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CHARACTERIZATION AND BIOASSAY OF RHIZOPHOSPHATE BACTERIA PRODUCING PHYTOHORMONE AND ORGANIC ACID TO ENHANCE THE MAIZE SEEDLING GROWTH

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Abstract. Rhizophosphate bacteria as biofertilizers is a low-cost and environment-friendly fertilizer for improving the nutrients status and fertilizers' efficiency on degraded agricultural or marginal soils. In this study, the characteristic and performance of selected rhizophosphate bacteria producing phytohormone and organic acid producers was investigated. Soils samples for beneficial rhizobacteria were taken from five maize (*Zea mays* L.) production area and forest ecosystems in Garut District, West Java Province, Indonesia. The rhizophosphate bacteria were isolated and grown in Pikovskaya medium. Bacterial colonies surrounded by clear zone were isolated and subjected to phosphate solubility and phosphatase activity test followed by bioassay. Based on the phosphatase activity, lactic acid production and indole acetic acid (IAA) production were obtained from three isolates of rhizophosphate. The isolates were identified as *Bulkholderia vietnamiensis*, *Enterobacter ludwigii*, and *Citrobacter amalonaticus* the best of which showed high phosphatase content and production of lactic acid, dissolved P and IAA.

Keywords: biofertlizer, superior strain, phosphatase

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INTRODUCTION

Maize is one of the important food crops that have a strategic role and high economic value in Indonesia and is considered a source of carbohydrate after rice. The demand for maize is increasing continually due to the versatility of its application in food industry. Maize stover is also used for cattle fodder in agricultural industry sector. Maize demand has reached 50% of national needs (Ministry of Agriculture 2014).

Since the adoption of green revolution, the efforts to boost maize productivity highly depend on the intensive use of inorganic fertilizers and other agrochemical products. Despite the production increase, an inorganic fertilizer also accelerates land degradation and hence causing environmental problems. Most Indonesian agricultural lands have been deprecated and exhausted. About 90% of dry land in Indonesia is marginal soil and categorized as sick soils with low organic carbon and high acidity (Simarmata *et al*. 2017). Indonesian dryland ecosystems are dominated by the Ultisols, Oxisols and Inceptisols. These soil orders have low pH, low nutrient content and phosphate, low organic matter content, and high metal (iron and aluminum) content (Sufardi *et al*. 2019, Husnain *et al*. 2014).

Phosphorus (P) is an essential element that plays an important role for plants growth. The need of P fertilizers is increasing along other major elements, such as nitrogen and potassium. The most common P fertilizers produced and used in Indonesia is super phosphate-36 or SP-36 (36% P_2O_5) and compound fertilizer "Ponska" (15% nitrogen, 15% P_2O_5 and 15% K_2O) (FAO 2005). Another alternative phosphorus source in agriculture is rock phosphate (RP) – a low-cost fertilizer is recently popular among farmers, contains about 28–32% of P_2O_5 but has a low solubility (Sanchez *et al*. 1997). RP can improve the chemical and physical properties and contain relatively high calcium content that could contribute to plant nutrition (Helall *et al*. 2019).

In the last three decades, the application of rhizophosphate bacteria has gained more attention due to the ability to improve the availability of fixed-P, P-solubility and to promote the environmentally-friendly agriculture (Singh and Purohit 2011). Rhizophosphate also produce phytohormone, such as indole acetic acid (IAA) (Fitriatin *et al*. 2020) and gibberellin (Khan *et al*. 2013). The use of rhizophosphate is expected to be able to increase the P fertilizer efficiency and maize productivity. This research focused on the selection and characterization of superior rhizophosphate isolates that potentially can be formulated as a phosphate solubilizing inducer and a phytohormone promoter for maize (*Zea mays* L.) as plant growth promoting rhizobacteria (PGPR).

