

Screening of Rhizosphere Bacteria from Clove (*Syzygium aromaticum*) in Tidore Island as Plant Growth Promoting Rhizobacteria

Ismat Ishak^{1*}, Tri Ardyati², Luqman Qurata Aini³

^{1,2}Departemant of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia

³Departemant of Agriculture, Faculty of Agriculture, University of Brawijaya, Malang, Indonesia

Abstract

Tidore Island in North Maluku Province is one of the clove (*Syzygium aromaticum*) producing regions. Clove plant fertility is maintained even though it is not given organic and inorganic fertilizers. This study aims to explore bacteria in the rhizosphere of the clove trees planted in Tidore island as candidate of biofertilizer agents have potency in Indol-3-Acetic Acid (IAA) production, phosphate solubilization and ammonium production. Bacteria were isolated from the rhizosphere of clove trees. Screening of the isolates was done according to qualitative and quantitative methods. Data collection of the qualitative method were based on medium color changes for each variable while that the quantitative method (i.e. the concentrations of produced IAA, solubilized phosphate, and produced ammonium) were obtained based on spectrophotometry. The results obtained 110 bacterial isolates, nineteen bacteria were potential as biofertilizer agents, of which isolate R11, R8P, and A1N showed the highest in IAA production ($8.71 \mu\text{gL}^{-1}$), phosphate solubilization ($8.37 \mu\text{gL}^{-1}$), and ammonium production ($11.71 \mu\text{gL}^{-1}$), respectively. The three isolates respectively have similarities to the genus of *Enterobacter*, *Burkholderia*, and *Stenotrophomonas*.

Keywords: Biofertilizer, Clove, Rhizobacteria, Rhizosphere.

INTRODUCTION

Total area of clove plantation in Tidore Island is 1,029 ha with the productivity of $500 \text{ kg}\cdot\text{ha}^{-1}$ [1]. The clove trees on Tidore Island grow naturally and the soil has never been supplemented with either organic or chemical fertilizers. However, crop productivity is naturally preserved. Clove commodity is selected because the plantation area of this commodity estimates increase. In addition, increases of soil fertility without contamination by chemical fertilizers and the need of other perspectives in order to exploit the potential of spice crops also became reasons. It has been reported that the bacteria from the clove rhizosphere were tested in-vitro capable of producing Indole-3-acetic acid [2] but have not revealed the potency of bacteria in the production of ammonium and phosphate solubilization. On the other side of field observes no research report yet on the use of clove rhizosphere bacteria as a biofertilizer agent.

Generally, crop productivity is supported by external factors such as climate and soil microorganism, especially bacteria. The climate

of Tidore is a humid tropical region influenced by sea breeze surrounded by the sea of Halmahera, Seram, and Maluku. Observations in this study recorded the type of soil in clove plantation commodity dominated by Inceptisol soil or Alluvial, although in some altitudes there is Mollisol soil. Bacteria found in the plant rhizosphere are called Plant Growth Promoting Rhizobacteria (PGPR). The bacteria use plant exudates as a source of nutrients for their growth [3].

The existence of PGPR on plant roots is beneficial for the plants. Some bacteria can support plant growth or act as agents of biocontrol for plant diseases [4]. Bacteria in the rhizosphere can stimulate the growth of plants through direct or indirect mechanisms. Direct mechanisms involve bacteria as a biofertilizer to stimulate the growth of plants, and as an agent for preventing plant stress, while indirect mechanisms include reducing the intensity of plant diseases, producing antibiotics, inducing systemic resistance, and competing for nutrition and space [5]. The main roles of PGPR are plant growth promoters including nitrogen fixation, production of plant growth hormones such as auxin and cytokinin, and solubilizing phosphate and iron from non-soluble form [6].

The use of PGPR as a biofertilizer agent was developed as an effort to maintain environmental health and prevent environmental damage due to the extensive use of synthetic

* Correspondence address:

Ismat Ishak

Email : ismatishak123@gmail.com

Address : Dept. Biology, Faculty of Mathematics and Natural Science, University of Brawijaya, Veteran Malang, Malang 65145.

chemical fertilizers. The use of PGPR has several advantages including 1) not polluting the environment, 2) not containing ingredients cause residues in the food chain; 3) not requiring repeated application as microbes can grow with a supportive environment; 4) as a companion for indigenous microbial plants, 5) can increase plant resistance against pathogens [7].

