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Characterization and potential of plant growth-promoting rhizobacteria on rice seedling growth and the effect on *Xanthomonas oryzae* pv. *oryzae*

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Abstract. *Rahma H, Nurbailis, Kristina N. 2019. Characterization and potential of plant growth-promoting rhizobacteria on rice seedling growth and the effect on* Xanthomonas oryzae *pv.* oryzae. *Biodiversitas 20: 3654-3661. Xanthomonas oryzae pv. oryzae* (*Xoo*), a major limiting factor in rice production, and the use of resistant *Xoo* varieties have failed to control the bacterial pathogens as well as increased yield. It is due to the diversity in pathotypes, caused by environmental factors, the nature of resistant variety used, and gene mutation. The aims of this study were to select rhizobacterial strains with the potential of suppressing *Xoo* growth and promoting the growth of rice seedlings. This experiment was conducted in a completely randomized design (CRD) using seven rhizobacterial isolates selected through a dual culture test, with four replications. There were four isolates that potential in inhibiting the growth of *Xoo*, namely KJKB5.4, LMTSA5.4, *Bacillus cereus* AJ34, and *Alcaligenes faecalis* AJ14, with inhibition diameters greater than 11.50 mm. Rhizobacterial supernatant of 4 potential isolates has a zone of inhibition ranging from 12.25 to 24.00 mm. Four potential isolates were also able to solubilize phosphate, produce indole acetic acid (IAA) growth hormone, and siderophore, as well as enhance the growth of rice seedlings. Based on the nucleic acid sequencing of LMTSA5.4, KJKB5.4, and RK12 isolates were identified as *Stenotrophomonas malthopilia* strain LMG 958 (99.13%) accession NR 119220.1, *Stenotrophomonas pavanii* strain LMG 25348 (95.84%) accession NR 118008.1 and *Ochrobactrum ciceri* strain ca-34 (92.91%) accession NR115819.1.

Keywords: Antagonists, biological agents, indole acetic acid, phosphate solubilization, siderophore

INTRODUCTION

Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a seed born and major pathogen in rice plants, An aid to overcome this disease for rice production in temperate and tropical rice-growing regions was needed, due to its high epidemic potential (Chithrashree et al. 2011). Yield losses caused by bacterial leaf blight can reach 60%, with a threshold level of damage reaching 20% in the two weeks before harvest. If above that threshold, every 10% increase in disease severity causes an increase in yield loss of up to 5-7% (Suparyono and Sudir 1992). The most effective control of bacterial leaf blight is by planting resistant varieties of rice. However, the use of resistant varieties is faced with a variety of *Xoo* pathotypes that cause its resistance was limited by time and place. So, varieties that are resistant to one season in one area may become vulnerable in another season and location. The resistance of a variety is very much influenced by the state of pathotype at a particular place and time (Sudir et al. 2015).

Plant growth-promoting bacteria or Plant Growth Promoting Rhizobacteria (PGPR)/Rhizobacteria are bacteria that colonize plant roots and are beneficial for plant growth. Rhizobacteria live and develop by utilizing exudates released by plant roots. Rhizobacteria are also able to use soil organic matters (Whipps 2001). Rhizobacteria have one or more beneficial roles for plants, depending on the species and strains. Rhizobacteria produce phytohormones, including indole acetic acid (IAA), cytokines, gibberellins, and ethylene production inhibiting compounds. As a biological fertilizer, rhizobacteria can increase the bioavailability of soil-borne nutrients by dissolving phosphates and increasing plant ability to extract iron (Fe3 +). Rhizobacteria can act as biocontrol agents by producing antibiotics to control pests and diseases of host plants and to induce plant resistance (Beneduzi et al. 2012). Rhizobacteria, such as *Alcaligenes faecalis, Serratia marcescens, Bacillus subtilis,* and *Pseudomonas fluorescens* have been widely used as biopesticides against plant pathogens. They are saprophytic bacteria that are capable of producing antibiotics, chelating Fe ions, dissolving phosphate, and potassium, subsequently enhancing the production of hormones that stimulate plant growth, including indole acetic acid (Hayat et al. 2012). According to Rahma et al. (2014), the application of endophytic microorganisms in maize and grassroots of plants, showed that several isolates from the bacterial group of *Alcaligenes faecalis* AJ14, *Bacillus subtilis* KJTSB7.2, and *Serratia marsescens* AR1 suppressed the progression of Stewart wilt and *Xoo* bacteria, subsequently promoting plant growth. Besides, the application of *A.*

faecalis AJ14, *S.marsescens* AR1, and *P. fluorescens* LPK1-9 increased seedling growth and suppressed the progression of Vascular Streak Dieback (VSD) disease in cocoa plants (Trisno et al. 2016). This study aims to obtain potential rhizobacteria strains that can suppress the development of *Xanthomonas oryzae* pv. *oryzae* in vitro, and stimulate the growth of rice plants.

