The effectiveness of plant growth promoting rhizobacteria isolated from Gunung Ciremai National Park forest to control Alternaria blight disease of tomato

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Abstract. Tomato is an important horticultural commodity in Indonesia. Alternaria Blight caused by Alternaria solani Ell. & Mart. is an important disease on tomato. Biological control using plant growth promoting rhizobacteria (PGPR) is a promising technique for controlling the disease. One potential source of PGPR is a natural ecosystem area such as forest. This research aimed to determine the effectiveness of 5 PGPR isolated from Gunung Ciremai National Park forest, i.e. AKBR, AKS, C71, KS1, and S2 for controlling Alternaria blight disease on tomato and to assess their effects on plant growth. PGPR was applied by seed coating before planting and bacterial suspension watering on the soils. The research showed that all tested PGPR isolates could delay incubation periods and inhibit the development of blight disease. AKBR and C71 isolates had the highest effectiveness in suppressing A. solani infection with effectiveness rate 68.08% and 62.01% respectively. All tested PGPR isolates increased seed germination and tomato seedling growth. Molecular identification using PCR, followed by sequencing resulted that C71 isolates has 99.8% homology with Lysinibacillus fusiformis (Accession Number: KY910256.1)

1. Introduction
Tomato is horticultural commodities that is cultivated throughout the world. Tomatoes are consumed in several ways such as fresh or mixed with other food. Tomatoes can be utilized as raw material in food production industry, cosmetic, and medicine. Tomato contains lycopene that helps to protect the body against cancer and heart disease. Beside lycopene, tomatoes also contain polyphenols that have antioxidants to extinguish free radicals. High tomato consumption can provide protection against oxidative damage to prevent mutations in the progress and initiation phase of cancer [1]. The consumption of fresh tomatoes and tomato refined is increasing in population and public awareness of the importance of proper nutrition. Higher production of tomato is therefore required to fulfill the ever-increasing demand [2].

Alternaria blight disease caused by Alternaria solani Ell. and Mart. is important disease on tomato. In some instances, annual economics yield losses due to Alternaria blight disease have been estimated 50 to 86% [3]. Symptoms of Alternaria blight occur on fruit, stem, and foliage of tomatoes. Alternaria blight symptoms typically begin on young tomato leaves as small necrotic spots that appear dry and papery. As lesions enlarge they usually produce concentric circle giving the lesion a target like appearance and often surrounded by a yellowing zone [3]. The symptoms of A. solani appear in leaf,
stem, and fruit. The critical period of plant against *A. solani* occur when tomato plant are in the generative phase [4]. Control measures for these diseases can be done by applications of fungicides, crop rotation, and the use of disease-free transplants. Fungicide treatments are generally the most effective control measure, but routine applications of fungicides can leave residue in the yields.

Plant growth promoting rhizobacteria (PGPR) are one of the most potential biological control agents in the plant diseases protection. PGPR is the bacteria from rhizosphere or portion of soil that has close contact with the plant roots. Rhizobacteria are known to successfully colonize the roots of host plant, compete with pathogenic organism and suppress their growth, by acting as a biofertilizer and/or antagonist (biopesticide) to pathogens [5]. Rhizobacteria have the ability to solubilize mineral phosphate, nitrogen fixation, among other soil nutrients [6]. The use of PGPR as biological control agents to controlling plant diseases is still limited to the origin of rhizobacteria isolated from around cultivated crops.

Other source of PGPR is natural ecosystem area such as the forest. One of the forests in Indonesia that still has a natural ecosystem is Gunung Ciremai National Park forest. Based on the Minister of Forestry decision, protection forest in Ciremai Mountain converted to become national park. This determination is based on existing potential that is the area of Gunung Ciremai National Park forest 15 383 areas are represents a united and unremarkable type of mountain forest ecosystem and possess an abundance of natural resources, among them are biological resources [7]. One of the biological resources from Gunung Ciremai National Park is soil microorganism such as bacteria. Many of these bacteria belongs to the group rhizobacteria that promote plant growth [8]. Bacteria with plant growth promoting activities present in the rhizosphere, are classified into genera *Azorarcus, Azospirillum, Azotobacter, Bacillus, Paenibacillus, Actinomyces, Clostridium, Enterobacter, Gluconacetobacter, Pseudomonas*, and *Serratia* [5].