MATERIALS AND METHODS

Thirty composite soils samples for the isolation of beneficial phosphate rhizobacteria were taken from five locations of maize plantation in Bandung (J_3B), Garut (J_2G), Tasikmalaya (J_3T), Majalengka (J_1M), and forest ecosystems in Garut, West Java Province (Indonesia). The rhizobacteria were isolated from plant rhizosphere and grown in Pikovskaya Agar (10 g glucose, 5 g $Ca_3(PO_4)_2$, 0,5 g $(NH_4)_2.SO_4$, 0,2 g KCl, 0.1 g $MgSO_4$, 0.1 g $MnSO_4$, 0.1 g $FeSO_4$, 0.5 g yeast extract, 10 g agar, 1 L distilled water). Bacterial colonies surrounded by clear zone (halozone) were isolated and subjected to phosphate solubility and phosphatase activity test, followed by bioassay. Three superior rhizophosphate bacteria isolates with the largest halo zone diameter were selected and characterized. Phosphatase activity, organic acid production, dissolved phosphate, phytohormone production and bioassay with maize seedling were further conducted. Total population, and colony diameter were recorded.

Phosphatase activity

Phosphatase enzyme activity was determined according to the Eivazi and Tabatabai method (Margesin 1996), *p*-nitrophenyl was added to the substrate to form *p*-nitrophenol compound through enzyme activity. Consecutively, it was stained by sodium hydroxide solution which can be detected by 400 nm spectrophotometer (Shimadzu Corp, Tokyo, Japan).

Organic acid and phyohormone production

The PSB isolates were grown for 48 h in Murphy liquid media (0.25 g $CaSO_4$ H_2O, 0.25 g KH_2PO_4 $2H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.08 g NaCl, 0.52 g KCl, 0.017 g $ZnCl_2$, 0.005 Cu $SO_4.5H_2O$, 0.025 $FeSO_4$, 10 g agar and 1 g aquadest) and incubated at 30°C. The type and quantity of released organic acid was measured by high performance liquid chromatography (HPLC) (Photodiode Array Detector, Singapore Product Waters 2998) at the Laboratory of Biomoleculer and Genetic Bioesources in Bogor, West Java, Indonesia. There was used the reverse phase HPLC method using GraceSmart™ C18 column at 40°C column temperature and wavelength 210 nm with potassium dihydrogen phosphate pH 2.8 as a mobile phase with rate of 0.7 ml per minute. Analysis was done in isocratic conditions (Nour *et al*. 2010).

The IAA production was determined using HPLC. Indoles extraction was conducted as follows: vacuum concentration of 100 mL of the liquid culture supernatant of each isolate using a lyophilizer to obtain a final volume of 10 mL. The pH was adjusted to 2.8 with 1N HCl and extracted three times with ethyl acetate (JT Baker, HPLC grade) (1:2 v:v) by vigorous shaking for 10 min-

utes. The following HPLC-grade indole standards was used IAA. After separation of the two phases using a separating funnel, the ethyl acetate fraction was evaporated in a rotoevaporator coupled to a vacuum pump, whereas the solid phase was suspended in 500 µL of absolute methanol and centrifuged at 10,000 rpm for 10 minutes. HPLC was performed by injecting 10 µL of an aliquot in an ULTRA C18 reverse phase column (150 × 4.6 mm; Restek, Bellefonte, Pennsylvania, USA) with a particle size of 5 µm, connected to an SLC 10A VP HPLC apparatus (Shimadzu, Japan), and the absorbance was monitored using an UV-visible detector (model SPD M10A VP) at a wavelength of 254 nm. The mobile phase consisted of water : acetonitrile : acetic acid (40 : 60 : 1), pH 2.8. the flow rate was 0.5 mL/min at a pressure of 7.5 MPa. The presence of IAA was confirmed by comparing the retention time of the commercial IAA and indoles standards. The eluates were quantified by comparing the areas of the peaks using CLASS-VP software (Shimadzu, Japan).

Bioassay

Bioassay of rhizophosphate bacteria was done in accordance with the Murphy method as follows (Murphy and Riley 1962): Reaction tube (100 mL) is filled with a 95 mL liquid Murphy medium (0.25 g $CaSO_4$ H_2O, 0.25 g KH_2PO_4 $2H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.08 g NaCl, 0.52 g KCl, 0.017 g $ZnCl_2$, 0.005 $CuSO_4.5H_2O$, 0.025 $FeSO_4$, 10 g agar and 1 g aquadest). Maize seedlings were sterilized with 0.2% $HgCl_2$ and 70% ethanol and added aseptically to sterilized petri dishes containing sterile moist paper. Maize seeds were germinated at 30°C for 72 h. Sprouts were grown in the medium with sterile gauze and buffer tubes and grown in screen house for 14 days. The growth of maize seedling, the content of phosphatase, organic acids and IAA were measured and subjected to statistical analyses.