MATERIALS AND METHODS

This research was conducted at the Laboratory of Biological Control, Department of Pests and Plant Diseases, Faculty of Agriculture, Andalas University, Padang, Indonesia, from April to June 2019.

Rhizobacterial propagation

Rhizobacteria (*Alcaligenes faecalis* AJ14, *Alcaligenes faecalis* ANO6, *Bacillus cereus* AJ34, *Serratia marscescens* AR1, KJKB54, LMTSA54, and RK12 isolate) were obtained from the Laboratory of Biological Control, Department of Pests and Plant Diseases, Faculty of Agriculture, Andalas University (Rahma et al. 2014). The isolates were cultured on Luria Bertani (LB) Agar media (10 g/L Tripton, 5 g/L NaCl, 5 g/L yeast extract, 15 g/L Agar, and 1000 1000 mL distilled water), at pH 7.3 and incubated for 48 hours. After incubation, one colony was transferred into 50 mL of LB Broth medium in a 250 ml Erlenmeyer flask and incubated on an orbital shaker at a speed of 150 rpm for 24 hours. Ten mL of each rhizobacterial suspension was transferred into 250 ml of sterile coconut water media with the addition of 10% glucose in Erlenmeyer flask (500 ml volume) and incubated 2 x 24 hours in the same way. Population density is determined by the degree of turbidity compared with McFarland's solution on a scale of 8 (considered 10^8 CFU/mL (Klement et al. 1990)

Xanthomonas oryzae pv. *oryzae* propagation

The *Xoo* isolates were obtained from the Indonesian Center for Rice Research (ICRR), Sukamandi, and cultured on *Wakimoto Agar* media (150 g/L potatoes, 15 g/L sucrose, 5 g/L peptone, 15 g/L Bacto agar, 0.5 g/L Ca $(NO_3)_2$. $4H_2O$, 0.5 g/L Na_2HPO_4, $12H_2O$, 1000 mL distilled water). And then, the *Xoo* isolates were grown on liquid Wakimoto media, incubated at 30ºC, and shaken at 150 rpm for 24 hours.

Antibiosis ability test

The selection of rhizobacteria for biological agents candidates was carried out by *Xoo* antibiotic testing using the double-layer method (Lisboa et al. 2006). Bacterial isolates were grown on Luria Bertani Broth medium and incubated for 48 hours on a rotary shaker (100 rpm). Suspension of *Xoo* bacteria with a density of 10^8 CFU/mL (McFarland's Solution on a Scale of 8) was used for the test. A total of 100 µl *Xoo* suspension is spread on the surface of Wakimoto agar media use glass beads and dried so that the suspension distributed evenly. Five sterile paper discs with a diameter of 5 mm are placed on the surface of the media. Four paper discs were impregnated with five µL of different rhizobacterial suspension, and one paper disc was used as a control without rhizobacterial suspension. Observations on the diameter of the clear zone around the paper disc indicated as an inhibitory reaction of the rhizobacteria against *Xoo* bacteria, and each test was performed four times. Antibiosis activity of supernatant was performed using paper disc diffusion method (Muharni et al. 2016; Rahma et al. 2018). Four biocontrols standard and rice growth promoter rhizobacterial isolates were used as positive control. In addition, a single rhizobacteria colony was placed on LB media, into the 15 mL LB Broth, further transferred into a 50 ml culture bottle, and subsequently incubated on a shaker at a rate of 120 rpm, at room temperature for 2 x 24 hours.

Moreover, bioactive compounds were obtained by separating rhizobacterial cells from their solvents by centrifugation at 10,000 rpm and 25ºC for 5 minutes, followed by sterilization, using a 0.22 µm millipore filter. Furthermore, the compound activity of the supernatant obtained was tested using the disc diffusion method by growing the *Xoo* suspensions in Wakimoto Agar media. The rhizobacterial supernatant (10 µL) was dispensed using pipette onto sterile filter paper (5 mm) and placed on four sides of the Petri dish, while sterile distilled water was used as control. Furthermore, inhibition was observed twelve days after treatments by measuring the clear zones in the supernatant, and this test was performed four times.