Some special genera of microbes have been found to be useful as PGPR. Anandaraj and Dinesh [8] reported several genera of bacteria as PGPR including *Acinetobacter*, *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium Rhizobium* and *Serratia*. Several studies have reported the positive effect of PGPR on plant growth such as PGPR has a positive effect on wheat crops [9], fruits [10], and vegetables [11].

Due to the enormous potential of PGPR in extensive plantations and plant health, in this study, exploration of the potential bacteria in the rhizosphere of clove plantation in Tidore Islands, North Maluku as biofertilizer- functioning PGPR was carried out.

MATERIAL AND METHOD

Soil Sampling

Soil sampling from a clove plantation on Tidore Island, was carried out at three location on different altitude. The rhizosphere soil samples were taken from several clove plant roots at a depth of 0-20 cm from the surface. Soil chemical compounds such as organic matter, C/N ratio, soluble phosphate, nitrogen content were analyzed in Faculty of agriculture, University of Brawijaya.

Isolation of Potential PGPR

Twenty-five grams of soil samples were suspended in 225 mL of 0.85% NaCl solution and shaken on rotary shaker for 60 min at 27 °C 120 rpm. Samples were diluted and transferred on Tryptic Soy Agar (TSA) medium containing 200 mg.L⁻¹ L-tryptophan for the production of Indole-3 acetic acid (IAA), and Pikovskaya (PVK) agar medium for phosphate solubilization assay containing Glucose 10g; NaCl 0.2g; Ca₃PO₄ 2.5g; KCl 0.2g; MgSO₄. 7H₂O 0.1 g; MnSO₄ 0.025 g; FeSO₄. 7H₂O 0.025 g; (NH₄)₂SO₄ 0.5g; Yeast Extract 1 g; and Bacto Agar 15 g in 1000 mL of Aquades. Semisolid Nfb medium [12] was used for nitrogen-fixing bacteria assay containing K₂HPO₄ 0.5g; MgSO₄. 7H₂O 0.2g; FeCl₂. 6H₂O 0.015g; NaCl 0.1g; DL-Malic Acid 5g; KOH 4.8g; Yeast Extract 1 g; 0.1% Bromothymol blue 5 mL;

Bacto Agar 3 g in 1000 mL Aquades [13]. Total plate count (TPC) was performed on each growth medium to determine the number of cells of each bacterial isolate.

IAA Production Assay

Production of IAA by bacteria was tested using Tryptic Soy Broth (TSB) and Salkowski reagents (1.05 g FeCl₃.6H₂O; 60 mL absolute H₂SO₄; 100 mL aquades). The bacteria were cultured in TSB medium containing L-tryptophan 200 mg.mL⁻¹ and incubated with a rotary shaker at 120 rpm in the dark at room temperature for 3 days. Then the culture was centrifuged at 12,000 rpm for 15 min to pellet cells and two mL of supernatant were added to 3 mL Salkowski reagent and incubated in the dark for 30 minutes at room temperature. The intensity of pink color, indicating positive IAA production, was measured using a UV-VIS spectrophotometer at a wavelength of 535 nm [14]. From the standard curve prepared with known IAA concentrations, the quantity of IAA in the culture is determined and expressed as mg.mL⁻¹ [15].

Phosphate Solubilization Assay

All bacterial isolates were first tested on the Pikovskaya agar medium to observe the phosphate solubilization index as described by Gaur [16]. Quantitative analysis of phosphate solubilization was done in liquid medium as described by King [17]. Isolates were cultured in 25 mL of Pikovskaya broth and incubated for 24 to 72 hours at room temperature 28 ± 2°C. Bacterial culture was centrifuged at 12,000 rpm for 15 min to pellet cells. One mL of the supernatant was mixed with 5 ml of chloromolybdic acid and added 0.25 mL chlorostannous. The bluish gray absorbance was read at 690 nm. The amount of dissolved phosphorus was calculated based on the KH₂PO₄ standard curve. The pH of medium was measured to determine the correlation of phosphate concentration to the medium acidity.

Nitrogen Fixation (Ammonium Production) Assay

The concentration of ammonium was estimated using Serra Ammonium detection Kit. The isolates were cultured in 25 mL Nfb broth medium. After centrifuging the liquid culture at 12,000 rpm for 10 min at room temperature, one mL of supernatant was transferred into a test tube. One drop of NH₄1, NH₄2, and NH₄3 was added to the sample and mix well and the sample was left at room temperature for 5 min. The

ammonium concentration was estimated using the provided color chart [18].

