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Abilities of phosphate solubilizing bacteria isolated from sandy soils under rubber tree plantations of different ages in Northeast Thailand

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Abstract

In order to understand the impact of land use and the change which occurs within ecosystem services in rubber plantations, we must address the absence of knowledge on the influence of agricultural practices over biological processes within the soil. This study describes the results of phosphate solubilizing bacteria (PSB) isolated from soil from different ages of rubber tree plantations in Northeast Thailand; where soils are mainly sandy, and retain a P deficiency. PSB isolates were tested using different P sources [Tri-calcium Phosphate ($\text{Ca}_3(\text{PO}_4)_2$) and Ferric Phosphate (FePO_4)] on specific culture media [National Botanical Research Institute Phosphate Growth Medium, (NBRIP)] under controlled conditions. Our results indicate that PSB isolated from rubber trees of different ages (5, 11, and 22 years) solubilized significantly higher amounts ($P \leq 0.01$) of FePO_4 (601.45-631.63 mgP/L) and $\text{Ca}_3(\text{PO}_4)_2$ (319.24-597.85 mgP/L). The highest activity of solubilization was achieved in FePO_4 , which is the main form of insoluble phosphates in acidic sandy soil. Indole acetic acid (IAA) production was also observed in different ages and locations of the rubber plantations. These results provide essential information for the influence of soil fertility within Thailand's rubber plantations.

Keywords: Bacteria, Phosphate Solubilization, Rubber, sandy soil

1. Introduction

Today, rubber is cultivated worldwide. The production is heavily concentrated throughout Asia, where over 90% of the world's natural rubber is being produced. The further expansion of rubber plantations has led to a major land use change in Asia. In Northeast Thailand, rubber has replaced other cash crops; like cassava, or sugar cane. In Laos, new rubber plantations are often established to the detriment of natural spaces (pioneer dynamics). Rapid expansion of rubber has caused large-scale loss of forest resources, watershed destruction, soil quality changes, and the incapacity of soil to function within ecosystem boundaries necessary to sustain biological productivity, and maintain environmental stability.

In the upland regions of Southeast Asia, agriculture has gone from being primarily "forest-subsistence agriculture" (until the 1960s); to a system dominated by increasingly intensive agricultural practices. FAO statistics indicate that in Thailand's natural forests (19 million ha), the harvested areas of rubber plantations occupy 2 million ha, and produce about 40% of the total rubber used in domestic industry (10 Mt). Contrary to common belief, smallholders represent the majority of rubber producers, with more than 1.5 million family owned farms; most of which have less than 4 ha of surface area [13]. Favorable conditions continue to expand the prospects for the development of new rubber plantations in Asia, thereby contributing to major land use changes. Strong market demand for natural rubber combined with an abundance of land has encouraged foreign investors to promote rubber planting in almost all regions of Lao PDR [1]. The steady increase in rubber prices, until March 2011, and the benefits of a long term perennial cash incentives suggest that rubber may be a worthwhile crop for Lao upland farmers.

However, the rapid rush to plant rubber, and the corresponding plummeting prices of natural rubber since early 2011, have awakened many environmental and social concerns. Today, in Northeast Thailand, the current ever-increasing demand for natural rubber coincides with the depleting availability of areas with suitable soil (sandy, acidic, low cation exchange capacity (CEC), low nutrient reserves, and low water holding capacity). Furthermore, the average annual rainfall of less than 1,300mm (with a severe dry season) contrasts the generally favourable biotope usually admitted for rubber tree development (generally about 1,800-2,000mm of rainfall). The uplands often present severe physical and/or chemical degradations, due to deforestation and the cultivation of both cassava and sugar cane.

The purpose of this research is to address the absence of knowledge regarding the influence of rubber plantation practices over soil quality changes, such as nutrient cycling, and microorganic services like phosphate solubilizing bacteria (PSB). PSB has been shown to enhance the solubilization of insoluble P compounds [16], and are therefore widely used as inoculants to increase P uptake and crop yield [3]. Our objective was to estimate the importance and variability of specific PSB isolates from plantations of different ages, in order to make suggestions regarding fertility management in the poor sandy soils of the region.

