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Laboratory and Greenhouse Assays of Phosphate Solubilizing Rhizobacteria to Improve Growth of *Falcataria moluccana* Seedling

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Abstract Phosphate solubilizing bacteria (PSB) play an important role as plant growth promoting, through phosphate solubilization and IAA hormone production. This study was aimed at screening P solubilizing bacteria from post-tin mining soils, and examining their activity in promoting *Falcataria moluccana* seedling growth. The methods include inorganic phosphate and organic phosphates solubilization characterization in solid and liquid media, and inoculation of PSB isolates into seeds of *F. moluccana* in laboratory and greenhouse assays. A total 11 isolates exhibited P solubilization (organic and inorganic phosphates) activity and IAA production. Highest inorganic phosphate solubilization was exhibited by *Bacillus* sp. strain P6 (9.749 µg/mL at 72 h). In addition, *Enterobacter cloacae* strain P2 and *Bacillus* sp. strain P6 produced highest seed vigor index (SVI) and seed germination, respectively. Inoculation of *E. cloacae* strain P2 to the seedlings of *F. moluccana* also increased dry weight (42.63 mg), enchanced P content of the seedling (2.26 mg/g) and available P (52.65 mg/g) in rhizosphere soil.

Keywords: *Falcataria moluccana*, IAA, P solubilization, rhizobacteria, rhizosphere

Introduction

Recovery of post-tin mining land usually involved various planting programmes, such as plant selection, fertilizer use, and cultivation method. Selected plants must possess fast growing character, drought-resistant, capable to grow on poor nutrients soils, and symbiotic to other microbes (Rahmawaty, 2002). *Falcataria moluccana* (Miq.) Barneby and J.W. Grimes (local name: *sengon*) is one of exotic plants distributed in Southeast Asia region suitable for post-tin mined revegetation program. This plant is fast growing and highly adaptable to various soils type such as poor nutrients soils, sandy soils, and basic soils (Krisnawati *et al*., 2011).

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Phosphate (P) is one of the most important macronutrients as well as being a limiting factor for plants growth and development. The phosphate provides several important roles in plant metabolism such as transfer of energy, stimulate nitrogen fixation in legumes, biosynthesis of nucleic acid and cell membranes, and regulation a number of enzymes (Saber *et al.*, 2005). Crops usually obtained P through fertilization as well as decomposition and mineralization of organic substance. In soils, P presents as insoluble organic and inorganic, and their existence is affected by soils type and pH. Aluminum phosphate and iron phosphate are main forms of P in acid soils, whereas calcium phosphate in alkaline soils (Whitelaw, 2000). About 15 to 80% of P in soils is available as organic forms (Singh and Satyanarayana, 2010). Although P is abundance in soils, however, majority of P is fixed, and therefore, P is scarcely available for plant.

In the rhizosphere, soil microbes such as phosphate solubilizing bacteria (PSB) play an important role in dissolving inorganic and organic P to be available for plants (George *et al*., 2009). Several investigators reported that aside from its potential of solubilizing P, PSB also provides plant growth promoting rhizobacteria (PGPR) such as improving the uptake of nutrients and stimulating the production of some phytohormones (Hariprasad and Niranjana, 2009, Singh *et al*., 2014). PSB, therefore, is likely to serve as an efficient bio-fertilizer to increase the overall performance of plants especially in P deficient areas.

In order to optimize utilization of *F. moluccana* in revegetation of post-tin mining land, we screened and selected various bacteria capable in solubilizing P and PGPR activities. In our long term program, these bacteria will be used as biofertilizer for *F. moluccana* in the revegetation program. Activities of the selected bacteria to promote *F. moluccana* were examined under laboratory and greenhouse conditions.

Materials and methods

Soil samples

Soils were collected from post-tin mining soil in Batu Belubang village, Pangkalan Baru, Bangka (S. 02° 11' 409' and E. 106° 11' 265'), Bangka Belitung province, Indonesia. The soil samples were taken from the top surface near the plant roots at the depth of 20 cm and transferred into plastic bags. The plastic bag caps were loosed to maintain aerobic bacteria and kept in cool container box. In the laboratory, all samples were kept at 4°C in refrigerator until the start of the experiments.