Bacterial isolates were tested for the production of ammonia in peptone water. Bacterial isolates were inoculated in 20 mL Nfb broth medium without Bromothymol Blue in each tube and incubated for 48-72 hours at 25 ± 2°C. One mL of the Nessler reagent was then added in each tube. Development of yellow color is a positive indication for ammonia production [19]. The absorbance of the developing color was read at 425 nm. The quantity of ammonium production was calculated according to the NH₄Cl standard curve.

Bacterial Identification

Identification was performed on bacterial isolates that produced the highest concentrations in IAA production, phosphate solubilization, and ammonia production (NH₃). The extraction of genomic DNA from bacterial cells harvested from a 24-hour culture on NA medium was performed using the i-genomic DNA Extraction Kit (Intron) according to the manufacturer's protocol.

DNA amplification of 16S rDNA sequence was performed by Polymerase Chain Reaction (PCR) using primers 27F (5'-GCC TAA CAC GTC ATG CAA GA-3') and 1496r (3'-CGT AAC ATT CGG GGC TGC TGG TGC-5'). The PCR process was performed according to Zarei et al. [20] with 35 cycles consisting of denaturation at 94°C 30 s annealing 55°C for 30 s and extension of 72°C for 65 s. The PCR products were visualized using 1% agarose gel to present the DNA band and then aligned using the O'genRuler™ Ladder Mix DNA.

The correct size of PCR products was sent to 1st BASE Malaysia for sequencing. The obtained sequences were aligned using the Nucleotide

BLAST to find the similarity to GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The results of BLAST and the reference strain were aligned and constructed by phylogenetic trees consisting of evolutionary relationships according to Saitou and Nei [21], Felsenstein's [22]. While similarity percentage with Tamura and Nei [23]. Evolutionary distances are shown using MEGA 6 [24].

Statistical Analysis

Quantitative data from each assay were subjected to analysis of variance (ANOVA) and means were compared by Tukey test α 0.05 using the SPSS ver. 16.

RESULT AND DISCUSSION

Soil Chemical Properties

Soil chemical properties of the sampling location (i.e. organic matter, total nitrogen, C/N ratio, available phosphate, organic matter, and acidity) are presented in Table 1. According to Table 1, Tdr₁C soil samples contain low organic matter (1.98%), moderate total N content (0.21%) and very low dissolved phosphate content (2.25 mg.kg⁻¹). Tdr₂R contains moderate soil organic matter (4.08%), moderate total N content (0.4), and low dissolved phosphate (3.1 mg.kg⁻¹). Tdr₃A contains high soil organic matter (6.5%), very high total N content (0.77%) and very low dissolved phosphate content (0.81 mg.kg⁻¹). Variation of chemical soil properties is influenced by soil nutrient cycle in nature, the process in this cycle includes physical, chemical and biology, soil formation, chemical elements that bind to soil molecules and immobilization of soil microorganisms in the process of soil nutrient formation [25].

Table 1. Chemical properties of soil sample from clove plantation

Code of Soil Sample	Content of soil chemistry								
	C Organic (%)	N. Total (%)	Categories*	Ratio of C/N	P. Bray1 (mg.kg ⁻¹)	Categories*	Organic Matter	Categories*	pH
Tdr ₁	1.98	0.21	moderate	9	2.25	very low	3.42	low	5.9
Tdr ₂	4.08	0.4	moderate	10	3.1	low	7.05	moderate	5.4
Tdr ₃	6.5	0.77	very high	8	0.81	very low	11.25	high	5.1

Note: *The categories according to CSR/FAO 1983 [26]

Total Plate Count of Rhizosphere Bacteria

Estimation of the rhizosphere bacterial population was done using total plate count (TPC) and MPN methods. The TPC and MPN results were analyzed using one way ANOVA with significance (α < 0.05) according to the Tukey test. The MPN method used semisolid Nfb medium to observe the activity of bacteria in nitrogen fixing.

This activity is characterized by white ring-shaped on the surface of medium (Fig. 2c). The highest number of colonies shown on semisolid Nfb medium was in the range of 6.8 x 10⁴-7.03 x 10⁷ cells.g⁻¹, TSA showed total bacteria in the range of 6.5 - 6.7 x 10⁵ CFU.g⁻¹ and the Pikovskaya medium shows the number of colonies in the range of 6.1 - 7.1 x 10⁵ CFU.g⁻¹ per gram (Fig. 1).