2. Materials and Methods

2.1. Sampling Site and Soil Sample Collection

The Kranuan district of Khon Kaen province, Northeast Thailand; is characterized by a tropical climate with acidic sandy soils. Soil samples were collected at a depth of 0-10cm, with four replicates ($n=4$), on rubber tree plantations of three ages: 4-5, 11, and 22-23 years; and were preserved in plastic bags at 4°C for chemical and physical analysis, and PSB isolation.

2.2. Isolation of Phosphate Solubilizing Bacteria by Enrichment Culture

In order to extract the PSB, we transferred 5g of each soil sample to the National Botanical Research Institute's Phosphate growth medium (NBRIP) [11]. This growth liquid medium (per liter) contained 10g glucose, 5g $MgCl_2 \cdot 6H_2O$, 0.25g $MgSO_4 \cdot 7H_2O$, 0.2g KCl, and 0.1g $(NH_4)_2SO_4$. A modified NBRIP media, containing either $FePO_4$ or $Ca_3(PO_4)_2$ 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 50ml of the medium with inoculants were incubated for three days at 30°C in an incubator shaker at medium speed (150 cycles/ min^{-1}). During the next three days, 5ml of the incubated medium with inoculants were again transferred into 50ml Erlenmeyer flasks with new liquid medium at 30°C, in an incubator shaker, again at medium speed. At the end of each three-day cycle of the NBRIP growth liquid media, aliquots of each dilution were spread over the NBRIP medium, and further incubated at 30°C for seven days. After six PSB isolations, colonies were selected from the plates on the basis of the appearance of a clear halo; 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.

2.3. Mineral Phosphate Solubilization

The phosphate solubilizing (PS) activity of each isolate was determined through four replicates by the molybdenum-blue method [9]. The isolates were grown in NBRIP liquid medium containing different insoluble forms of phosphate [$Ca_3(PO_4)_2$ and $FePO_4$] for three 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 minutes 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.

2.4. Indole Acetic Acid Production (IAA)

Selected PSB strains, based on their ability to solubilize P, were analyzed for IAA production [12]. The selective bacterial strains were grown in 50 ml of Luria-Bertani medium (LB), containing 10 g/l Tryptone, 5 g/l NaCl, and 5 g/l yeast extract; at 30°C for two days. The 5 μ l PSB solutions were tested through the reaction with 1ml of Tris-TMRT reagent, containing 10 g/l D-mannitol, 0.2 g/l yeast extract, 0.2 g/l $CaCl_2 \cdot 2H_2O$, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 1.21 g/l Tris-base, and 0.061 g/l L-Tryptophan; at 28°C for ten days. The 2 ml mixtures were

synthesized with 0.01 M FeCl_3 in 35% HClO_4 for 30 minutes 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 minutes, at 25°C , in dark conditions. We 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 from 0-150 $\mu\text{g/ml}$ [12].

2.5. Statistical Analysis

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

3. Results and Discussion

3.1. Soil chemical and physical analysis

The results of the analysis of certain chemical properties within the soil in all sites are shown in table 1. 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 $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$; were not significant different given the increase of rubber tree age; whereas the electrical conductivity (EC) demonstrated a significant difference. The electrical conductivity is felt to have increased over time due to the falling of rubber tree leaves, which accumulate as organic matter within the soil. Moreover, the soil moisture, as well as the soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, also demonstrated an increase commensurate with the aging of the rubber trees (Table 1).

The relevant chemical properties of soil are: (i) soil reaction (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 [14]. 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. Sound 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 [14].

3.2. PSB Isolation

The screening strategy enabled the nine PSB isolates to solubilize FePO_4 far better than the $\text{Ca}_3(\text{PO}_4)_2$, (Table 2); which are the major elements in tropic soil [5]. This preliminary observation suggests the existence of bacterial isolates, exhibiting different degrees of PS efficiencies in the collected soil samples. Seangsanga [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 [8]; as well as the associated bacterial communities [18]. 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 [2].

3.3. Phosphate solubilization efficiency

The isolates were based upon morphological observation and biochemical characterization (Table 2). For the nine purified isolates, our 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 six isolates that solubilize $\text{Ca}_3(\text{PO}_4)_2$ and the nine isolates that solubilize FePO_4 are the main forms of insoluble phosphates in acidic sandy soils (17). After evaluating their P solubilization capacities, we have concluded that each of the nine isolates solubilize FePO_4 better than $\text{Ca}_3(\text{PO}_4)_2$; especially the isolates found in location A (5A, 11A, and 22A), location B (11B, 22B, and 5B); and location C (22C and 11C).

