The Ability of Phosphorhizobacteria Isolates to Produce Organic Acid and Promote Phosphatase Activity to Increase The Growth of Maize (Zea mays L.) in Selected Medium

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Abstract. Utilization of phosphorhizobacteria has the potential to increase the growth of maize by producing organic acid and promoting its phosphatase activity. The research to investigate the capability of phosphorhizobacteria isolates to produce organic acids to convert organic P into inorganic P so that P is available and can increase the nutrient uptake by plants. The experiment was conducted at the Soil Biology Laboratory in Department of Soil Science, Faculty of Agriculture, Universitas Padjadjaran. It was arranged as complete randomized factorial design, consisted of 16 treatments (combination of singular and consortia of phosphorhizobacteria isolates: J1M, J3T and J5H and two concentrations) with 3 replications. The suspension of phosphorhizobacteria isolates were inoculated into media Murphy then planted the maize seedlings into it. The produced organic acid, phosphatase enzyme and seedling growth were measured at 14 days after planting. Results showed that phosphorhizobacteria isolates has the ability to produce oxalic acid, tartaric acid, acetic acid, lactic acid and maleic acid along with maize seedlings. The interaction of consortia phosphorhizobacteria isolates (J1M+J3T) capable to increase maize biomass 21.87% higher than control and P uptake by 3.13 mg/100 g. The J5H isolate can significantly promote the highest phosphate solubilizing index and phosphatase activity.

1. Introduction
Maize (Zea mays L.) is an important food crop in Indonesia as a source of carbohydrates. From 2011 to 2015, the productivity and production of maize crops have increased. Maize production is still lower than the government's program production target of 20.33 million tons [1]. Production of maize plants that are not optimum yet and one of the reason is due to the decreasing of harvested maize crop area. In 2015, the maize crop harvesting area decreased by 1.29% compared to the previous year, due to the change of maize crops into land for other food commodities and the conversion of agricultural land to non-agricultural [2].

The effectiveness of inorganic P fertilizer in Ultisols is very low i.e. only 10-30% of P fertilizer is given so that 70-90% of P fertilizer remains in the soil and it is difficult to absorb for the plant [3]. Phosphorus is very easy to bind with metals present in Ultisols especially with Al and Fe which form insoluble Al-P and Fe-P bonds. These bonds cause the P element to be unavailable to the crop and fertilizer becomes inefficient [4].

The low availability of P nutrients in Ultisols becomes a barrier to the cultivation of maize in this soil. Utilization of phosphorhizobacteria can be done to dissolve P nutrients becomes available to plants. Phosphorhizobacteria dissolves phosphate through two mechanisms by producing phosphatase enzymes that convert P organic into P inorganic and by removing organic acids that can form stable complexes with binding cations of P in the soil and breaking the Al-P or Fe-P bond [5, 6], so that the P element becomes available and can be absorbed by the plant.
The utilization of phosphorhizobacteria can be done through single inoculation or consortium. Inoculation of the phosphorhizobacteria consortium was able to promote better plant growth and P uptake compared to single inoculation [7]. Therefore, compatibility test of phosphorhizobacteria isolates with plants was required for phosphorhizobacteria inoculum consortium formulation. Some of the previously selected phosphorhizobacteria isolates were obtained from rhizosphere in several districts of West Java. J1M obtained from maize rhizosphere of Majalengka, J3T from maize rhizosphere of Tasikmalaya and J5H from rhizosphere of natural forest of Garut are expected to be compatible and capable of supporting plant growth.

2. Materials and Method

2.1. Design and procedure of experiment

The experiment was conducted from October 2017 until March 2018 at the Soil Biology Laboratory in Department of Soil Science, Faculty of Agriculture, Universitas Padjadjaran, Bandung, Indonesia. The experiment was arranged as completely randomized block design, consisted of 16 treatments (one control and fifteen combination of singular and consortia of PSB isolates with two concentration). The three selected isolates (J1M, J3T and J5H) were maintained in Pikovskaya media and stored inside the Laboratory of Soil Biology Department of Soil Science and Land Resources, Faculty of Agriculture, Universitas Padjadjaran, Bandung. Phosphaterhizobacteria were inoculated in Pikovskaya medium and incubated in 150 rpm rotary shaker for 72 h at 28 °C to obtain the population density about 10^8 CFUs m/L. The TPC method (Total Plate Count) was used to enumerated the bacteria population. Subsequently, the suspension of phosphorhizobacteria isolates was applied to maize seedlings in liquid planting medium Murphy.

Before treatment, maize seeds (BISI 2) were sterilized in 0.1% mercuric chloride solution for 1 min, then sterilized in 70% ethanol, washed with 4 times with sterilized water and allowed to dried (air-dried). Preparation of maize seedlings is done by planting seeds of BISI 2 variety on paper towel. Two sheets of paper towel were moistened using aquadest and planted maize seeds with a wide spacing and then covered with other paper and then rolled with a plastic then kept in dark conditions for four days to grow roots then used for bio-test (bioassay).