The highest number of bacteria colonies were found in the Tdr₃A soil samples which is correlated with the abundance of N total content.

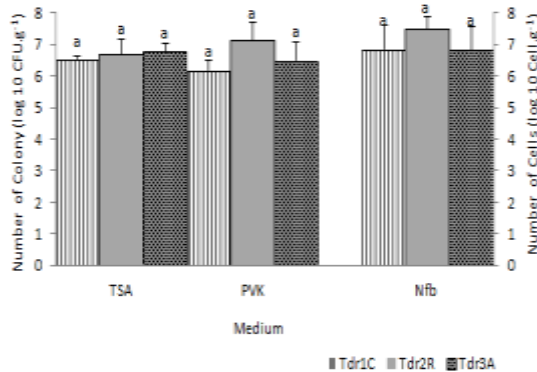


Figure 1. The bacteria population on the soil samples grouped by type of media TSA, PVK and Nfb. The same letter shows no significant different (P <0.05) based on Tukey's test.

Qualitative and Quantitative Assay of PGPR Bacteria as Biofertilizer

IAA Production Assay

Qualitative assay of IAA were carried out using TSB medium containing L-tryptophan with a concentration of 200 mg.L⁻¹ as a precursor of IAA synthesis. The Salkowsky reagent added to the supernatant of bacterial cultures resulting in red color indicated the production of IAA (Fig. 2a). The results of the screening, obtained 7 isolates able to produced IAA.

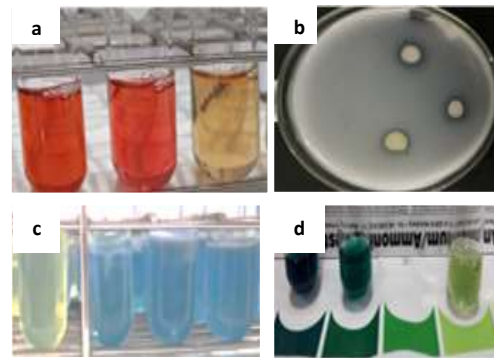


Figure 2. Qualitative and Quantitative Test of PGR. a. Results of qualitative test of IAA Production b. qualitative test of Phosphate solubilization c. the indicator of Nitrogen-Fixing bacteria, and d. qualitative test of ammonium production.

Isolation of Potential PGPR

A total of 110 rhizospheric bacteria isolates have been obtained from all soil samples. In TSA medium was obtained 65 isolates, Pikovskaya medium was 37 isolates and Nfb medium was 9 isolates. Qualitative and quantitative selection resulted 7 potential isolates as IAA producing, 7 isolates as phosphate solubilization and 5 isolates as nitrogen fixation (Table 2).

The seven isolates were then tested quantitatively based on the IAA standard curve. The results of the analysis obtained different concentrations of IAA for each isolate. The highest yielding IAA isolate was R11 with a concentration of 8.71 µg.L⁻¹, while the lowest in IAA production was A3 isolate with IAA concentration of 3.26 µg.L⁻¹ (Fig. 3).

Table 2. Number of Bacterial Isolates has Potency as Biofertilizer

Code of Soil Sample	Medium	Number of Isolates	Number and Code of Potential Isolates		
			IAA Production	Phosphate solubilization	Nitrogen-Fixation (NH ₃)
Tdr ₁ C	Tryptic Soy Agar	23	2 (C4, C8)		
	Pikovskaya	12		2 (C2.2P, C2.3P)	
	Nfb Medium	3			2 (C8N,C3N)
Tdr ₂ R	Tryptic Soy Agar	23	2 (R8, R11)		
	Pikovskaya	14		2 (R4P, R8P)	
	Nfb Medium	1			1 (R9N)
Tdr ₃ A	Tryptic Soy Agar	19	3 (A2, A3, A10)		
	Pikovskaya	11		3 (A4.2P, A4.3P, A6P)	
	Nfb Medium	3			2 (A1N, A5N)
Total Number of Isolates		110	7	7	5

Source: Research Analysis

Ahmad et al. [27] reported that there were different concentrations of IAA production in each type of bacteria with tryptophan at different concentrations. The using of 200 mg.L⁻¹ concentration as the optimal precursor for IAA synthesis in this study, the presence of tryptophan consumption will affect the high

levels of IAA concentrations produced by bacteria. Glickmann and Dessaux [15] reported the use of 2.5 mg.L⁻¹ L-tryptophan which led to a decrease in IAA production. Kesulya et al also reported 100 mg.L⁻¹ L-tryptophan obtained a maximum concentration of 5.82 mg.L⁻¹ [28].