Posphate solubilizing bacteria (PSB) isolation

Soils samples were composited, air dried, and sieved (<2 mm or mesh no. 10). Tens grams of sieved soils were dissolved in an Erlenmeyer flask containing 90 mL of 0.8% sterile saline solution and shaken at 120 rpm for 30 minutes. Serial dilution of 10^{-1} to 10^{-7} was made from the samples. A total 0.2 mL of each dilution were evenly dispersed on Pikovskaya (PVK) agar medium [yeast extract 0.5 g, dextrose 10 g, $Ca_3(PO_4)_2$ 5 g, $(NH_4)_2.SO_4$ 0.5 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, $MnSO_4$ 0.0001 g, $FeSO_4$ 0.0001 g, agar 20 g, distilled water 1000 mL] (Gaur, 1981), and incubated for 7 days at room temperature. The bacterial colonies capable to solubilize tricalcium phosphate were indicated by halo zone around the colonies.

Analysis of inorganic phosphate solubilization

Solubilization of inorganic P was qualitatively performed by inoculating culture of bacteria isolates on PVK agar medium containing 0.5% $Ca_3(PO_4)_2$. Using drop plate method, the medium was inoculated with 10 µL of selected bacterial isolate, and then incubated for 7 days at room temperature. All bacterial treatments were replicated four times. Phosphate solubilizing index (SI) was measured according to Premono and Vleck (1996) [SI = (colony diameter + halozone diameter)/colony diameter]. Quantitative analysis was conducted using PVK liquid medium. Soluble P and pH were measured at 24, 72, 144, and 192 h according to method described by Olsen and Sommers (1982).

Analysis of organic phosphate solubilization

All PSB isolates were qualitatively examined for their ability to produce phytase by using phytase screening medium (PSM) agar (L^{-1}): 0.5% calcium phytate as substrate, 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.01% NaCl, 0.05% KCl, 0.001% $FeSO_4$, 0.01% $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ 0.01%, 0.001% $MnSO_4$, 1.5% agar, pH 6.5 (Kerovuo *et al.*, 1998). 10 µL of bacterial culture was inoculated at the center of PSM agar medium and then incubated for 7 days at room temperature. Bacterial colonies capable of hydrolyzing calcium phytate (extracellular phytase) are characterized by halo zone surrounding the colony. The colony diameter and halo zone were measured after 7 days incubation. PSB isolates with halo zone was further examined for their phytase activities in PSM liquid medium. One enzyme unit (U) is defined as amount of enzyme that liberates 1 µmol inorganic phosphate in one minute (Kumar *et al.*, 2013).

IAA production

Indole Acetic Acid (IAA) production was performed using trypticase soy broth (TSB) medium (20 mL) containing L-Tryptophan (200 µg/mL) at 24, 48, and 72 h based on method described by Pattern and Glick (2002).

Seed germination

One mL (1×10^8 cfu/mL) of each selected PSB isolates was transferred to 100 mL Erlenmeyer flask containing 25 mL of PVK liquid medium. The flask was further incubated on a rotary shaker (125 rpm) for 24 hours at room temperature. Seeds of *F. moluccana* were obtained from plant collection managed by Bogor Botanical Garden. The seeds were sterilized by soaking in 1% sodium hypochlorite for 2 mins, and then rinsed with sterile distilled water three times. The seeds were soaked in the flask containing 25 mL of each suspension bacteria.The seeds soaked in 25 mL of sterile distilled water used as negative control.