Table 1 Soil properties in different ages of rubber tree plantations in the Kranuan district, Khon Kaen province, Thailand.

Soil properties	Rubber Tree age (Years)			F-test
	5	11	22	
pH (1:1 H ₂ O)	5.48	5.42	5.40	ns
Total N (%) (micro-Kjeldahl)	0.03	0.03	0.05	ns
Available P (mg/kg) (Bray II method)	16.78	9.17	7.39	ns
Exchangeable K (mg/kg)	9.05	9.45	11.70	ns
Exchangeable Ca (mg/kg)	14.17	18.38	16.55	ns
OM (%)	0.49	0.69	1.02	ns
EC (MS/Cm)	0.008c	0.011b	0.014a	**
NH ⁺ ₄ -N (mg/kg)	4.96	4.96	8.17	ns
NO ⁻ ₃ -N (mg/kg)	7.87	9.92	14.88	ns
Moisture (%)	5.67	9.67	11.71	ns
Density (g/cm ²)	1.51	1.52	1.44	ns

ns = not significant

** = significant difference at $P \leq 0.01$.

Different letters indicate a significant difference, at $P \leq 0.01$.

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

PSB-Isolates/ Rubber Tree age (Years)/Areas	Solubilized Phosphate (mgP/l) from		F-test	SD
	Ca ₃ (PO ₄) ₂	FePO ₄		
5A	598a	616a	**	1.66/3.32
11A	452b	577b		38.22/4.06
22A	91c	620a		7.55/5.44
5B	572a	574c	**	8.54/6.50
11B	0b	632a		0/4.38
22B	0b	616b		0/9.02
5C	0c	293c	**	0/10.80
11C	22b	582b		2.21/4.49
22C	319a	601a		8.30/3.26

** = significant difference at $P \leq 0.01$.

Different letters indicate a significant difference at $P \leq 0.01$.

A, B and C = Rubber tree plantations

5, 11 and 22 years = Rubber tree ages

3.4. IAA production of PSB isolates

The microorganisms isolated from the root rhizosphere of various crops have an ability to produce IAA as secondary metabolites. IAA is generally considered to be the most important native auxin on root growth in seedlings, especially root elongation. This group of bacteria is known as plant growth promoting bacteria (PGPB). IAA production contributes to plant growth through phytostabilization, by increasing the root and shoot biomass [6], which are involved in nutrient uptake [4]. In this study, the plant growth promoting effects of selected PSB were evaluated through the analysis of IAA production. IAA production was observed in PSB isolates; numbers 22A, 11B, 5C, 11C, and 22C, which indicated that these strains could utilize l-tryptophan as a precursor to growth. Phosphate solubilizing bacteria in isolated numbers 11B, 22A, and 5C achieved the highest IAA production (649, 634, and 625mg/L, respectively (Table 3). Similar to the research work of Seangsanga, by screening the nitrogen fixing bacteria (NFB) from the rhizosphere of the rubber (*Hevea brasiliensis* Mull-Arg), we produced a

phytohormone, indole-3-acetic acid (IAA); isolating NRRU-L11, -T11, and T-8; which exhibited high IAA production levels of 607.67, 501.67, and 459.33 µg/ml; respectively. Mutluru and Mallaiah reported that IAA production in bacteria varies among different species and strains; and is also influenced by 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 [7].

Table 3 Indole acetic acid production properties of PSB isolates.

PSB-Isolates/ Rubber Tree age (Years)/Areas	IAA production (mg/l)
22A	634a
11B	649a
5C	625a
11C	488b
22C	480b
F-test	**
C.V.(%)	11.38

** = significant difference at $P \leq 0.01$.

Different letters indicate a significant difference at $P \leq 0.01$.

A, B and C = Rubber tree plantations

5, 11 and 22 years = Rubber tree ages

4. Conclusions

Different ages and locations of rubber plantations throughout Northeast Thailand significantly affect the microorganic services, like phosphate solubilizing bacteria (PSB) on phosphate solubilisation and IAA production in vitro condition. The PSB isolates from rubber plantation A demonstrated the capacity to solubilize insoluble P and IAA production, more than the other rubber plantations 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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