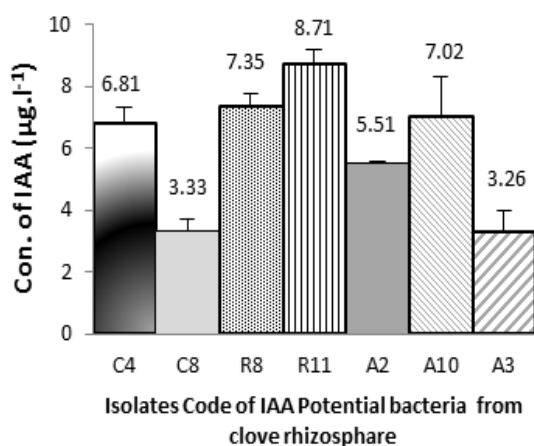


Figure 3. IAA producing by Some Isolates from Rhizospheric Soil of Clove Plantation

Some microorganisms require tryptophan to produce IAA from the IAA biosynthesis mechanism [29]. PGPR is known to affect plant growth because it can produce phytohormones, such as the auxin or in this study called IAA that has been known to stimulate cell lengthening and cell division differentiation [27] and gene regulation [30]. The results of molecular identification using the 16S rDNA gene showed that the rhizosphere bacterium isolate R11 had similarities to the *Enterobacter hormaechei* species. The construction of phylogenetic trees showed a similarity of 98.5% (Fig. 4). It has been reported that bacteria from the soil of rhizosphere have similarities with *Enterobacter sp.* and capable of producing IAA (5.09 mg.L⁻¹) as a biofertilizer agent [31].

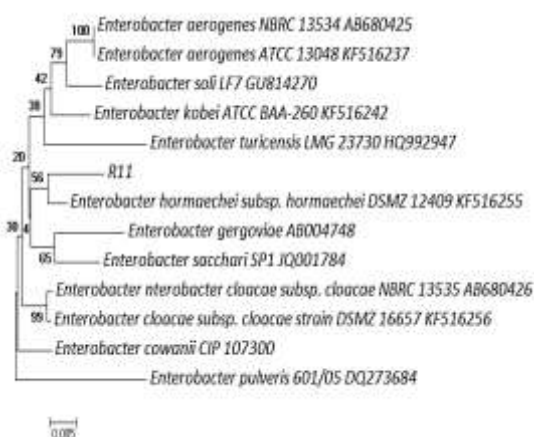


Figure 4. The Phylogenetic Tree of R11 Isolate based on 16s rDNA Gene Sequence

Phosphate Solubilization Assay

The clear zone around bacterial colonies on Pikovskaya agar medium containing Tricalcium phosphate (Ca₃PO₄) shows the presence of

phosphate dissolving activity. From qualitative selection, there were 7 isolates of bacteria showing solubilization activity of phosphate. Each isolate showed a phosphate solubilization index that varied in the range of 10 to 13 mm. Tukey test analysis with significance α 0.05 showed that each isolate showed significantly different in solubilization of phosphate (Table 3). The phosphate solubility index was obtained based on the clear zone around the bacterial colony. Clear zone formation is caused by the production of organic acids, the production of polysaccharides, or the activity of phosphatase enzyme from phosphate solubilizing bacteria [32]. Quantitative tests with Pikovskaya broth medium at pH 7.0 were performed to measure the concentration of solubilized phosphate (PO₄) based on a standard KH₂PO₄ curve.

Table 3. Phosphate Solubilization Index of Bacteria Isolates from Rhizospheric Soil of Clove Plantation

Isolat Code	Solubilization Index \pm SD
C2.2 P	13.68 \pm 3.15 ^c
C2.3P	13.02 \pm 2.18 ^{bc}
R4P	10.34 \pm 0.7 ^a
R8P	11.45 \pm 1.07 ^{ab}
A4.2P	12.89 \pm 1.78 ^{bc}
A4.3P	12.69 \pm 2.55 ^d
A6P	13.75 \pm 3.28 ^c

The assay results showed that pH medium varied in each isolate (Fig. 5). In the isolate R8 there was a correlation between the higher PO₄ concentration (8.37 μ g.L⁻¹) followed by a decrease in the pH of the media to 4.09.