Germination test was carried out using paper towel method in completely randomized design with four replicaties. A total 100 seeds/petridish were prepared. Each petridish contained sterile filter paper moistened with 10 mL sterile distilled water. Seed germination was observed every day, and in order to retain the media moisture during the ongoing germination process, the filter paper was watered with sterile distilled water. Root and shoot length of seedling, and percentage of germinated seeds were calculated after 14 days. Seedling vigor index (SVI) was calculated according to the method described by Abdul Baki and Anderson (1970) with the following formula:

SVI = Germination percentage × [Mean root length (mm) + Mean hypocotyl length (mm)]

Greenhouse experiment

Pot experiment was conducted at greenhouse managed by Microbiology Division, Research Center for Biology, LIPI, Cibinong, West Java, Indonesia. For the evaluation of PSB activities in promoting *F. moluccana* seedling growth, the PSB isolates was tested in post-tin mining soil collected from Bangka Belitung province. Soil was autoclaved twice for 20 minutes at 120^{o}C and then fed into the pot ± 300 grams.

Each pot contains three PSB inoculated seeds. The pots were arranged in completely randomized design. The experimental treatment consists of: 1. Plant

without PSB inoculant; 2. Plants inoculated with PSB isolates (P2, P5, P6, P7, P8, and P10). Each treatment was repeated three times.

Plants were harvested after 30 days. Several growth parameters such as root length (cm), shoot length (cm), plant fresh weight and dry weight (mg), and P content were measured. Soil samples from rhizosphere area from each treatment were analyzed to determine the available P (mg/g). Data obtained from this experiment were analyzed using SPSS, and significant differences of treatments were determined by Least Significant Difference (LSD) ($P<0.05$).

Results and Discussion

Posphate solubilizing bacteria (PSB) isolation

A total of 11 PSB isolates were isolated and characterized for their ability in solubilizing organic and inorganic P (Table 1). In addition, all these PSB isolates positively produce indole acetic acid (IAA) hormone. Highest PSI (2.60) showed by *Enterobacter* sp. strain P8. Inorganic phosphate solubilizing index (PSI) of these isolates varied between 2.12 – 2.60. However, variation of PSI among PSB isolates was not significantly different. Therefore, it is necessary to conduct quantitative assay. In qualitative assay of organic phopshate solubilization assay, the clear zone around colonies on PSM medium ranged from 2.15 – 2.61. All PSB isolates also produced IAA hormone (Table 1).

Table 1. Inorganic P solubilization and gowth hormone production assay of PSB isolates.

Isolates	Inorganic phosphate qualitative assay (mm)	Organic phosphate qualitative assay (mm)	Maximum IAA production (µg/mL)
Unidentified bacterium (strain P1)	2.38±0.07 ^{abc}	2.19±0.06^{a}	2.028±0.03^{c}
Enterobacter cloacae strain P2	2.42±0.05^{abc}	2.38±0.11^{abc}	10.47±0.04^{f}
Unidentified bacterium (strain P3)	2.42±0.05^{abc}	2.56±0.10^{bc}	18.88±0.04^{g}
Unidentified bacterium (strain P4)	2.43±0.14^{abc}	2.61±0.09^{c}	0.147±0.02^{a}
Enterobacter sp. strain P5	2.12±0.02^{a}	2.22±0.11^{ab}	19.98±0.054^{h}
Bacillus sp. strain P6	2.51±0.05^{bc}	2.48±0.06^{abc}	7.73±0.19^{c}
Enterobacter sp. strain P7	2.25±0.00^{abc}	2.38±0.11^{abc}	10.27±0.11^{f}
Enterobacter sp. strain P8	2.60±0.06^{c}	2.48±0.20^{abc}	3.56±0.05^{d}
Unidentified bacterium (strain P9)	2.19±0.11^{ab}	2.15±0.09^{a}	1.51±0.075^{c}
Bacillus sp. strain P10	2.36±0.13^{abc}	2.60±0.15^{c}	0.906±0.044^{b}
Unidentified bacterium (strain P11)	2.37±0.17^{abc}	2.40±0.06^{abc}	1.479±0.072^{c}

Note: Means in the same group followed by the same letter in the coloums are not significantly different ($p \leq 0.05$) as determined by the least significant different (LSD) test.

Solubilization of inorganic phosphate

Fluctuation of pH medium during P solubilizing activity at the period of 24 – 192 h incubation was showed in figure 1. In all experiments, pH medium changed into acid (3.72 to 5.68) during incubation. In all PSB isolates (strains of P1-P11), lowest pH was found at 72 h incubation (pH ranged about 3-5). At the early stage of incubation, pH decreased from 7 to 6, except P1 isolate (pH 5). At 144 h and 192 h incubation, pH medium increased in all isolates.