2. Methods
The experiment was arranged in completely randomized design (CRD) with 6 treatments and 4 replications. Bacterial isolates used in this experiment were bacterial collections of Plant Clinic, Department of Plant Protection. Bacterial isolates used were originated from bacteria isolated from Gunung Ciremai National Park forest, i.e. AKBR and C71 from bamboo roots; AKS and KS1 from roots and rhizosphere of kaliantras plant; and S2 from roots of Sonokeling plant. Pure cultures of bacteria were plated on NA medium by streaking method. These culture plates were incubated for 24 hr to 48 hr. The bacteria to be used for treatment is a single bacterial culture with concentration $10^6$ cfu/mL. The experiment was carried out in plant mycology laboratory and green houses, while the molecular identification of bacteria was carried out in Indonesian Center for Biodiversity and Biotechnology.

2.1. Seed growth test
Seeds of tomato var. Karina were surface sterilized with 1% natrium hypochlorite, rinsed in sterile distilled water and dried. Before planting, seed of tomato was soaked in bacteria suspension for 30 min. Seeds were then planted to the growing medium of mixed sterilized soil and compost manure (1:1). The plant germination, plant height, and root length of tomato seedlings were observed and recorded after 2 wk.

2.2. The effect of PGPR to the growth of tomato plants
PGPR-primed tomato plants were evaluated and plant growth stages were monitored and measured under greenhouse conditions. The tomato plants were transplanted in 35 x 35 cm diameter plastic pots 4 wk after seedling. The growing media contains of mixture soil and manure (2:1). The treatments of PGPR were 2 times applied by watering bacterial suspension to the soils, i.e. 1 wk and 3 wk after planting. The bacteria to be used for treatment was a single bacterial culture with concentration $10^7$ cfu/mL. The dosage of bacterial suspension used was 100 mL/plant or equivalent to $\pm 10^6$ cfu/mL. As check control plants were treated using sterilized water. The agronomic aspects such as plant height,
trunk diameter, root length and fresh weight roots of plant was observed 4 wk after application of PGPR.

2.3. The effect of PGPR to the alternaria blight disease
Inoculation of A. solani was done by spraying the mycelium suspension on tomato plants 10 wk after transplanting. The concentration of mycelium suspension for inoculation was 10⁴ fragmen/mL, or each plant was sprayed by 10 mL of mycelium suspension. The tomato plants were monitored for incubation period, diseases incidence, and severity. Observation of incubation period were made every day until the symptoms in each plant appeared. Diseases incidence and severity was observed at 7, 14, and 21 d after inoculation (DAI).

2.4. Characterization test of PGPR isolates
Bacterial isolates were characterized by Gram staining test, chitinolytic test, and antibiosis test. The isolated bacteria were subjected to Gram staining test to differentiate Gram-negative and positive bacteria. The test of Gram staining was performed used KOH 3% on the preparat glass. Chitinolytic test was performed by plating pure cultures of bacteria on 1% chitin medium. These culture plates were incubated for 48 hr. The clear zone around the bacteria showed that the bacteria isolate had the chitinase enzyme.

2.4.1. Antibiosis test. Pure culture of pathogenic fungi was a collection of mycology laboratory, Department of Plant Protection. Antagonistic activities of bacterial isolate on A. solani were carried out according to the dual culture assay. The fungus was tested as a plug of mycelium at the center of a petri dish (9 cm) of half strength TSA using sterile corked borer 0.5 cm in diameter. Bacterial isolates were spotted on the agar near the outer edge of the disk. Plates were incubated at 27 °C and inhibition zones were measured after 7-d. Only those isolates that produce a clear inhibition zone were considered positive.

2.4.2 Molecular identification. Bacterial isolates will be selected to those with the highest effectiveness in increasing the plant growth and suppressing A. solani infection. This selected isolated was further identified by molecular method. The species identity of the bacterial isolates was determined by sequencing the 16S rRNA genes of the isolates, which were amplified by polymerase chain reaction (PCR) using the universal primers 27F (5’-AGAGTTTGATCCTGGCTC AG-3’) and 1492R (5’- TACGGTTACCTTGTTACGACTT-3’). DNA of the bacteria isolates was extracted and amplification was proceeded following protocol kit of KOD FX Neo (Toyobo 2017). Purified process, sequencing, and adjusting the nitrogen base were done by sending result of DNA amplification to a sequencing company. Sequencing data analysis was done using software molecular evolutionary genetic analysis (MEGA) v 7.0. The DNA sequences were analyzed utilizing the BLASTn program to determined similarity of the sequence DNA and compared with database of sequence DNA in site of NCBI (National Center for Biotechnology Information). A neighbor-joining phylogenetic tree was then determined using MEGA v. 7.0.