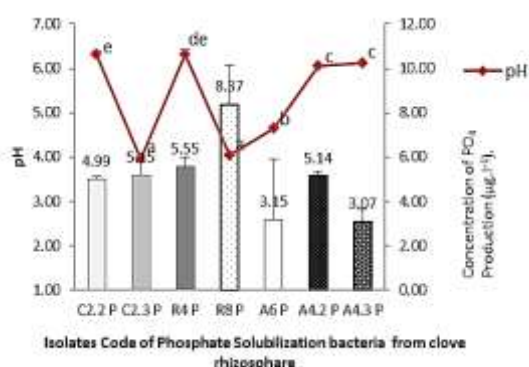


Figure 5. Correlation of pH and PO₄ Concentration

Other isolates showed significant variations based on statistical analysis. Acid levels in bacterial media indicate the production of organic acids which are common mechanisms of phosphate dissolution [32,33]. Molecular identification was performed on isolates with the

highest phosphate concentration in R8P isolates. Identification using a sequence of 16S rDNA genes showed that R8P isolate had similarities with *Burkholderia multivorans* at 95.5% (Fig. 6). *B. multivorans* WS-FJ9 has been known to dissolve phosphate and decrease pH from initial pH [34].

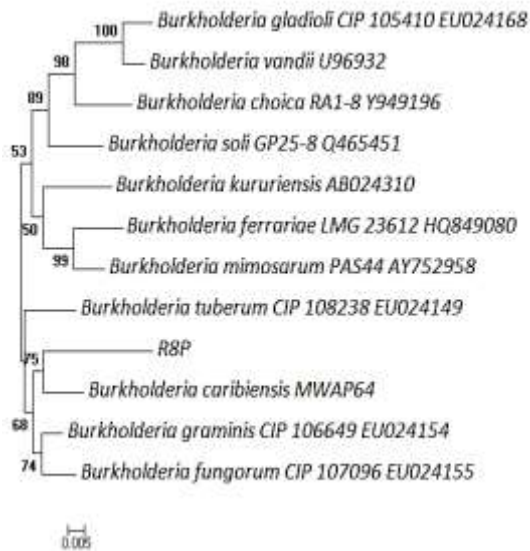


Figure 6. The phylogenetic tree of A1N isolate based on 16S rDNA gene sequence

Nitrogen Fixation (Ammonium Production) Assay

The nitrogen fixation assay shows the change of media color from green to blue and has a white follicle on the sub-surface medium (Fig. 2c). The TPC results showed that nitrogen fixation bacteria had the highest number of cells in the range of 3.5×10^5 - 6.5×10^6 cfu per gram of soil (Fig. 1). The result of the qualitative test using Serra ammonium test obtained 5 isolates of nitrogen fixation bacteria produce ammonium with varying concentration that is blue and dark green color showed high ammonium content while light green contains low ammonium concentration (Fig. 7). Qualitative test of ammonium concentration based on NH_4Cl standard curve obtained 5 isolates with different concentration. The isolate that produces the highest concentration is A1N.

Results of molecular identification based on the sequence of 16S rDNA gene showed that A1N isolate had a similarity of 98.7% with *Stenotrophomonas maltophilia* species (figure 8). It is known that *S. maltophilia* bacterial strains are capable of producing ammonium with various carbon sources [18].

Bacteria associated with clove plants in the rhizosphere are able to improve nitrogen fixation that has been widely reported as a plant growth promoter [35]. The activity of nitrogen fixation organisms is generally active in plant roots.