Solubilization of $Ca_3(PO_4)_2$ activity in liquid medium by PSB isolates is showed in figure 2. Highest inorganic phosphate solubilization was exhibited by *Bacillus* sp. strain P6 (9.749 µg/mL). Maximum inorganic phosphate solubilization occurred at 72 h in almost all isolates, except strain P3, *Bacillus* sp. strain P6, and *Bacillus* sp. strain P10 (highest activity at 144 h). P solubilization activity of all PSB isolates decreased after 144 h.

Capacity of PSB isolates in solubilizing inorganic phosphate in solid and liquid medium was varied in this study. This is probably due to availability of substrate, strain or species of bacteria, and pH medium (Kumar *et al.*, 2013). Our data showed that inorganic P solubilization activity in liquid medium was in line with decrease of pH in almost all PSB isolates (Figs. 1 and 2). Previous studies reported that pH declining during P solubilization activity was due to organic acids release (e.g. citric acid, oxalic acid, lactic acid, gluconic acid, etc.) by bacterial metabolism during the solubilization process (Alikhani *et al*, 2006, Schoebitz *et al.*, 2013). Organic acids secretion is one of important mechanisms for PSB isolates to dissolve insoluble P in soil. The roles of organic acids in mechanism of mineral phosphate solubilization involves lowering of pH, chelation of cations, and increasing competing capacity of microbes with P for adsorption sites in the soil. Organic acids may form soluble complexes with metal ions associated with insoluble P, such as Ca, AI, and Fe. Therefore, P is released (Perez *et al*., 2007; Khan *et al.*, 2007; Sashidhar and Podile, 2010).

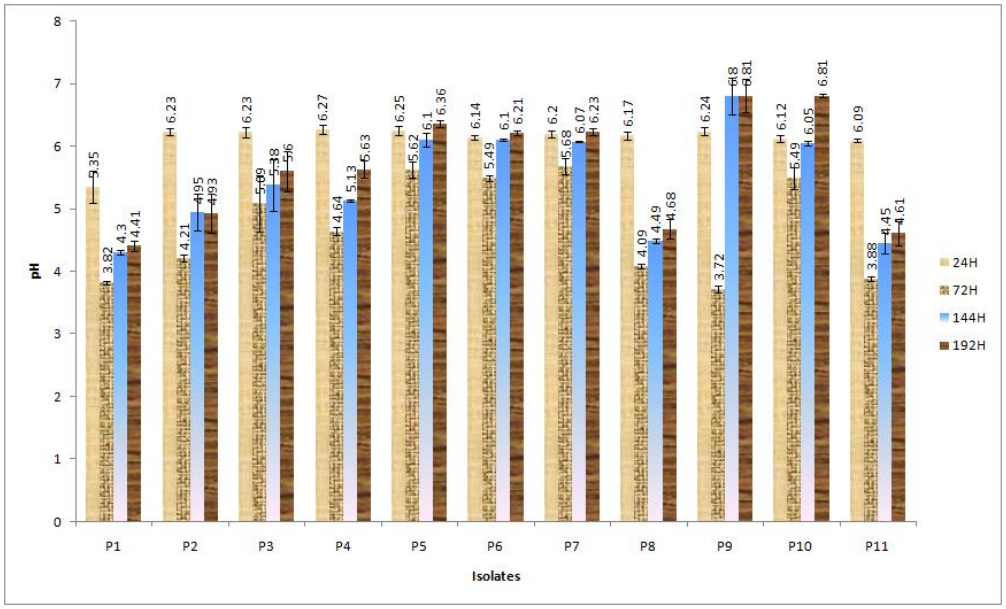

Figure 1. pH change during inorganic P solubilization by PSB isolates at different time interval.

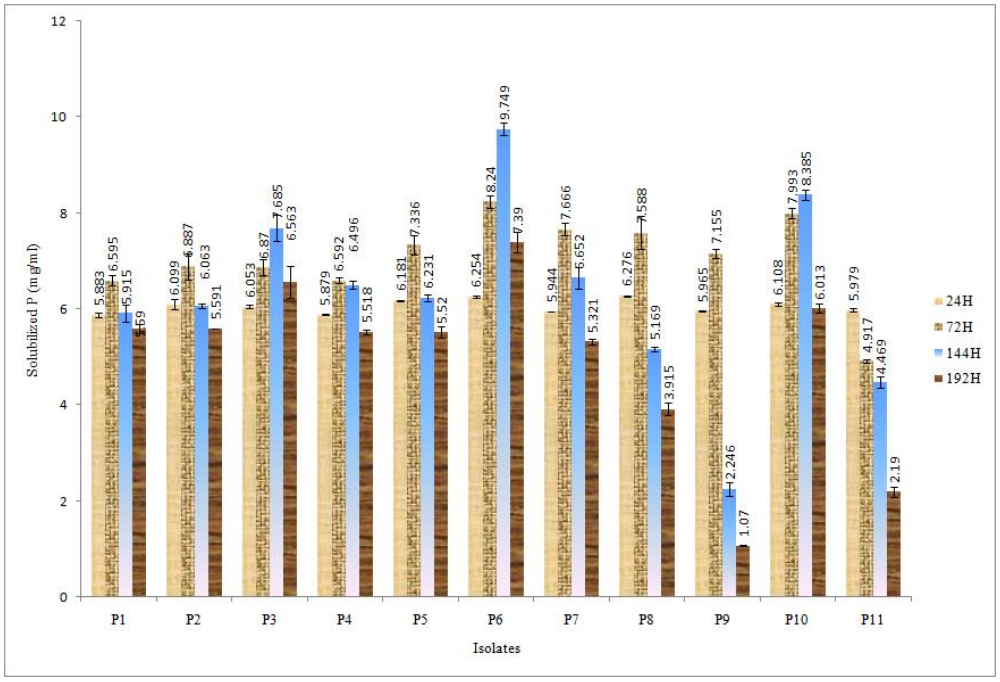

Figure 2. Inorganic P solubilization activity by PSB isolates at different time interval.

Highest inorganic P solubilization activity by majority PSB isolates occurred at pH 6, except inidentified bacterium strain P1. However, maximum P solubilization activity varied among bacterial isolates, and depends on the source of the bacterial isolates. For example, previous study noted that most of PSB isolates from mangrove ecosystem exhibited highest P solubilization activity at pH 5 (Behera *et al*., 2016). In addition, there are also correlation between initial pH and organic acids production during P solubilization activity by bacterial isolates. Marra *et al*. (2015) reported that initial pH affects organic acids production during P solubilibilization, but did not correlate to P solubilization activity of bacteria.

Solubilization of organic phosphate

Phytase plays an important role in dissolving organic P. Inoculation of phytase-producing bacteria can improve plant growth, P content of seedling, and available P in the soil (Kumar *et al*., 2013; Ranjan *et al*., 2013). In this study, all PSB isolates exhibited phytase production (Table 1). Phytase activity ranged between 0.04 – 0.67 U/mL. Highest phytase production was obtained by unidentified bacterium strain P1 (0.67 U/mL at 72 h incubation) (Fig. 3). This concentration is relatively high for PSB bacteria. Other studies reported that phytase production was about 0.14–0.2 U/mL (Acuna *et al.*, 2011; Kumar *et al.*, 2013). Almost all PSB isolates exhibited phytase production at 72 h, except *Bacillus* sp. strain P6 (0.04 U/mL) and *Bacillus* sp. strain P10 (0.38 U/mL). Highest phytase production by unidentified bacterium strain P1, *Enterobacter cloacae* strain P2, strain P3, *Enterobacter* sp. strain P7, unidentified bacterium strain P9, *Bacillus* sp. P10, and unidentified bacterium strain P11 isolates occurred at 72 h, and then decreased at 144 h and 192 h. However, highest phytase production by unidentified bacterium strain P4, *Enterobacter* sp. strain P5, *Bacillus* sp. strain P6, and *Enterobacter* sp. strain P8 was obtained at 144 h. In another study, highest level phytase activity was also obtained at 72 h incubation (Alhadi *et al.*, 2015).