Effect of rhizobacteria on the growth of rice seedlings

Rice seeds of cultivar Batang Piaman were surface-sterilized for two minutes using 70% alcohol and rinsed for five times in sterile distilled, modified from (Rudolph et al. 2015), subsequently and then soaked in rhizobacterial suspension in coconut water media (coconut water, glucose 10%) (10^8 cfu/mL) for 15 minutes. Fifty seeds were planted on moist stencil paper and incubated for 10 days. The potential rhizobacteria isolates were applied for subsequent tests.

Characterization of Rhizobacteria as PGPR

The rhizobacteria were evaluated for their plant growth-promoting activity, including hypersensitive reactions, chitinolytic activity, phosphate solubilization, and siderophore production.

Hypersensitivity reaction test

Rhizobacteria candidates for biological agents were tested for their pathogenicity by hypersensitive testing on tobacco leaves. Bacteria were cultured in LB Broth media for 24 hours, agitated at 100 rpm. Rhizobacteria with a density of 10^8-10^9 CFU/mL (McFarland's solution scale 8) were infiltrated using a syringe in leaf tissue on the lower surface. The response of plants in the form of necrotic symptoms was observed 24-48 hours after inoculation.

Indole Acetic Acid (IAA) production

IAA growth hormone production by rhizobacteria was determined by the colorimetric method, using a spectrophotometer at a wavelength of 510 nm (Glickmann

and Dessaux 1995). Rhizobacteria were inoculated into 15 mL of LB media with 0.1 mM tryptophan under the dark condition for 48 hours at room temperature, agitated at 110 rpm. Three mL of each rhizobacteria suspension was taken, placed in two Eppendorf (1.5 mL each), and centrifuged at 10,000 rpm for 15 minutes, then 1 mL of supernatant was placed in a test tube and added with 4 mL of Salkowski reagent (150 mL of concentrated H2SO4, 7.5 mL of FeCl3.6H2) 0.5 M, 250 mL of distilled water),subsequently incubated for 30 minutes. The absorbance was measured at a wavelength of 510 nm. The IAA concentrations of rhizobacterial culture were determined based on standard curves, with a concentration range of 0, 10, 20, 30, 40, 50, and 60 µg/mL of pure IAA.

Phosphate solubilization

Phosphate solubilization test was carried out by cultivating rhizobacterial isolates on Pikovskaya agar (10 g/L glucose; 5.0 g/L Ca_3PO_4; 0.5 g/L $(NH_4)_2SO_4$; 0.2 g/L KCl; 0.1 g/L $MgSO_4.7H_2O$; 0.01 g/L $MnSO_4.H_2O$; 0.5 g/L yeast extract, and 0.01 g/L $FeCl_3.6H_2O$; 1 L distilled water, pH 7.0). Phosphate solubilization activity was measured by comparing its diameter with that of the colony after two weeks incubation period at room temperature (Thakuria 2004).

Siderophore production

The estimation of siderophore production was carried out using the Simple Double-Layered Chrome Azurol Sulfonate Agar (SD-CASA) according to the method by (Hu and Xu 2011). 60.5 mg Chrome Azulol Sulfonate (CAS) was dissolved in 50 mL distilled water, and mixed with 10 mL iron (III) solution (1 mmol/L FeCl3 .6H20, 10 mmol/L HCl). While stirring, this solution was slowly added to 72.9 mg of HDTMA dissolved in 40 mL water. The dark blue mixture was diluted 20-fold and autoclaved at 121°C for 15 min. Agar (2%, w/v) was used as a gelling agent. The test is done by placing a 5 mm sterile filter paper that has been dipped in rhizobacterial suspension. One filter paper is dipped in sterile distilled water as a control and placed in the center of a petri dish. The culture was incubated for 24 hours. Isolates capable of producing siderophore are characterized by the appearance of orange zones around the growing rhizobacteria colony. Observations were made after rhizobacteria were incubated for 24 hours.