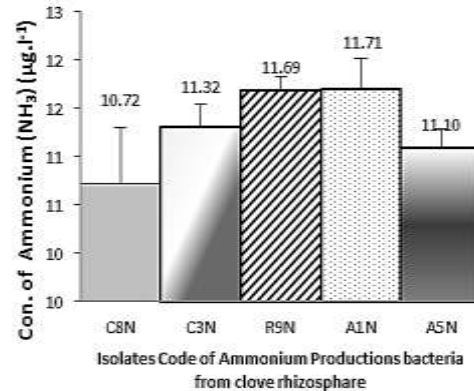


Figure 7. NH₃ Production of Bacterial Isolates

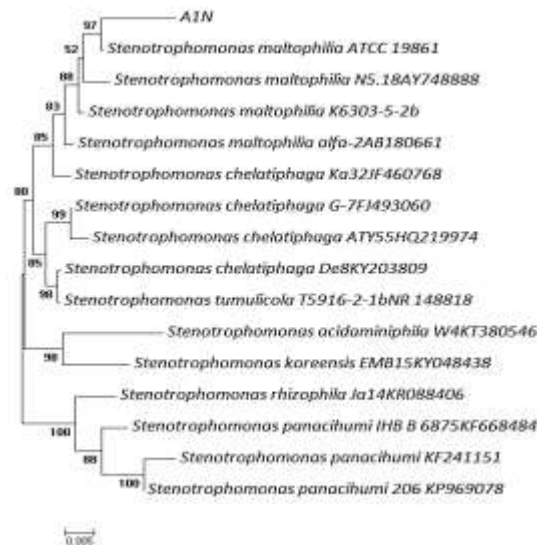


Figure 8. The phylogenetic tree of R8P isolate based on 16S rDNA gene sequence

CONCLUSION

This study obtained *Enterobacter hormaechei*, *Burkholderia multivorans* and *Stenotrophomonas maltophilia* that are potential for biofertilizer agents. The recommendation for further research is Application of synergism or antagonism then the isolates perform as consortium and prepare for biofertilizer and apply to plants of three isolates in a greenhouse to determine their effect on crops, without exception to commodity crops such as cloves. In addition, a biochemical and physiological characterization and identification of encoding genes are necessary to support the identified molecular data of bacterial species.

ACKNOWLEDGEMENT

We thank to Indonesia Endowment Fund for Education (LPDP) for giving funding in this study, and all of the member in working grup in Laboratorium of Microbiology, Biology Department of Brawijaya University.

REFERENCES

- [1] Directorate General of Plantations. 2016. Statistik perkebunan Indonesia 2015-2017. Sekretariat of Directorate General of Plantations. Ministry of Agriculture. Jakarta.
- [2] Sukmadewi D.K.T., Suharjono, S. Antonius. 2015. Uji Potensi Bakteri Penghasil Hormon IAA (*Indole Acetic Acid*) dari Tanah Rhizosfer Cengkeh (*Syzigium aromaticum* L.). *Biotropika*. 3(2). 91-94.
- [3] Burdman, S., E. Jurkevitch, Y. Okon. 2000. Recent advances in the use of plant growth promoting rhizobacteria (PGPR) in agriculture. In: Subba-Rao, N.S., Y.R. Dommergues. *Microbial Interactions in Agriculture and Forestry Science Vol III*. Science Publishers, Inc. Enfield, USA. 229-250.
- [4] Kloepper, J.W., J. Leong, M. Teintze, M.N. Schroth. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature*. 286. 883-884.
- [5] Lugtenberg, B., F. Kamilova. 2009. Plant-growth-promoting rhizobacteria. *Ann. Rev. Microbiol.* 63. 541-56.
- [6] Abdulwareth, A.A., U.K. Kaleem, Z.N. Bin-Li, A. Mumtaz, C.I. Yang, L.X. Guan. 2014. Tomato plant growth promotion and antibacterial related-mechanisms of four rhizobacterial *Bacillus* strains against *Ralstonia solanacearum*. *Symbiosis*. 63. 59-70.
- [7] Gloud, W., J.W.Klopper, S. Tuzun. 1990. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by selected strain of Plant Growth Promoting Rhizobacteria. *Phytopathology*. 81. 1508-1512.
- [8] Anandaraj, M., R. Dinesh. 2008. Use of microbes for spices production. In: Parthasarathy V.A., K. Kandiannan, V. Srinivasan (Eds). *Organic spices*. 101-132.
- [9] Shaharoon, B., M. Arshad, Z.A. Zahir, A. Khalid. 2006. Performance of *Pseudomonas* spp. containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. *Soil Biol. Biochem.* 38. 2971-2975.
- [10] Kavino, M., S. Harish, N. Kumar, D. Saravanakumar, R. Samiyappan. 2010. Effect of chitinolytic PGPR on growth, yield and physiological attributes of banana (*Musa spp.*) underfield conditions. *Appl. Soil Ecol.* 45. 71-77.
- [11] Kurabachew, H., K. Wydra. 2013. Characterization of plant growth promoting rhizobacteria and their potential as bioprotectant against tomato bacterial wilt caused by *Ralstonia solanacearum*. *Biol. Control*. 67. 75-83.
- [12] Baldani, J. I., V. L. Baldani, Seldin, J. Dobereiner. 1986. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *Int. J. Syst. Bacteriol.* 1(36). 86-93.
- [13] Adesina, M.F., A. Lembke, R. Costa, S. Arjen, K. Smalla. 2017. Screening of bacterial isolates from various European soils for in vitro antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: Site-dependent composition and diversity revealed. *Soil Biol. Biochem.* 39. 2818-2828.
- [14] Sawar, M., R.J. Kremer. 1995. Determination of bacterially derived auxins using a microplatemethod. *Lett. Appl. Microbiol.* 20. 282-285.
- [15] Glickmann, E., Y. Dessaux. 1994. A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol.* 61. 793-796.
- [16] Gaur, A.C. 1990. Physiological functions of phosphate solubilizing microorganisms. Scientific Publishers. 16-72.
- [17] King, J.E. 1932. The colorimetric determination of phosphorus. *Biochem. J.* 26 (2). 292-297.
- [18] Yu, SS., E.P. Kyaw, TM. Lynn, Z.K. Lan, A. Aung, T.M. Sev, M.T. New, W.W. Mon. 2017. The correlation of carbon source and ammonium accumulation in culture broth by nitrogen-fixing bacterial isolates. *J. Sci. Innov. Res.* 6(2). 63-67.
- [19] Cappuccino, J.C., N. Sherman. 1992. *Microbiology: a laboratory manual*, 3rd Ed. Benjamin/Cummings. New York. 125-179.
- [20] Zarei, S. Aminzadeh, A. Ghoroghi, A.A. Motalebi, J. Alikhajeh, M. Daliri. 2012. Chitinase Isolated from Water and bacteria