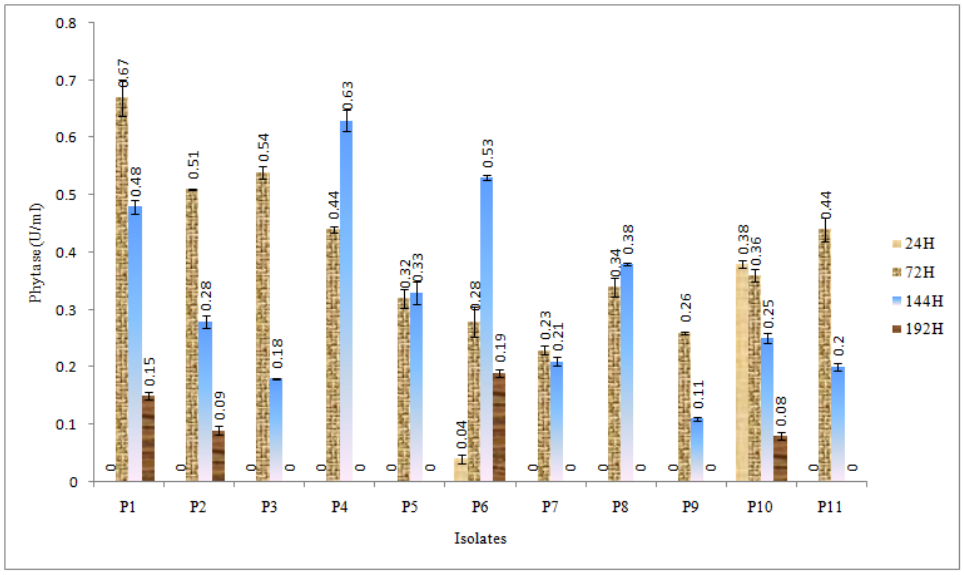

Figure 3. Phytase activity by PSB isolates at different time interval.

IAA production assay

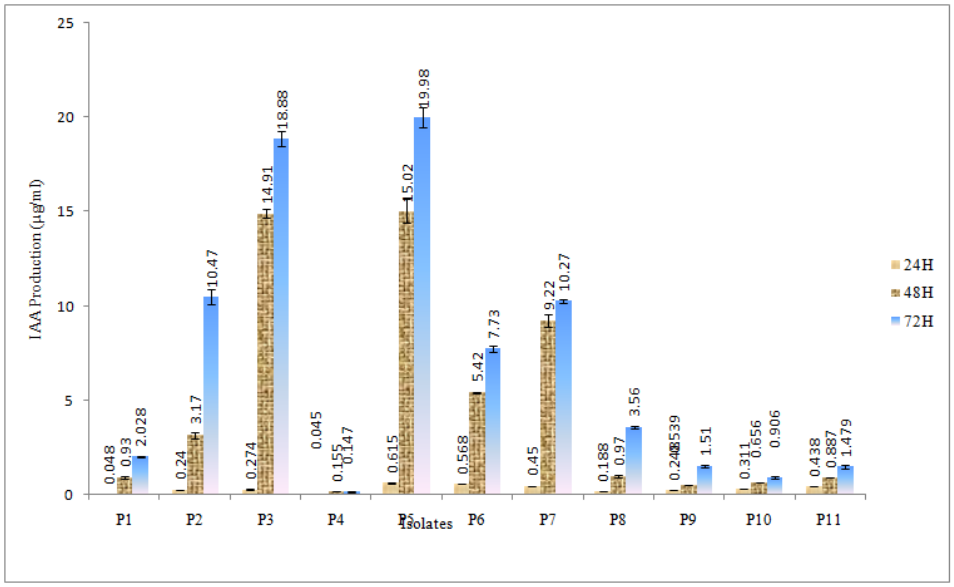

Figure 4. IAA production by PSB isolates at different time interval.

IAA production assay showed that all PSB isolates capable in producing IAA hormone during incubation period. Concentration of IAA produced by PSB isolates varied from 0.045 to 19.98 µg/mL. IAA production was initiated

at 24 h incubation and still increasing until 72 h (Fig. 4). *Enterobacter* sp. strain P5 showed highest IAA production (19.98 µg/mL) at 72 h incubation, followed by Unidentified bacterium strain P3 (18.88 µg/mL). Lowest IAA was produced by Unidentified bacterium strain P4 (0.15 µg/mL). IAA concentration produced by PSB isolates in this study is relatively low in contrast to other studies. For exmple, IAA concentration at 34 µg/mL (Kumar *et al*., 2013) and 58.34 µg/mL (Inui-Kishi *et* al., 2012) after 72 h incubation were reported. Production of IAA in the artificial medium is apparently influenced by culture conditions, growth stage, and substrate (Mirza *et al*., 2001).

Seed germination

Based on P solubilization activity and IAA production, six PSB isolates (*E. cloacae* strain P2, *Enterobacter* sp. strain P5, *Bacillus* sp. strain P6, *Enterobacter* sp. strain P7, *Enterobacter* sp. strain P8, and *Bacillus* sp. P10) were selected for seed germination assay under laboratory and greenhouse conditions (Table 2). The results showed that six PSB isolates influenced root length, shoot length, seed germination, and seed vigor index (SVI) of *F. moluccana* (Table 2). Almost all isolates, except *Enterobacter* sp. strain P8, improved seed germination and SVI of *F. moluccana* compared with negative control (without PSB inoculant). Percentage of seed germination and SVI ranged between 52–86% and 206–773.72, respectively. *Enterobacter cloacae* strain P2 and *Bacillus* sp. strain P6 exhibited highest SVI and seed germination, respectively.

Seeds inoculation by using PGPR isolates can improve seed germination, seedling vigor, and seedling emergence in a variety of plant seeds as a result of bacterial activity in promoting plant growth (Demissie *et al*., 2013). In addition, the use of PGPR bacteria such as *Azospirillum lipoferum*, *Azotobacter chroococcum*, *Pseudomonas fluorescence*, and *B. megaterium* also indicated positive influence on germination rate and vigor index of various crops (Lenin and Jayanthi, 2012). Plants inoculation with phosphate solubilizing bacteria, in particular, provides benefits in the growth of root by induction of phytohormones production such as auxin (IAA), which can stimulate cell division and extension of roots (Pattern and Glick, 2002).

Table 2. Effect of PSB isolates inoculation on several seed germination parameters.

Isolates	Root length (cm)	Shoot length (cm)	Total (cm)	Seed germination (%)	Seed vigor index (SVI)
Control	1.45±0.18^{b}	4.64±0.14^{bc}	6.098±0.30^{b}	64±2.45^{b}	390.00±14.00^{b}
Enterobacter cloacae strain P2	3.51±0.13^{e}	5.95±0.19^{d}	9.44±0.20^{f}	82±2.00^{ef}	773.72±22.88^{e}
Enterobacter sp. strain P5	3.61±0.31^{e}	4.28±0.31^{b}	7.89±0.27^{e}	70±3.16^{bcd}	549.36±16.15^{cd}
Bacillus sp. strain P6	2.26±0.09^{c}	4.41±0.11^{bc}	6.67±0.10^{bc}	86±2.45^{f}	567.54±18.25^{d}
Enterobacter sp. strain P7	2.54±0.13^{c}	4.59±0.13^{bc}	7,13±0.11^{cd}	74±2.45^{cde}	527.02±16.97^{cd}
Enterobacter sp. strain P8	0.43±0.01^{a}	2.18±0.14^{a}	2.63±0.13^{a}	78±3.74^{def}	206.4±18.14^{a}
Bacillus sp. P10	4.31±0.25^{f}	4.87±0.17^{c}	9.18±0.7^{f}	52±2.00^{a}	492.02±5.06^{c}

Note: Means in the same group followed by the same letter in the coloums are not significantly different ($p<0.05$) as determined by the least significant different (LSD) test.