Rhizobacteria identification based on 16S rRNA sequences

Rhizobacteria identification was carried out on LMTSA 5.4, KJKB 5.4, and RK12 isolates. Rhizobacterial culture on LB + glycerol media was rejuvenated using LB media and incubated for 24 hours at 28°C (room temperature). DNA isolation was carried out using an extraction Kit (Geneaid). Amplification of 16S rRNA gene by Polymerase Chain Reaction (PCR) technique using 27F universal primers (5'-AGAGTTTGATCCTGGCTCAG-3 ') and 1492R (5'-GGTTA CCTTGTTACGACTT-3' (Galkiewicz and Kellogg 2008). The PCR mixture contains KAPA Taq Ready Mix) (KAPA Biosystem) 12.5 µL, 20 pmol for each primer, genomic DNA, and ddH_2O until the reaction volume is 25 µL. The PCR process was carried out under initial denaturation conditions at temperatures. 95° C for 5 minutes, 30 cycles of denaturation at 95 ° C for 1 minute, primer attachment at 55 ° C for 1 minute, DNA elongation at 72 ° C for 1.5 minutes, and the final stage at 72 ° C for 5 minutes using GeneAmp PCR System 9700 (Applied Biosystems, USA). The PCR product was then analyzed by agarose 1% gel electrophoresis under an ultraviolet transilluminator with the size markers (1 kb DNA ladder, Geneaid). The DNA sequence was aligned by using BLAST-N to determine the similarity of the isolates tested with homologous species available in a gene bank. Related sequences were identified using the BLAST search program, National Center for Biotechnology Information (NCBI), National Library of Medicine, USA (http://www.ncbi.nlm.nih.gov/) (Altschul et al. 1997). Sequence alignments were performed by BioEdit 7.2.1 (Hall 2011), and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) using *MEGA6* software (Kumar et al. 2004).

RESULTS AND DISCUSSION

Antimicrobial activity assay

Rhizobacteria antagonist test against *Xoo* showed that all isolates were able to inhibit the growth of *Xoo*, with KJKB5.4 has the most significant inhibition (diameter of growth inhibition was 16.50 mm), while *Alcaligenes faecalis* ANO6 has the lowest growth inhibition (3.25 mm) (Table 1). The use of biological agents, including the rhizobacteria to suppress the development of plant pathogens, has been widely reported. Also, the results of previous studies prove the effectiveness of rhizobacterial isolate in suppressing Stewart wilt disease, and subsequently promoting the growth of corn plants (Rahma et al. 2014). Rahma et al. (2018) reported the capability of KJKB5.4 and LMTSA5.4 isolates to inhibit the development of the *Diplodia maydis* fungus with growth inhibition of more than 50%

The capability of rhizobacterial isolate in inhibiting the growth of *Xoo* was related to the various mechanisms of controlling different pathogens, including producing antibiotic compounds, competition, producing bioactive compounds, secreting extracellular enzymes, and other secondary metabolite compounds, inducing resistance and increasing plant growth. Rhizobacteria isolate occasionally portrays more than one inhibitory mechanisms. Dual culture test showed that there were four isolates of rhizobacteria (KJKB5.4, LMTSA5.4, *B.cereus* AJ34, and *A.faecalis* AJ14) that have the potential in inhibiting the growth of *Xoo*. Growth inhibition of *Xoo* was indicated by the formation of clear zones, production of antibiosis and extracellular enzyme. Glick et al. (2007) reported that the production of one or more antibiotics by bacteria usually associated with the ability of rhizobacteria to act as a controlling agent for plant pathogens. Istiqomah and Kusumawati (2018) reported that mechanisms of pathogenic inhibition occur through the production of

various related metabolite compounds, including siderophore production (Dimkpa 2016), chitinase, and antibiotics (Couillerot and Moe 2009), bacteriocin (Riley 1993) and the induction of systemic resistance. Agustiansyah et al. (2013) reported that *Pseudomonas diminuta* A6, *Pseudomonas Aeruginosa* A54, *Bacillus subtilis* 11/C, and *Bacillus subtilis* 5/B were able to suppress *Xanthomonas oryzae* pv.*oryzae* by producing siderophore compounds, phosphatase enzymes, IAA and produce HCN compounds. However, microorganisms under certain conditions tend to produce secondary metabolite compounds to survive. Zhou et al. (2013) reported that *Xoo* produces membrane-bound yellow pigments, referred to as xanthomonadins. This pigment protects these bacteria from photodamage and host-induced peroxidation damage, and required for epiphytic survival and successful host plant infection.

