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Screening of endophyte bacteria for phosphate solubilization from organic rice

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Abstract

Endophytic bacteria in root zone are capable of increasing the availability of soil phosphorus to vegetation and improve plant growth. The aims of this study were to isolate endophytic bacteria and study their efficient phosphate solubilizing *in vitro*. Endophytic bacteria were extracted from rice tissue of 3 varieties such as RD6, Chainat 1 and Black glutinous rice cultivated in the 4th year of organic paddy field in Han Tao Village, Muang District, Udon Thani. Rice plants of each variety were sampled in 1 m^2 for 4 replications. They were washed by clean water. Samples were surface-sterilized, grinded and diluted to 10^{-1}- 10^{-6} before pouring plate in TSA and incubated at 30 ◦C for 24 hours. Endophytic bacteria were isolated using different characteristics of colony, cell shape and gram's strain. The potential of phosphate solubilization was screened by spotting in Pikovskaya's Agar and incubated at 30 ◦C for 7 days. Colony diameter and clear zone diameter were measured. Phosphate solubilisation index and solubilization efficiency were calculated. The amount of soluble phosphate was determined by culture in Pikovskaya's broth for 24, 48 or 72 hours. Cells were separated by centrifuging at 3,000 g for 5 mins. The supernatants were added with 1 ml vanadate solution (0.25% in 35% HNO_3) and 1 ml of molibdate solution (50% in water), respectively. Then they were incubated for 5 mins or until red color appeared and the absorbance measured at 420 nm with spectrophotometer. The amount of soluble phosphate was calculated from standard curve. The number of isolates and colonies forming unit of endophytic bacteria extracted from tissue of three varieties showed no significant differences. They had similar colony characteristics such as big, round, white and rim. Cells were rod shape. Gram's stains were either positive or negative. The isolates of CHR2I02, CHR3I01, CHR4I07, BRR1I04 and BRR3I01 can solubilize tricalcium phosphate in Pikovskaya's Agar. The amount of soluble phosphate in the supernatant of isolates increased the highest at 48 hours after inoculation with the range from 52.9 - 98.9 mg l^{-1}.

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Keywords: endophytic bacteria, phosphate solubilization

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1. Introduction

Adequate soil phosphorus (P) is essential for optimal crop yields. Phosphorus enables a plant to store and transfer energy, promotes root, flower and fruit development, and allows early maturity [1]. However, most of it is in insoluble compounds that are unavailable to plants [1] [2]. The soil pH range at which maximum P availability occurs is between 6.0 and 7.0. Soils with a pH of 7.5 and higher typically have a high calcium concentration that binds P as calcium-phosphate creating an insoluble compound that is not available to plants [1]. Organic phosphorus is a part of all living organisms, including microbial tissues and plant residue. It is the principal form of phosphorus in the manure of most animals. About two-thirds of the phosphorus in fresh manure is in the organic form [3]. The release of inorganic phosphate from organic phosphates is called mineralization and is caused by microorganisms breaking down organic compounds [4]. Inorganic P is negatively charged in most soils. Because of its particular chemistry, P reacts readily with positively charged iron (Fe), aluminum (Al), and calcium (Ca) ions to form relatively insoluble substances. When this occurs, this process fixes P in an unavailable form [5].

Farmers in Han Thao Village, Kud Jab District, Udon Thani Province, Thailand cultivates rice (*Oryza sativa* L.), organically. Bio-products from microbial were required to replace agrochemical for reaching higher growth and yield. Soil type of organic farm in Han Thao Village is sandy loam. Available P in soil was indicated to 0.89 $mg \cdot kg^{-1}$ or very low [6]. The use of organic fertilizers is an essential component to maintain or improve soil fertility. If soil P levels are declining or if the soil type is just inherently too low in P for the planned cropping, then organic farmers supplement plant-available P with manures, green waste compost or mineral rock phosphate [7]. In organic farming organizations manufactured phosphorus fertilizers such as superphosphate or ammonium phosphate fertilizers such as MAP or DAP are restricted. To improve available P in soil, rock phosphate is allowed to apply in organic farming [1]. However the use of rock phosphates instead of the water soluble phosphorus fertilizers will result in less production. It requires the activity of microorganisms to change the soil phosphorus into an available form for uptake by a plant [8]. Therefore, soil microorganisms were applied for increasing the availability of P from mineral phosphates to plants. The ability of soil fungi to solubilize iron and aluminum phosphates was indicated with the ability to solubilize the inorganic phosphates in culture [9]. In addition, many groups of bacterial were isolated for phosphate solubilization. For example, *P. fluorescens* complex has been reported on superior phosphate solubilizing potential [10]. Bacterial groups from rhizophere soil of various crops in Korea were isolated. Out of several hundred colonies of bacteria that grew on Pikovskaya's medium 13 best isolates were selected based on the solubilization of insoluble phosphates in liquid culture and further characterized and identified. They were clustered under the genera *Enterobacter*, *Pantoea* and *Klebsiella* [11]. Three hundred actinomycete isolates originating from these mines were tested for their ability to grow on a synthetic minimum medium (SMM) containing insoluble rock phosphate as unique phosphate source [12]. Use of phosphorus solubilizing bacteria as inoculants increases P uptake. These bacteria also increase prospects of using phosphatic rocks in crop production. Greater efficiency of P solubilizing bacteria has been shown through co-inoculation with other beneficial bacteria and mycorrhiza [13]. The cold tolerant mutants of *Pseudomonas fluorescens* strains based on 'P' solubilization ability and subsequent effect on plant growth promotion under *in vitro* and *in situ* conditions was conducted. Under *in vitro* conditions at 10 °C, these cold tolerant mutants exhibited increased plant growth indicating their functionality at low temperature. Subsequently, greenhouse trials using high 'P' solubilizer microorganisms showed a significant increase in root (30 and 20%) and shoot length (20 and 24%) of mung bean, both in sterilized and unsterilized soil, respectively. On the contrary, low 'P' solubilizer microorganism did not stimulate plant growth. Furthermore, sand experiments indicated that tricalcium phosphate served as better phosphorus source for treated mung bean seeds [14]. In addition, *Rahnella aquatilis* is a Gram-negative bacterium that can fix atmospheric nitrogen and also has the ability to solubilize mineral phosphate [15].

Phosphate solubilizing bacteria was isolated from the rhizosphere of chickpea and alkaline soils and demonstrated diverse levels of phosphate solubilization activity under in vitro conditions in the presence of various carbon and nitrogen sources [16]. *Pseudomonas* sp. strain PAC, *Serratia* sp. strain CMR165 can solubilize inorganic tricalcium phosphate and organic calcium magnesium inositol hexaphosphate. Rice plants inoculated with *Pseudomonas* sp. strain PAC or *Serratia* sp. strain CMR165 had higher concentrations of phosphates than those inoculated with *Azospirillum brasilense* strain FT326 and plants that were not inoculated. Glucose was the only sugar identified in rice root exudates. PAC and CMR165 promoted plant growth and uptake of phosphate and could be used as biofertilizers to optimize phosphate fertilization [17]. In Argentina, tricalcium phosphate solubilzing activity was employed in order to screen beneficial bacteria associated to peanut in the main cultivation area of this crop [18]. Isolation of phosphate-solubilizing bacteria (PSB) was isolated from aerobic rice grown in Penang Malaysia. The highest P solubilizing activity (69.58%) was found in PSB9 strain grown in NBRIP plate. A number of PSB isolates belong to the *Bacillus* sp. PSB inoculants with their beneficial traits would be considered as potential biofertilizer for the sustainable aerobic rice cultivation system [19]. Isolation of phosphate solubilizing bacteria was inoculated in aerobic rice (*Oryza sativa* L.) under rock phosphate (RP) treatments. PSB strains PSB9 and PSB16 solubilized significantly high amounts of P compared to non-inoculated treatments. The higher amounts of soluble P in the soil solution increased P uptake in plants and resulted in higher plant biomass. PSB strains also increased plant height and improved root morphology in aerobic rice. [20].

The objective of this study was to isolate endophytic bacteria from 3 varieties of rice plants grown by organic cultivation and studied on their efficient phosphate solubilizing *in vitro* for using as bio-fertilizer**.**

2. Meterial and Methods

Organic rice were sampled from the 4th year organic paddy fields which grown rice cultivars RD6, Chainat 1 and Black Glutinous, in Han Tao Village, Tambon Pako, Muang District, Udon Thani. Samples of each variety were taken in 1 m^2 area for 4 replications. Roots and stems of samples were cut and washed by clean water. Samples were surface-sterilized by soaking in 70% ethanol for 30 seconds, 0.1% $HgCl_2$ for 3 minutes and washed by sterilized distilled water for 10 minutes in 3 times. Sterilized samples were grinded and diluted to 10^{-1}- 10^{-6} before pouring plate in TSA medium and incubated at 30 ◦C for 24 hours. Endophytic bacteria were isolated using different characteristic of colony. Cell shape and gram's strain were investigated. Screening of their phosphate solubilizing ability was revealed by spotting in Pikovskaya's Agar for 4 replications and incubating at 30 ◦C for 5 days. Colony diameter and clear zone diameter were measured. Solubilization index (SI) was calculated using following formula [21]

$$SI = \frac{\text{colony diameter + halozone diameter}}{\text{colony diameter}}$$

Solubilization efficiency was calculated. Then amount of soluble phosphate in broth culture were determined. Each isolation was cultured in Pikovskaya's broth for 24, 48 or 72 hours. Cells were separated by centrifuging at 3,000 g for 5 mins. Their supernatants were added with 1 ml vanadate solution (0.25% in 35% HNO_3) and 1 ml. of molibdate solution (50% in water), respectively. Then they were incubated for 5 mins or until red color appear. Then the absorbance at 420 nm was measured with spectrophotometer. Amount of soluble phosphate were calculated from standard curve.

3. Results and Discussion

The results indicated that the number of isolates and colonies forming unit of endophytic bacteria extracted from tissue of three varieties of organic rice was no significant difference. Average number of isolates from RD6, Chainat 1 and Black Glutanious were 1.5, 3.5 and 3.5, respectively. Totally, there were all 36 isolates from 4 replications. Average CFU of RD6, Chainat 1 and Black Glutanious were 3.7, 5.4 and 3.4 $\times 10^4$ CFU/ml, respectively (Table 1). They had similar colony characteristic such as big, round, white, rim and etc. Cell was rod shape. Gram's stain was either positive or negative. Endophytic bacteria can symbiosis in all kinds of plants.

Some of them infect in plant as without symptoms and show benefit activities for plant such by enhancing plant growth, nitrogen fixation, induction of antagonism to plant pathogen [22]. Endophytic bacteria were isolated from many varieties of rice. Some isolates from stem tissue and seeds were identified as *Bacillus subtilis* and *Bacillus cereus* [23].

Table 1. The number of isolates and colonies forming unit of endophytic bacteria extracted from tissue of three varieties of organic rice

Varieties	**Number of isolates**	**Colony forming unit ($\times 10^4$ CFU/ml)**
RD6	1.5	3.7
Chainat1	3.5	5.4
Black Glutinous	3.5	3.4
F-test	ns[1/]	ns
CV (%)	40.6	26.4

[1/]No significant difference at $P \leq 0.05$

The result indicated that 5 isolates of endophytic bacteria can solubilize tricalcium phosphate in Pikovskaya's Agar. The solubization index of CHR3I01, BRR1I04, BRR3I01, CHR4I07 and CHR2I02 were 10.0, 7.0, 3.9, 3.2 and 2.7, respectively. The solubilization efficiency of CHR3I01, BRR1I04, BRR3I01, CHR4I07 and CHR2I02 were 800.0, 504.0, 186.1, 120.3 and 72.3, respectively (Table 2). These results were in accordance with the report of *in vitro* solubilization of inorganic phosphate by phosphate solubilizing microorganisms (PSM) from maize rhizosphere. The phosphate solubilizing bacteria grown *in vitro* for seven days on Pikovskaya,s medium showed Phosphate solubilization index of these isolates ranged from 1.63-3.29. Drop in pH of the medium ranged from 7 to 3.2 with the continuous growth of these isolates for seven days [24].

Amount of soluble phosphate in the supernatant of isolates CHR2I02, CHR3I01, CHR4I07, BRR1I04 and BRR3I01 increased highest in 48 hours after inoculation at 58.4, 98.9, 53.4, 57.3 and 52.9 mg l^{-1}, respectively. Then they decreased except isolates BRR1I04. Amount of soluble phosphate in CHR4I01 cultivation was the highest (Fig. 1). This is similar to the report of *in vitro* solubilisation of tricalcium phosphate and production of IAA by phosphate solubilising bacteria isolated from tea rhizosphere of Darjeeling Himalaya [25]. A number of rhizosphere associated microorganisms was isolated from the tea plants of Darjeeling hills and screened for the solubilisation of tricalcium phosphate (TCP) *in vitro*. Eight of the 62 isolates were able to solubilize TCP in Pikovskaya's agar. The isolates of rhizosphere associated microorganism were also screened for phosphate solubilization in liquid medium. The amount of solubilized phosphorus by these strains was measured after 7 days of incubation using 5.0 g l^{-1} TCP in liquid culture. Amount of phosphate solubilized ranged from 40.62 ± 1.1 to 136.73 ± 1.7 mg l^{-1}.

Table 2. Phosphate solubilization efficiency of endophytic bacteria isolated from tissue of three varieties of organic rice

Isolate	**Solubilization index**	**solubilization efficiency (%)**
CHR2I02	2.7^{d}[1/]	72.3^{d}
CHR3I01	10.0^{a}	800.0^{a}
CHR4I07	3.2^{cd}	120.3^{cd}
BRR1I04	7.0^{b}	504.0^{b}
BRR3I01	3.9^{c}	186.1^{c}
F-test	*	*
C.V. (%)	16.1	25.6

[1/]Mean within the same column followed by the same letter are not significantly difference by DMRT at P=0.05

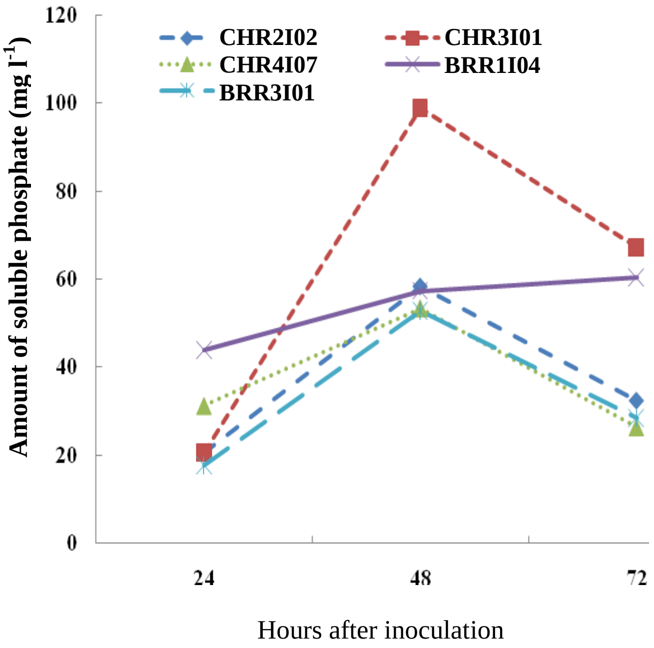

Fig. 1. Amount of soluble phosphate in supernatant of Pikovskaya's broth after endophytic bacteria inoculation for 24, 48 or 72 hours

4. Conclusions

The number of isolates and colonies forming unit of endophytic bacteria extracted from tissue of three varieties of organic rice; RD 6, Chainat 1 and Black Glutanious were no significant difference. The isolates of CHR2I02, CHR3I01, CHR4I07, BRR1I04 and BRR3I01 can solubilize tricalcium phosphate in Pikovskaya's Agar. Amount of soluble phosphate in the supernatant of isolates increased highest in 48 hours after inoculation and ranged from 52.9 - 98.9 mg l^{-1}.

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