Greenhouse experiment

In greenhouse experiments, application of six PSB inoculants on *F. moluccana* seed significantly increased root length, shoot length, fresh and dry weight, P content of seedling, and P available in soil (Table 3). The increase in length of roots and shoots varied between 19 -112% and 12-27%, respectively. Seedling inoculated with *E. cloacae* strain P2 showed highest gain in dry weight (42.63 mg). While the dry weight of seedlings inoculated by *Bacillus* sp. strain P6 and *Enterobacter* sp. strain P8 were not significantly different with control. Inoculation of *E. cloacae* strain P2 to the seedling also enchanced P content of the seedling (2.26 mg/g) and available P (52.65 mg/g) in rhizosphere soil.

Table 3. Effect of PSB isolates inoculation on several seed germination parameters at 30 days old seedling.

Isolates	Root length (cm)	Shoot length (cm)	Fresh weight (mg)	Dry weight (mg)	Seedling P content (mg/g)	Available phosphate (mg/g)
Control	3.5±0.58[a]	7.87±1.04[a b]	147.67±6.67 [ab]	27.80±1.14 [a]	1.13±0.03 [a]	29.70±0.65[a]
Enterobacter cloacae strain P2	6.67±0.93 [ab]	10.00±0.00[b]	193.30±3.33c	42.63±1.81 [c]	2.26±0.01 [f]	52.65±1.92[f]
Enterobacter sp. strain P5	7.67±0.73 [b]	9.27±0.65[a b]	161.67±19.22[a bc]	32.10±0.67 [ab]	1.29±0.00 [b]	35.26±0.70[b c]
Bacillus sp. strain P6	4.17±1.17 [ab]	9.50±0.76[a b]	140.00±5.77[a]	25.30±1.46 [a]	1.82±0.00 [e]	34.75 ±0.64[b]
Enterobacter sp. strain P7	7.43±0.72 [b]	9.93±0.58[a b]	173.33±11.67[a bc]	37.63±1.73 [bc]	1.76±0.01 [d]	39.34±1.84[b c]
Enterobacter sp. strain P8	4.67±0.93 [ab]	8.93±0.23[a b]	140.00±0.00[a]	28.53±1.33 [a]	1.84±0.00 [e]	37.13±0.67[b cd]
Bacillus sp. P10	5.17±1.92 [ab]	8.83±0.60[a b]	180.00±10.00[b c]	36.60±5.25 [bc]	1.64±0.03 [c]	38.85±0.18[c d]

Note: Means in the same group followed by the same letter in the coloums are not significantly different ($p \leq 0.05$) as determined by the least significant different (LSD) test.

This study found that inoculation of PSB isolates enhanced available P in soils. This finding supports previous report on application of PSB that enhanced available P in rhizosphere tomato plants (Hariprasad and Niranjana, 2009). Escalation of available P in rhizosphere is probably due to solubilization process of insoluble phosphates in the soils by inoculated bacteria. Supply of inorganic P and organic P in soils can promote plant growth and immune system (Rodriguez and Fraga, 1999; Mahdi *et al.*, 2011).

An increase in plant growth is generally attributed to solubilization activity of inorganic P and organic P as well as production of IAA phytohormon in rhizosphere (Beneduzi *et al.*, 2008). In the study of PSB

inoculation into maize and tomatoes (Beneduzi *et al.*, 2008; Hariprasad and Niranjana, 2009), the use of PSB inoculants not only improve the growth and yield, but also maintain and improve soil fertility. It is due to microbial inoculants response directly to soils condition including organic substances, temperature, humidity, aeration, and available nutrients.

Although all PSB isolates in this study exhibited plant growth promoting activity in both laboratory and greenhouse assays, however, there was no correlation between the results in both assays. Hariprasad and Niranjana (2009) and Collavino *et al*. (2010) noted that the results of plant growth promoting study in both laboratory and greenhouse settings were not necessarily related to each other, because activity of PSB isolates will be different depends on different condititions.

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