Rhizophosphate bacteria identification and phylogenetic analysis

Genomic DNA was isolated by the CTAB method (Winnepenninckx *et al*. 1993). PCR amplifications of 16 rRNA were performed by using universal forward and reverse primers P1 (5'-CGggatccAGAGTTTGATC-CTGGTCAGAACGAAC-3'), P6 (5'-CGggatccTACGGCTACCTTGTTACGACTTCACC-3') for prokaryotes (Tan *et al*. 1997). A PCR reaction of 50 µl was prepared by using Taq polymerase (5U) 0.5 µl, Taq buffer (10X) 2 µl, $MgCl_2$ (25 mM) 2.5 µl, dNTPs (2.5 mM) 2 µl, 2 µl each of forward and reverse primer (10 pmol), 36 µl of dd H_2O and 3 µl of template DNA. First denaturation step was performed at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension step was at 72°C for 10 min, as described by Tan *et al*. (1997). PCR products were analysed by using 1% aga-

rose gel and purified by using GeneJET PCR Purification Kit (K0702 – Thermo Fisher Scientific, Germany). Purified PCR products were sequenced by using forward and reverse primers (Eurofins, Germany).

Acquired sequences were assembled and analyzed with the help of Chromus Lite 2.01 sequence, using the Technelysium Pty Ltd. software (Australia). The gene sequences were compared to those deposited in the GenBank nucleotide database in BLAST software (NIH, USA). Sequences were aligned using the Clustal X 2.1 software and phylogenetic tree was constructed using a neighbor-joining method (Saitou and Nei 1987). Bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*. 2004) and in units of number of base substitutions per site. All positions with gaps and unavailable data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*. 2011). There were 1,457 positions in the final dataset. The sequences were submitted to NCBI GenBank data base under the accession number LT703516.

Statistical analysis

The experiment was arranged as a randomized block design consisting of six treatments (one control and five of rhizophosphate isolates) with five replicates. The data were analyzed using analysis of variance (ANOVA) and continued with Duncan's multiple range test at the 5% significance level.

RESULTS

Characteristics of rhizophosphate bacteria

Based on the characteristics and the diameter of the clear zone, there were selected five best rhizophosphate bacteria isolates for phosphatase, organic acids, bioassay on maize crop and the IAA production test. Five rhizophosphate bacteria isolates were selected based on the clear zone diameter and phosphate dissolution index (Table 1). The isolates were subjected to the phosphatase, organic acids, bioassay, and IAA production test to determine superior isolates.

Table 1. Characteristics of various rhizophosphate bacteria isolates

Isolate	Clear zone diameter (b) (cm)	Colony diameter (a) (cm)	Phosphate dissolution index
J_3B	1.2	0.8	1.50
J_2G	0.9	0.7	1.28
J_3T	1.1	0.7	1.57
J_5H	1.6	0.8	2.00
J_1M	1.2	0.7	1.71

Note: J_3B (Bandung), J_2G (Garut), J_3T (Tasikmalaya), J_5F (Garut; virgin forest), J_1M (Majalengka).

Table 1 shows that the relatively large clear zone diameter of J_5H, J_1M and J_3B isolates is 1.6 and 1.2 cm, then in the relatively large diameter of colony that is in isolate J_5H and J_3B was 0.8 cm, while the highest phosphate dissolution index of the isolates J_5H was 2.00 and J_1M was 1.71. Clear zone was a qualitative indicator of the bacterial ability to dissolve P from the insoluble phosphate (Pande *et al*. 2017). Based on the clear zone diameter, it was obvious that the phosphate solubilizing ability varies.

Phosphatase enzyme and dissolved P

The solubility capacity of dissolved P due to the activity of rhizophosphate bacteria through the phosphatase enzyme, and P-soluble from various PSB isolates is shown in Table 2. The bacterial isolates with high phosphatase enzyme production were J_1M isolate (63.25 µg $pNPg^{-1}h^{-1}$), J_5H isolate (62.84 µg $pNPg^{-1}h^{-1}$) and J_3T isolate (51.69 µg $pNPg^{-1}h^{-1}$).