2.5. Statistical analysis
Observational data was tabulated using Microsoft Excel program version 2010. Data were analyzed statistically using analysis of variance according to SAS version 9.0. The differences of means were identified by SNK (Student Newmann Keul) Test at 0.05 levels.

3. Results and discussion

3.1. Effect of seed treatment using PGPR on seedling performance
The growth of tomato seed was better with PGPR treatment than those on check control. In general, all tested PGPR isolates increased seed germination, except S2 isolate. This is an indication that PGPR
isolates stimulated seed growth with the rate 88 to 99% [9]. Furthermore, the effect of PGPR can be seen from the growth of the stem and the roots. C71 isolate significantly increased the growth of the stem, while AKS isolate significantly increased the growth of the roots (Table 1).

Table 1. Effect of PGPR treatments on the seedlings performance.

| Treatment | Seed germination (%)<sup>a</sup> | Seedlings height (cm)<sup>a</sup> | Root length (cm)<sup>a</sup> |
|-----------|---------------------------------|---------------------------------|-----------------------------|
| AKBR      | 96.87a                          | 7.08ab                          | 2.29b                       |
| AKS       | 98.44a                          | 7.18ab                          | 2.67a                       |
| C71       | 98.43a                          | 7.78a                           | 2.40b                       |
| KSI       | 96.09a                          | 6.87b                           | 2.11b                       |
| S2        | 94.53ab                         | 7.15ab                          | 2.14b                       |
| Control   | 89.85b                          | 6.44b                           | 2.29b                       |

<sup>a</sup> Means in the same column followed by the same alphabet are not significantly different according to SNK Test (p≤0.05).

3.2. Effect of PGPR treatments on growth of tomato plants
There was no significant difference in the vegetative growth of tomato plants treated with PGPR isolates and the control (Table 2). It was accordance with the previous research [10] which suggested that PGPR did not have significant effect to the height of the plant, the number of leaves, the wet weight of headings, the length of the leaves, and the width of the corn plant leaves planted in the plastic pots.

Table 2. Effect of PGPR treatments on growth of tomato plants.

| Treatment | Plant height (cm)<sup>a</sup> | Stem diameter (mm)<sup>a</sup> | Root length (cm)<sup>a</sup> | Root fresh weight (g)<sup>a</sup> |
|-----------|--------------------------------|--------------------------------|-----------------------------|---------------------------------|
| AKBR      | 94.70a                          | 7.42a                          | 46.15a                      | 7.07a                           |
| AKS       | 94.96a                          | 6.52a                          | 46.43a                      | 6.24a                           |
| C71       | 98.46a                          | 6.98a                          | 43.70a                      | 6.37a                           |
| KSI       | 101.36a                         | 7.24a                          | 43.33a                      | 6.30a                           |
| S2        | 91.29a                          | 6.86a                          | 39.98a                      | 6.39a                           |
| Control   | 94.29a                          | 7.19a                          | 41.68a                      | 7.05a                           |

<sup>a</sup> Means in the same column followed by the same alphabet are not significantly different according to SNK Test (p≤0.05).

Visually, the growth of the plant with PGPR treatment was taller than the control treatment. It might be due to the association between plants with PGPR increased the synthesis of gibberelin hormones on plants. Gibberelin is a hormone that can control stem enlargement and crop tops and regulated the reproductive process in plants [11]. The dosage of PGPR isolates used in this research probably too low so they need more time to colonize the roots; as consequences, it was not able to increased plants growth significantly. Based on previous research [5] the density of PGPR isolates (1x10<sup>8</sup> cfu/mL) was able to increase the growth of tomato plants significantly to plant height, number of leaves, wet and dry weight heading and roots of tomato plant under greenhouse condition.