The most significant inhibition was shown by the KJKB5.4 supernatant, with a diameter of 24.00 mm, while AJ14 has the smallest diameter of growth inhibition (12.25 mm) (Table 3). The antibiotic test assessment showed a various inhibitory of the isolates, and the disk diffusion method was observed to possess a more significant activity in contrast with the dual culture method, due to the high content of bioactive compounds in rhizobacteria supernatants. In this study the level of emphasis of bioactive compounds showed the ability to suppress *Xoo* higher than dual culture, this might be due to environmental factors that play a role in testing. According to Berendsen et al. (2012), the production of compounds of microbial origin is influenced by several factors including temperature, culture medium (nutrients), pH, the growth phase of the microbial colony, stress conditions, and the presence of or stimulation from other microorganisms. Adriansyah et al. (2015) reported a possibility of increasing the number of secondary metabolites of rhizobacteria supernatant by adding pathogens in the cell propagation because of the competition between both of microorganisms. Pathogens may stimulate bacterial growth and subsequently leading to the increased production of anti-microbial compounds.

Kanthaiah and Velu (2019) reported that PGPR *P. aeruginosa* VRKK1 was isolated from Cowpea rhizosphere soil was the best biocontrol agent. this strain produced the biometabolite such as octadecanoic acid 2-oxo methyl ester. At the concentration of 100 µL in diluted octadecanoic acid 2-oxo methyl ester compound completely reduces the *X. campestris* which is a causative agent of bacterial blight disease in cowpea plant. In vitro seed germination study with different concentrations of compounds showed suppression in the pathogen survival and also lessen the pathogenicity in the 60-100 µL of compound range. Germination rate also increased with the increased concentration of the compound added. Furthermore, (Rabbee et al. 2019)) reported the propensity of *Bacillus* spp. to yield volatile organic compounds, metabolites (siderophore and bacteriocin), and further stimulate plant growth. The study by Kannahi and Senbagam (2014) showed that rhizobacterial that produced siderophores has the ability not only improve rhizosphere colonization of bacterial strain but also play an important role in iron nutrition of plant and antagonism against phytopathogens.

Tabel 1. The diameter of *Xoo* growth inhibition by rhizobacterial isolates

Rhizobacteria isolates	Inhibitory zone (mm)
KJKB5.4	16.50a
LMTSA5.4	15.50ab
B.cereus AJ34	12.25abc
A.faecalis AJ14	11.75abc
RK12	11.50 bc
S. marcescens AR1	10.50c
A. faecalis ANO6	3.25d

Note: The numbers followed by the same letter in the same column are not significantly different at the 5% level (LSD)

Table 3. The diameter of *Xoo* growth inhibition by rhizobacterial supernatant

Supernatant's isolate	Inhibitory zone (mm)
KJKB5.4	24.00 a
B.cereus AJ34	22.25 ab
LMTSA5.4	20.00 b
A.faecalis AJ14	12.25 c
S. marcescens AR1	8.75 d
RK12	7.50 d
A.faecalis ANO6	3.00 e

Note: The numbers followed by the same letter within similar columns are not significantly different at the 5% level (LSD)

Table 2. The effect of rhizobacteria isolates on radicle and plumule length of rice seedlings

Rhizobacteria isolates	Radicular length (cm)	Effectiveness (%)	Plumule length (cm)	Effectiveness (%)
B. cereus AJ34	8.25 a	6.87	6.65 a	21.13
LMTSA5.4	7.98 ab	3.37	6.61 a	20.40
KJKB5.4	7.92 ab	2.59	6.67 a	21.49
A. faecalis AJ14	7.82 ab	1.29	6.66 a	21.31
Control	7.72 ab	00.00	5.49 b	00.00
S. marsescens AR1	7.52 ab	-2.59	7.01 a	27.67
RK12	7.46 b	-3.37	5.15 b	-6.19
A. faecalis ANO6	6.67 c	-13.60	5.54 b	0.91

Note: The numbers followed by the same letter within similar columns are not significantly different at the 5% level (LSD)

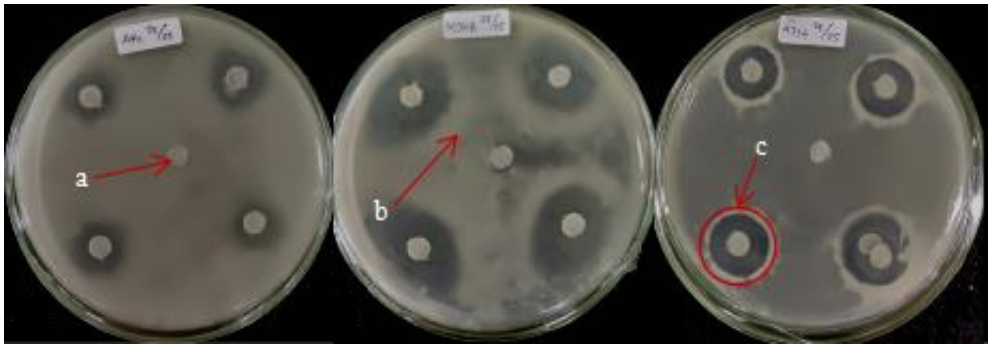


Figure 1. Inhibitory zone of rhizobacteria (A) LMTSA5.4, (B) KJKB5.4, and (C) *B. cereus* AJ34 against *Xoo* growth. Arrows: (a) control (distilled water sterile), (b) *Xoo*, and (c) clear zone, (12 days after treatment)