Table 2. Rhizophosphate bacteria ability to produce phosphatase enzymes and dissolved P

Isolate	Phosphatase (µg $pNPg^{-1}h^{-1}$)	Dissolved P (ppm)
J_3B	19.78	45.56
J_2G	11.27	46.35
J_3T	51.69	49.61
J_5H	62.84	75.42
J_1M	63.25	66.24

Phosphatase is an enzyme that will be produced when the availability of phosphate is low (Lidbury 2022). In the mineralization process of organic matter, organic phosphate compounds are broken down into inorganic phosphate forms available to plants with the help of phosphatase enzymes (Paul and Clark 1989). Phosphatase enzymes may break the phosphate bound by organic compounds into the available form and can be absorbed by the plant.

PRODUCTION OF IAA AND ORGANIC ACID

Production of IAA and organic acids produced by rhizophosphate bacteria from various isolates are shown in Table 3. J_1M isolate showed the highest ability to produce IAA, followed by J_3T and J_5H. As can be seen, obtained J_1M isolate is considered the most potential in producing IAA. Compared to other isolates, bacteria have the ability to synthesize tryptophan to IAA faster.

Table 3. IAA production capability and the type of organic acid

Isolate	IAA (ppm)	Lactic acid ($\mu m\ mL^{-1}\ h^{-1}$)	Pyruvic acid ($\mu m\ mL^{-1}\ h^{-1}$)	Succinic acid ($\mu m\ mL^{-1}\ h^{-1}$)	Malic acid ($\mu m\ mL^{-1}\ h^{-1}$)
J_3B	24	11	16	11	12
J_2G	29	10	24	36	37
J_3T	35	11	17	24	18
J_5H	34	18	18	26	18
J_1M	37	11	17	19	29

Maize seedling growth

Biological test results of the influence of various rhizophosphate bacteria on plant height and root length are shown in Table 4.

Table 4. Influence of rhizophosphate bacteria on plant height and root length of maize seedling

Isolates/Treatments	Plant height (cm)	Root length (cm)
Control	10.94a	12.26a
J_3B	12.46a	11.60a
J_2G	12.96a	11.28a
J_3T	13.70a	12.72a
J_5H	13.96a	18.90ab
J_1M	14.40a	22.20b

Note: Average values followed by the same letter within the column do not differ significantly, according to Duncan's multiple range test ($\alpha \leq 0.05$).

Table 4 shows that the application of phosphate solubilizing bacteria (PSB) did not differ significantly between the treatments of the five rhizophosphate bacteria in terms of plant height, although J_1M isolate tends to increase the height of the maize, but there is a significant difference in root length.

As can be seen in Table 5, J_5H and J_1M isolates have a significant effect on a leaf dry weight and root of maize. Rhizophosphate bacteria tended to increase root dry weight, and the increase was significantly higher than that of control. The ratio of the shoot-root describes the development of plant toward the can-

opy or root. It seems that maize with inoculated rhizophosphate bacteria (J_5H) had a greater root weight when compared to canopy.

Table 5. Effect of rhizophosphate bacteria on maize seedling growth

PSB isolates	Shoot dry weight (g)	Root dry weight (g)	Shoot-root weight ratio
Control	34a	420a	0.35a
J_3B	50ab	500ab	0.57b
J_2G	50ab	500ab	0.44ab
J_3T	54b	530ab	0.54ab
J_5H	56b	604b	0.44ab
J_1M	68b	660b	0.51ab

Note: Average values followed by the same letter within the column do not differ significantly, according to Duncan's multiple range test ($\alpha \leq 0.05$).

Genotypic identification and phylogenetic analysis

The BLAST search against GenBank revealed a large number of similar 16S rRNA gene sequences. The blast results of most promising bacterial isolates showed >99% similarities between available GenBank entries in which J_3T isolate was identified as *Bulkholderia vietnamiensis*, J_1M was identified as *Enterobacter ludwigii* and J_5H was identified as *Citrobacter amalonaticus*. The results are shown in Table 6.

Table 6. Molecular characterization of J_3T, J_1M and J_5H isolates

Isolate	Most closely related organism		
	Species	Similarity (%)	Sequence query coverage (%)
J_3T	*Burkholderia vietnamiensis*	99	97
J_1M	*Enterobacter ludwigii*	99	97
J_5H	*Citrobacter amalonaticus*	99	96

DISCUSSION

Some microbes that live freely in the soil have the ability to produce extracellular enzymes, the group of phosphatase enzymes that can mineralize organic P into inorganic P so as to provide high P for plants (Rao 1994). The phosphatase belongs to the group of hydrolase enzymes that are enzymes that can hydrolyze organic phosphoric compounds (phosphoric ester hydrolysis) into inorganic phosphorus compounds (George *et al*. 2002, Sarapatka 2003, Zhongqi *et al*. 2004).

Acid phosphatase activity will actively work at low pH or high acidity. Phosphatase activity will also work as the number of organic P, the high value of phosphatase activity is suspected because rhizophosphate works by actively hydrolyzing organic P (Whitelaw 2000). According to Sarapatka (2003), phosphatase activity is strongly influenced by the content of nitrogen media. It is further explained that an increase in the nitrogen content of the medium may increase its phosphatase activity. The results of research conducted by Fitriatin *et al*. (2008) show that the pH of the medium affects its phosphatase activity. In addition, the experiments showed that isolates with relatively high soluble P content were J_5H (75.42 ppm), J_1M (66.24 ppm) and the lowest was J_3B (45.56 ppm). Rhizophosphate bacteria releases enzymes and organic compounds that can release bounded phosphate and increase phosphate availability for plants (Fitriatin *et al*. 2014).

The organic acid content produced by some rhizophosphate bacteria are lactic, pyruvic, succinic, and malic acid. Organic acid production depend greatly on the type of microorganism, adaptability, and ability to produce enzymes. Besides the acids mentioned above, also formic acids, acetates, propionate, lactonate, glycolate, and fumarate, can form aluminium chelate compounds and iron cations. This will cause higher P solubility and its availability for plants.

There was a significant difference between the controls as for the application of rhizophosphate isolates J_3T, J_5H, and J_1M. It is possible that the activity of rhizophosphate bacteria is more likely to release the growth hormone that is IAA which participates in root extension. In addition, there can be observed the activity of other growth hormones, e.g. gibberellins, and increased root growth after the application of PSB isolates. J_1M PSB are able to independently increase root length. Root length is a more determining factor than root weight in absorbing nutrients, because long roots will easily absorb nutrients found in the soil. IAA or auxin can promote root extension and nutrient absorbing ability in plants. They can be synthesized as secondary metabolites under suboptimal growth conditions or with the presence of tryptophan. Similar result were obtained by Ahmad *et al*. (2005), where PSB from the *Pseudomonas* genus can synthesize up to 32.3 ppm of IAA after 5 days of incubation. Rhizophosphate bacteria inoculation promotes root elongation, indicated by the low root-and-shoot weight ratio. The shoot-and-root dry weight ratio referred to the development of the plant toward the canopy or root (Tolley and Mohammadi 2020).

P dissolved from inorganic P due to the activity of organic acids produced by J_5H isolate which is absorbed by plant roots can increase root weight. Based on the favorable traits, three isolates: J_3T, J_1M, and J_5H were chosen as superior strains. They were subjected to genotype identification and phylogenetic analysis to determine the closest species.

These sequences were submitted to the NCBI database and the accession numbers were obtained. The phylogenetic tree included the isolates (J_3T, J_1M and J_5H) taken from this study and some closely-related sequences obtained from

NCBI. Two distant phylogenetic groups corresponded to the following genera: *Burkholderia* sp., *Enterobacter* sp., and *Citrobacter* sp. In the phylogenetic group of the *Burkholderia* genus, isolate J_3T was closely related to *Bulkholderia vietnamiensis*, isolate J_1M – to *Enterobacter ludwigii*, and isolate J_5H – to *Citrobacter amalonaticus.*

CONCLUSIONS

The five selected rhizophosphate bacteria had shown a different characteristic and ability to improve the solubility of P and production of organic acid and phytohormone. Based on the phosphatase activity, lactic acid production, and IAA production, there were obtained three rhizophosphate bacteria, the most potential isolates that could be used for the formulation of phosphate and plant growth biofertilizers. The isolate of J_3T, J_1M and J_5H were the superior isolate in a liquid culture with maize.

These isolates were superior in phosphatase, lactic acid and IAA production, and P-solubilization. Three bacterial strains J_3T, J_1M, and J_5H were identified as *Burkholderia vietnamiensis*, *Enterobacter ludwigii*, and *Citrobacter amalonaticus*.

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