The planting of maize seedlings on Murphy media was carried out on test tubes of 20 mm diameter and 300 mm sterilized sizes. Adding liquid media Murphy according to each treatment. In the control treatment, add the Murphy media as much as 100 ml into the test tube without adding a bacterial suspension. At a 5% phosphorhizobacteria concentration treatment, added a liquid medium of Murphy as much as 95 ml and a bacterial suspension of 5 ml. At the phosphorhizobacteria treatment of 10%, added a media of Murphy as much as 90 ml and a bacterial suspension of 10 ml. Then plant the maize seedlings by soaking the roots of maize plants into the liquid planting media Murphy and the top of the plant is supported by plastic and floxoc pipe. Then arranged and stored in a greenhouse.

2.2. Determination of organic acid and phosphatase activity

The type and quantity of released organic acid into liquid planting media Murphy was analyzed by high performance liquid chromatography (Singapore), in the Laboratory Central, Universitas Padjadjaran. The organic acid standard to be used in this study was analyzed by reversed phase chromatography using Grace smart RP 18.5 μ column, and read at λ = 210 nm. Before injection into HPLC, organic acid standards and samples were first filtered with 0.18 μm Whatman paper. The analysis was carried out at isocratic conditions at 40 °C using 50 mM phosphate solution as a mobile phase (dissolving 6.8 g of potassium dihydrogen phosphate in 900 ml water, pH value adjusted by adding phosphoric acid to 2.8 using pH meters, then adding water up to 1000 ml), then filtered with Whatman paper 0.45 μm. The flow rate of the mobile phase is set to 0.7 ml/min. Prior to injection, the device must be stabilized first[8].

Phosphatase activity was determined according to Eivazi and Tabatabai method by giving p-nitrophenyl substrate so that p-nitrophenol compound formed by enzyme activity was then stained by sodium hydroxide solution which could be detected by 400 nm spectrophotometer [9, 10].
2.3. Statistic data analysis

Statistical analysis were processed by SPSS software ver. 21 (Information Technology Service, Universitas Padjadjaran). Data were analyzed using one-way analysis of variance (ANOVA) and the difference between treatments was tested by Duncan’s multiple range test at p ≤ 0.05.

3. Results and Discussion

3.1. Organic acid production

Phosphorhizobacteria produces organic acids that react with AlPO_4, FePO_4, and Ca_3(PO_4)_2 in releasing a P bound. From these reactions are formed organic chelates of Al, Fe, and Ca so that P is liberated and soluble and available for plants [11]. Organic acids such as acetic acid, formic acid, lactic acid, oxalic acid, malic acid and citric acid are organic acids produced by the microbes. The order of organic acid ability in dissolving phosphate is: citric acid>oxalic acid=tartrate acid=malic acid>lactic acid=formic acid[12]. Organic acids that form a more steady complex with metal cations will be more effective in removing Ca, Al and Fe minerals so the soil will release a larger P. Ease of phosphate released following the order of Ca_3(PO_4)_2> AlPO_4> FePO_4 [13-15].

The results of the analysis were not statistically processed. Based on the result indicated that average of all isolate of phosphorhizobacteria either single or consortium at concentration 5 and 10% able to produce organic acids, which are oxalic acid, maleic acid, tartaric acid, acetic acid and lactic acid with different amount in each treatment (Table 1). Consortium inoculum of the phosphorhizobacteria isolate produces more organic acids that are more effective to dissolve P [16]. The organic acids were produced by phosphorhizobacteria from glucose as the primary metabolite used for cell survival [17]. Production of organic acids by phosphorhizobacteria is also capable of lowering pH, as an anion exchanger with phosphate and as chelating agent P absorbent [18].

| Treatments | Oxalic acid (ppm) | Maleic acid (ppm) | Lactic acid (ppm) | Tartaric acid (ppm) | Acetic acid (ppm) |
|------------|------------------|-------------------|-------------------|---------------------|------------------|
| b0k1       | 7.33             | 0.15              | 10.86             | 12.89               | n/d              |
| b1k1       | 9.48             | 0.16              | 18.00             | n/d                 | n/d              |
| b2k1       | 9.20             | 0.16              | 116.19            | 2.77                | n/d              |
| b3k1       | 8.95             | 0.15              | 10.20             | n/d                 | 1207             |
| b4k1       | 9.86             | 0.16              | 7.61              | n/d                 | 0.18             |
| b5k1       | 7.72             | 0.16              | 42.79             | n/d                 | 1.40             |
| b6k1       | 15.21            | 0.18              | 42.17             | n/d                 | n/d              |
| b7k1       | 7.83             | 0.15              | 7.30              | n/d                 | 1.41             |
| b0k2       | 24.95            | 0.16              | 6.68              | n/d                 | n/d              |
| b1k2       | 14.76            | 0.15              | 21.68             | n/d                 | n/d              |
| b2k2       | 9.64             | 0.21              | 7.52              | n/d                 | n/d              |
| b3k2       | 15.98            | 0.16              | 6.49              | 0.53                | 55.91            |
| b4k2       | 11.15            | 0.16              | 7.35              | n/d                 | 3.53             |
| b5k2       | 7.61             | 0.15              | 6.56              | n/d                 | 62.69            |
| b6k2       | 7.86             | 0.16              | 9.17              | n/d                 | 54.97            |
| b7k2       | 18.27            | 0.16              | 6.62              | n/d                 | 17.88            |

n/d = number of organic acids not detected.
3.2. Phosphatase activity

The results of statistical analysis showed that there were no interaction between single or consortium phosphorhizobacteria isolates with phosphorhizobacteria concentrations but showed independent effect on each treatment (Table 2). The use of single isolate and consortium of phosphorhizobacteria was not significantly different from control, but in treatment b3 the use of single isolate J5H was significantly different from the treatments of b2, b5 and b6 which showed higher phosphatase activity value and followed by different notation of Duncan’s Multiple Range Test.

Enzyme phosphatase is an enzyme that will be produced when the availability of phosphate is low [19]. Phosphatase enzymes can break phosphate bonds with organic compounds into available forms and can be absorbed by plants. Phosphatase activity will work as the number of P organic, the high value of phosphatase activity is suspected because phosphorhizobacteria works by actively hydrolyzing organic P [3].

Table 2. Phosphatase Activity in Liquid Planting Media Murphy Resulting from Provision of Various Isolates and Concentrations

| Treatments                  | Phosphatase Activity (µg pNP/g/h) |
|-----------------------------|----------------------------------|
| b₀ = control                | 1.735 ab                         |
| b₁ = J1M                    | 1.814 ab                         |
| b₂ = J3T                    | 1.526 a                          |
| b₃ = J5H                    | 2.103 b                          |
| b₄ = J1M + J3T              | 1.885 ab                         |
| b₅ = J1M + J5H              | 1.576 a                          |
| b₆ = J3T + J5H              | 1.463 a                          |
| b₇ = J1M + J3T + J5H        | 1.760 ab                         |

Concentration

| k₁ = 5%                     | 1.600 a                          |
| k₂ = 10%                    | 1.865 b                          |

Figures followed by the same notation are not significantly different based on Duncan Multiple Range Test at 5% real level.

Table 3. Plant Height and Root Length of Maize Seedlings due to Provision of Various Isolates and Concentrations

| Treatments                  | Plant Height (cm) | Root Length (cm) |
|-----------------------------|-------------------|------------------|
| b₀ = control                | 20.917            | 10.883           |
| b₁ = J1M                    | 23.783            | 10.867           |
| b₂ = J3T                    | 23.558            | 10.567           |
| b₃ = J5H                    | 25.483            | 11.083           |
| b₄ = J1M + J3T              | 22.383            | 11.667           |
| b₅ = J1M + J5H              | 25.275            | 10.067           |
| b₆ = J3T + J5H              | 21.383            | 9.800            |
| b₇ = J1M + J3T + J5H        | 20.817            | 9.567            |

Concentration

| k₁ = 5%                     | 22.396            | 10.550           |
| k₂ = 10%                    | 23.504            | 10.575           |

Figures followed by the same notation are not significantly different based on Duncan Multiple Range Test at 5% real level.
3.3. Maize seedlings growth promotion

Single or consortium inoculation and phosphorhizobacteria concentrations did not have a significant effect on plant height and root length and no interaction between them on plant height and root length of maize seedlings at 14 days after planting (Table 3). The use of phosphorhizobacteria isolates with different concentrations provides various plant growth responses.

The growth of plant height and root length is influenced by the availability of nutrients in the planting medium. The increase in plant height and root length is the effect of bacteria that can convert unavailable elements into available for plants [20]. The phosphate solubilizing bacteria isolated from the maize plant rhizosphere is capable of producing plant growth promoting substances and is capable of producing phytohormones in assisting plant growth [21, 22]. The average plant height and root length of maize crops with 10% phosphorhizobacteria concentration during the experiment was better than the phosphorhizobacteria concentration of 5%, this is because the phosphorhizobacteria population contained larger.

4. Conclusion

All isolates of phosphorhizobacteria either single or consortium at concentration 5 and 10% able to produce organic acids, which are oxalic acid, maleic acid, tartaric acid, acetic acid and lactic acid with different amount in each treatment. The use of single isolate J5H showed higher phosphatase activity value than other isolates either in single or consortium. The use of phosphorhizobacteria isolates with different concentrations provides various plant growth responses. Therefore, further research are needed to develop and formulate these phosphorhizobacteria of biological agent or biofertilizer to improve the growth and yield of maize.

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