Table 4. Phosphate solubilization, siderophore and IAA production by Rhizobacteria

Rhizobacteria isolates	Phosphate Solubilizing Index	Sidero-phore	IAA conc. (µg/mL)
LMTSA5.4	35.25	+	26.58
KJKB5.4	28.25	-	50.51
B.cereus AJ34	24.25	+	42.34
A. faecalis AJ14	22.50	+	32.81
S. marsescens AR1	12.00	+	17.45
A.faecalis ANO6	12.00	-	7.50
RK 12	10.00	+	3.75

The inhibitory effect of the active compound from rhizobacteria against *Xoo* was observed based on the diameter of the clear zones formed around the paper discs that were supernatant impregnated (Figure 1). The ability of rhizobacterial supernatant to inhibit the growth of pathogens caused by the presence of antimicrobial compounds in the supernatant

The capability of rhizobacteria to stimulate rice seedling growth

The ability of rhizobacteria in stimulating the growth of rice seedling was assessed based on the indicators of radicle and plumule length. Based on the radicle length, it showed that *B.cereus* AJ34 has the highest effectiveness by 6.87%, and the most elevated plumule extension was obtained from *S.marsescens* AR1 by 27.67% (Table 2). The rhizobacterial isolates can promote the length of radicle and plumule of rice seedlings, as well as provide and mobilize the absorption of various nutrients in the soil. Rhizobacteria also able to enhance capacity in synthesizing and modifying the concentration of numerous phytohormones, dissolving P elements, and producing the Indole Acetic Acid hormone (Bhattacharyya and Jha 2012). Besides, they are also capable of promoting growth-regulating hormones, encompassing IAA, phosphate solubilization, and Siderophore production (Rahma et al. 2014).

Microorganisms can enhance the capacity of plants to acquire P from soil through various mechanisms such as: increased root growth through either an extension of existing root systems by hormonal stimulation of root growth, alteration of sorption equilibria that may result in increased net transfer of orthophosphate ions into soil solution or facilitate the mobility of organic P either directly or indirectly through microbial turnover, through induction of metabolic processes that are effective in directly solubilizing and mineralizing P from sparingly available forms of soil inorganic and organic P (Richardson and Simpson 2011). Rahni (2012) reported that the bacteria of the genus *Pseudomonas, Azotobacter, Bacillus*, and *Serratia* were identified as phytohormone-producing PGPR. The ability of rhizobacteria as phytohormone-producing PGPR is indicated by their ability to produce IAA and ACC deaminase, nitrogen fixation, increase the availability of P nutrients and other nutrients, and produce siderophore.

The mechanism of growth enhancement due to bacterial-plant interactions is related to the ability of bacteria to produce Indole Acetic Acid (IAA). IAA is a group of auxin, a plant growth hormone, involved in physiological processes of plant growth such as cell elongation and division, tissue differentiation, and root initiation (Gravel et al. 2007). This hormone is usually produced by soil microbes, both rhizobacteria and endophytic bacteria (Sessitsch et al. 2004). According to Duca et al. (2014), Indole-3-acetic acid (IAA) is an essential phytohormone with the capacity to control plant development in both beneficial and harmful ways. The ability to synthesize IAA is an attribute that many bacteria, including both plant growth-promoters and phytopathogen possess.