3.3. Effect of PGPR treatments on alternaria blight disease
PGPR isolates were used not only to promote plant growth but also to trigger systemic resistance, the so-called induce systemic resistance (ISR). The used of PGPR isolates was expected can suppressing infection of alternaria blight disease. Based on observations of incubation period, AKBR, C71, and S2 isolates could delay the development of symptoms of alternaria blight disease and significantly difference with check control (Table 3). The other isolates, AKS and KS1 had the potential to delay...
the development of Alternaria blight disease though no different from control. However, disease incidence was not significantly different although it can reduce disease incidence on 7 DAI.

Table 3. Effect of PGPR treatments on incubation period and diseases incidence.

| Treatment | Incubation period (day) | Diseases incidence (%) |
|-----------|-------------------------|------------------------|
|           | 7 DAI | 14 DAI | 21 DAI | 7 DAI | 14 DAI | 21 DAI |
| AKBR      | 8.13a | 18.75a | 100.00a | 100.00a |
| AKS       | 7.25ab | 50.00a | 100.00a | 100.00a |
| C71       | 8.36a | 12.50a | 100.00a | 100.00a |
| KS1       | 7.67ab | 25.00a | 100.00a | 100.00a |
| S2        | 8.00a | 31.25a | 100.00a | 100.00a |
| Control   | 5.88b | 62.50a | 100.00a | 100.00a |

*Means in the same column followed by the same alphabet are not significantly different according to SNK Test (p≤0.05).*

PGPR produces secondary metabolic compounds which may cause disease inhibition by decreasing the inhibitory effect produced by various pathogens [12]. Based on observation on the diseases severity, all PGPR isolates were effective in suppressing the development of diseases severity significantly compared to untreated control on 14 DAI. (Table 4). Isolates AKBR and C71 had the highest effectiveness in suppressing A. solani infection. AKBR and C71 could suppress diseases severity 68.08% and 62.01%, on 7 until 14 DAI and on 7 until 21 DAI, respectively.

Table 4. Effect of PGPR treatment on diseases severity of Alternaria blight on tomato.

| Treatment | Diseases severity (%) |
|-----------|------------------------|
|           | 7 DAI | 14 DAI | 21 DAI |
| AKBR      | 4.06b | 21.66b | 28.61ab |
| AKS       | 16.25ab | 29.65b | 35.40ab |
| C71       | 2.47b | 22.26b | 26.62b |
| KS1       | 8.33ab | 20.42b | 28.72ab |
| S2        | 6.55ab | 24.77b | 33.36ab |
| Control   | 26.47a | 44.66a | 48.58a |

*Means in the same column followed by the same alphabet are not significantly different according to SNK Test (p≤0.05).*

Previous study [12] reported that roots and rhizosphere of corn plant and bamboo plant have association with beneficial bacteria, which mostly belong to genera *Bacillus* sp. and *Pseudomonas* sp. Both of these bacteria can be utilized as promoting the growth of plants and to controlling plant diseases. Antagonistic bacteria *P. fluorescens* P60 increased the content of phenol compounds such as glycosides, saponins, and tannins in tomato plant tissue. These chemical compounds are secondary metabolites which are produced as chemical resistance of plants to prevent the growth and development of pathogens. Secondary metabolites can be toxic and inhibit the growth of pathogens but do not inhibit plant growth.

3.4. Characterization of PGPR isolates

Four PGPR isolates, AKBR, AKS, C71, and KS1 were classified as Gram positive bacteria, while S2 was Gram negative. (Table 5). Among the 5 PGPR isolates only AKS isolate have chitinolytic activity. Chitinolytic activity was marked by clear zone around the bacteria isolates. Chitinase enzyme was able to degrade the chitin on media. Chitin was a component cell membrane of pathogens such as fungi and
Chitinase was an enzyme to cut off $\beta$-1, 4, homopolymer N-acetylglucosamin bond on chitin to monomer N-acetylglucosamin [13].

**Table 5.** Characterization of PGPR isolates.

| Bacterial isolates | Gram$^a$ | Chitinolytic activity$^b$ | Antibiosis test$^c$ |
|-------------------|--------|--------------------------|-------------------|
| AKBR              | +      | -                        | -                 |
| AKS               | +      | +                        | +                 |
| C71               | +      | -                        | -                 |
| KSI               | +      | -                        | -                 |
| S2                | -      | -                        | +                 |

$^a$ (+) = Gram-positive, (-) = Gram negative.
$^b$ (+)= Able to produced chitinase enzyme, (-) = unable to produced chitinase enzyme.
$^c$ (+) = Able to produced inhibition zone, (-) = