production (mg L^{-1})
PSB-M-01	-
PSB-M-02	962.53[a]
PSB-B-01	112.13[b]
PSB-C-01	-

M: *Mucuna bracteata*, B: Banana and C: Cassava, different letters indicate a significant difference at $p \leq 0.01$

IAA production of PSB isolates: The IAA is generally considered to be the most important native auxin on root growth in seedlings, especially root elongation. The microorganisms isolated from the root rhizosphere of banana, cassava and *M. bracteata* have an ability to produce IAA as secondary metabolites. This group of bacteria is known as Plant Growth Promoting Bacteria (PGPB). The IAA production contributes to plant growth through phytostabilization, by increasing the root and shoot biomass[18], which are involved in nutrient uptake[19]. In this study, the plant growth promoting effects of selected PSB were evaluated through the analysis of IAA production. The IAA production was observed in PSB isolates; numbers M-02 and B-01, which indicated that these strains could utilize L-tryptophan as a precursor to growth. The PSB in isolated numbers M-02 achieved the highest IAA production (962.53 mg L^{-1}) (Table 3). Mutluru and Mallaiah[20] reported that IAA production in bacteria varies among different species and strains and is also influenced by

Table 4: Identification of PSB isolates from soil samples of paddy fields and eggplant fields in Kochi by 16S rRNA sequencing after inoculation

Isolate code	Length of 16S rRNA gene sequenced	GenBank accession no	Most closely related organism/species (Strain)	Accession No.	Gene identity (%)
PSB-M-01	578	Lcl/53891	*Klebsiella* sp., SCAUS56	KF836054	94
PSB-M-02	875	Lcl/185803	Bacterium 5-2 (2013)	KC753506	99
PSB-B-01	879	Lcl/80489	*Klebsiella variicola* strain XF13	KC853306	99
PSB-C-01	826	Lcl/244355	*Nguyenibacter vanlangensis* strain TN01LGI	NR125459	99

M: *Mucuna bracteata*, B: Banana and C: Cassava

culture condition, growth stage and substrate availability. Additionally, the production of IAA was greater in YMD than in LB media and the YMD media with tryptophan proved more suitable for IAA production, compared with YMD without tryptophan[21].

Identification of PSB isolates: Nucleotide sequencing of PCR-amplified 16S rRNA genes and sequence comparison with available data in the GenBank using the BlastN program[11] allowed us to identify the majority of the PSB isolates (Table 4). Based on a sequence identification of 94% or greater, they were all affiliated to the β or γ sub-divisions of the proteobacteria: Two isolates were similar to species of the *Klebsiella* genus, another one was similar to bacterium 5-2 (2013) and one was closely related to *Nguyenibacter vanlangensis* strain TN01LGI. *Klebsiella* sp., SCAUS56 in the *M. bracteata* intercropping with rubber trees field solubilize $Ca_3(PO_4)_2$ better than $AlPO_4$. Moreover, *Klebsiella* sp., SCAUS56 isolate code M-01 can solubilize $AlPO_4$ as well.

CONCLUSION

Different intercropping with rubber plantations throughout Northeast Thailand significantly affect the microorganic services, like Phosphate Solubilizing Bacteria (PSB) on diversity, phosphate solubilisation and IAA production *in vitro* condition. The PSB isolates from *M. bracteata* intercropping with rubber trees field demonstrated the capacity to solubilize insoluble P and IAA production, more than the other intercropping tested. This indirectly confirms the involvement of bacterial isolates in enhancing plant growth through the synthesis of IAA. In further investigation, these bacteria will be characterized for their inoculative effects on plant growth, by increasing the abundant population of active and effective microorganisms in the root activity zone and increasing the rubber tree's ability to uptake more nutrients.

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SIGNIFICANT STATEMENT

Phosphorus deficiency is a major constraint to crop production in many tropical countries. Although soils generally contain a large amount of total P, only a small proportion is immediately available for plant uptake. 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. Free-living bacteria and fungi can mobilize orthophosphate (predominantly as HPO_4^{-2} and $H_2PO_4^-$) from either organic or inorganic P sources such as Phosphate Rock (PR). Phosphate Solubilizing Microorganisms (PSM) are characterized by their capacity to solubilize precipitated forms of P such as tri-calcium phosphate (TCP), the main P ingredient in PR and could be good bio-fertilizers for improving phosphorus plant nutrition.

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