Phosphate solubilization, siderophore, and IAA hormone production

The results showed variations in the phosphate solubilization of four selected rhizobacterial isolates. The ability to dissolve phosphate is characterized by the presence of clear zones around the rhizobacteria colony (Figure 2.A). George et al. (2002) state that phosphate solubilizing bacteria will dissolve phosphate in the form of PO_4 using phosphatase enzymes so that clear halo formed around the colonies of phosphate solubilizing bacteria. The

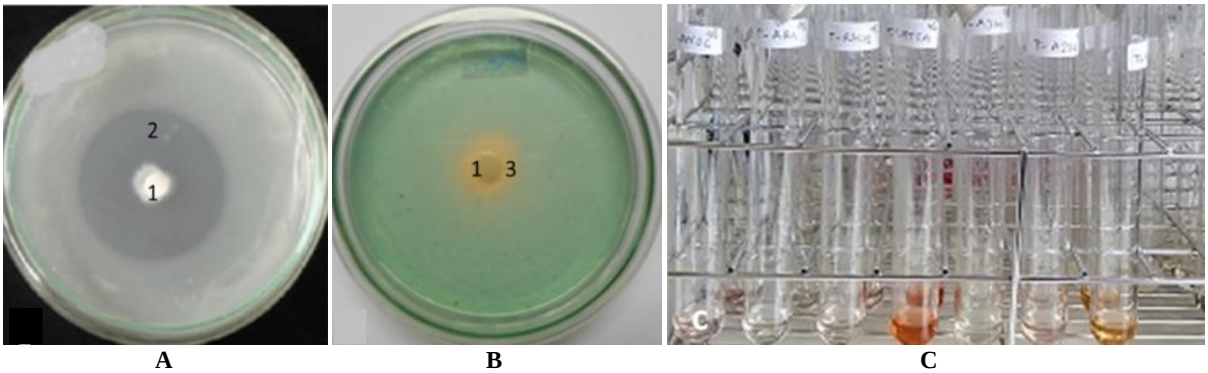
phosphate solubility index (IPF) of each isolate varied from 22.50 to 35.25. The highest IPF was shown by LMTSA 5.4 isolates. Midekssa et al. (2016) stated that clear halo was qualitatively showed the ability of bacteria to dissolve phosphate. The clear halo on solid media cannot measure the amount of dissolved P contributed by each bacterium, although the diameter of clear halo may indicate the size of the bacteria to dissolve phosphate. The results also showed that the selected rhizobacteria were also able to produce IAA hormone. KJKB5.4 showed the highest production of IAA (50.51 µg/mL), while LMTSA5.4 was the lowest production of IAA (26.58 µg/mL) (Table 4).

Rhizobacteria are also able to produce siderophore, which is characterized by changes in color from green to orange on CAS Agar media. Color changes occur because rhizobacteria can chelate iron in the media. Orange zones around rhizobacteria colonies showed the ability to produce siderophore (Figure 2.B). The ability of microorganisms to produce Siderophore is commonly detected using chrome azurol sulphonate (CAS) assay, as given by Schwyn and Neilands (1987). CAS Agar media are also used to essential siderophore production qualitatively (Raaska et al. 1993). CAS assay can identify the absorption of iron between siderophore and iron complexes from CAS (CAS-iron-detergent complex) dyes. Siderophore is a potent iron chelator that removes iron from iron-dyes and free dyes in the media that result in color changes from blue to orange (Louden et al. 2011). Several rhizobacteria such as the genus of *Pseudomonas, Azotobacter, Bacillus, Enterobacter, Serratia, Azospirillum,* and *Rhizobium* can produce siderophore as a chelating agent of the element Fe (Ali and Vidhale 2013).

Rhizobacteria identification based on 16S rRNA sequences

Identification based on 16SrRNA genes from isolates LMTSA 5.4, KJKB5.4, and RK12 revealed that the size of the 16S rRNA gene that can be amplified is ± 1500 bp. Based on DNA sequencing, it is considered that LMTSA 5.4 was closely related to *Stenotrophomonas maltophilia* strain IAM 12423, and KJKB 5.4 isolate was *Stenotrophomonas pavanii* strain LMG 25348, while RK 12 isolate was *Ochrobactrum cicero* strain ca-34. with similarity values of 95.57%, 99.46% and 92.91% respectively (Table 3).

Phylogenetic tree topology shows two main groups. The first group was KJKB 5.4 and LMTSA 5.4., while the second group is divided into two subgroups, namely the *Ochrobactrum* sp. group together with *Mycoplana* sp. and RK12 isolate, then the second subgroup namely *Stenotrophomonas* sp. together with *Pseudomonas* sp. In the first group, isolates KJKB 5.4 and LMTSA 5.4 showed that they were not closely related to the second group, although the results of BLAST showed a similarity of 95.57-99.46% with *Stenotrophomonas.* It suggests that the two isolates (KJKB 5.4 and LMTSA 5.4) are different species from bacteria in the second group. According to Clarridge (2004) if there is a difference between BLAST analysis, which is local alignment and phylogenetic tree analysis, which is global alignment, then the determination of identity is recommended based on global alignment. The similarity between sequence queries and their equivalent species on GenBank is > 94%, so they assumed to be the same species.