- in Shring Ponds. *Iran J. Fish Sci.* 11(4). 911-925.
- [21] Saitou, N., M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4. 406-425.
- [22] Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* 39. 783-791.
- [23] Tamura, K., M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10. 512-526.
- [24] Tamura, K., G. Stecher, D. Peterson, A. Filipski, S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30. 2725-2729.
- [25] Delgado, A., J. A. Gómez. 2016. The Soil. Physical, chemical and biological properties. Principles of Agronomy for Sustainable Agriculture. Chapter 2. DOI: 10.1007/978-3-319-46116-8_2.
- [26] CSR/FAO. 1983. Reconnaissance land resources surveys 1 : 250.000 Scale, Atlas Format Procedures. Ministry of Agriculture and FAO/UNDP. Bogor. 106.
- [27] Ahmad, F., I. Ahmad, M.S. Khan 2008. Screening of free living rhizobacteria for their multiple plant growth promoting activities. *Microbiol.* 163. 173-181.
- [28] Kesaulya, H., Baharuddin, B. Zakaria, S.A. Syaiful. 2015. Isolation and physiological characterization of PGPR from potato plant Rhizosphere in medium land of Buru Island. *Procedia Food Sci.* 190-199.
- [29] Ul, H., A. Bano. 2015. The stimulatory effects of L-tryptophan and plant growth promoting rhizobacteria (PGPR) on soil health and physiology of wheat. *J. Soil Sci. Plant Nutr.* 15(1). 190-201.
- [30] Ryu, R., C.L. Patten. 2008. Aromatic amino acid-dependent expression of indole-3-pyruvate decarboxylase is regulated by 4 TyrR in *Enterobacter cloacae* UW5. *Am. Soc. Microbiol.* 21. 1-35.
- [31] Rodrigues, A.A., M.F. Forzani, R. de S. Soares, Sibov, J.G.D. Vieira 2016. Isolation and selection of plant growth-promoting bacteria associated with sugarcane. *Pesq. Agropec. Trop., Goiânia.* 4(6). 149-158.
- [32] Watanabe, F.S., S.R. Olsen. 1965. Test of an ascorbic acid method for determining phosphorous in water and NaHCO₃ extracts from soil. *Soil Sci. Soc. Am. J.* 29. 677-678.
- [33] Vassilev, N., A. Medina, R. Azcon, M. Vassileva. 2006. Microbial solubilization of rock phosphate on media containing agroindustrial wastes and effect of the resulting products on plant growth and P uptake Plant Soil. *Appl. Environ. Microbiol.* 287. 77-84.
- [34] Ma, Y., M. Rajkumar, H. Freitas. 2009. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *J. Environ. Manag.* 90. 831-837.
- [35] Zeng, Q., X. Wu, J. Wang, X. Ding. 2017. Phosphate solubilization and gene expression of phosphate-solubilizing bacterium *Burkholderia multivorans* WS-FJ9 under different levels of soluble phosphate. *J. Microbiol. Biotechnol.* 27(4). 844-855.