A **B** **C**

Figure 2. Characterization of rhizobacteria as PGPR. A. The ability of LMTSA5.4 isolate to dissolve phosphate in Pikovskaya media, B. Siderophore production by LMTSA5.4 isolate on SD-CASA media, C. IAA production in the Luria Bertani Broth medium + L-tryptophan and Salkoswky reagent

Table 3. Identification of rhizobacterial isolates based on 16S rRNA gene sequences

Isolate code	Query length (bp)	Related species	Identitas max (%)	Accesion number
LMTSA5.4	1584	*Stenotrophomonas malttophilia* strain IAM 12423	95.57	NR014577.1
KJKB5.4	1509	*Stenotrophomonas pavanii* strain LMG 25348	99.46	NR118008.1
RK12	1569	*Ochrobactrum ciceri* strain ca-34	92.91	NR115819.1

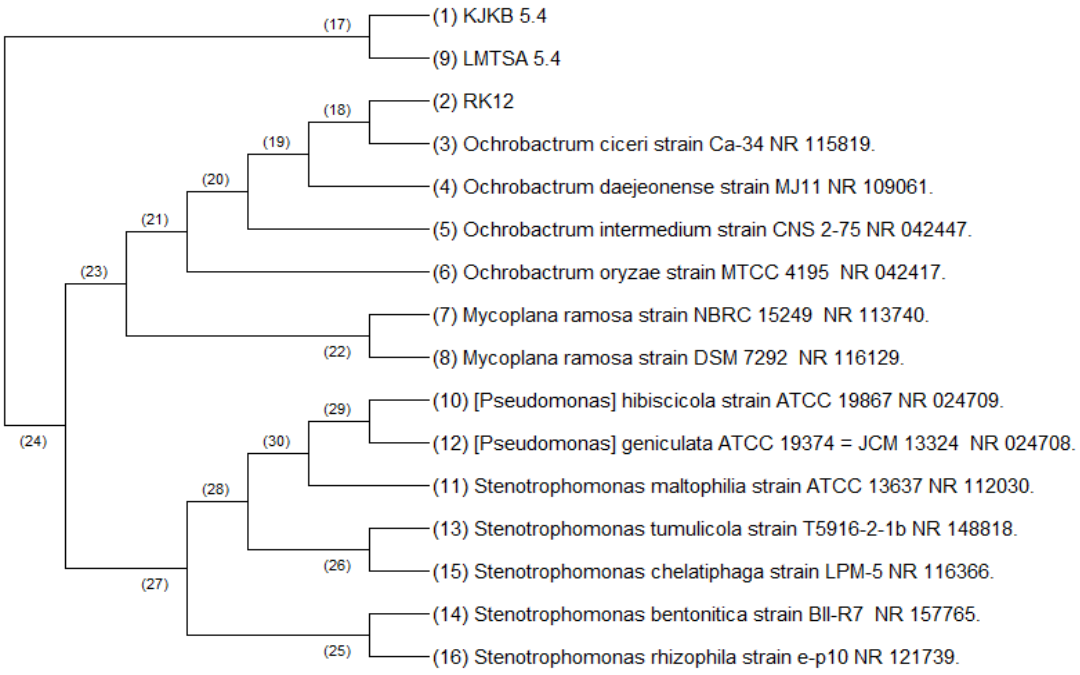


Figure 3. The phylogenetic tree based on sequencing of 16S rRNA gene using MEGA6 software (Kumar et al. 2004)

In conclusion, Rhizobacteria antagonist selection by dual culture test result in 4 potential isolates, namely KJKB5.4, LMTSA5.4, *B.cereus AJ34, and A. faecalis AJ14*, with growth inhibition was > 11.50 mm. The disc diffusion method showed that the diameter of growth inhibition was higher than in dual (12.25-24.00 mm). All selected isolates were able to dissolve phosphate, produce Indole Acetic Acid (IAA) hormone, produce siderophore except KJKB5.4, and stimulate rice seed growth, therefore, so they were considered as potential biological agents for controlling plant pathogens. Based on nucleic acid sequencing, LMTSA5.4, KJK5.4, and RK12 isolates were identified as *Stenotrophomonas malthopilia*, *Stenotrophomonas pavanii* and *Ochrobactrum ciceri* respectively.

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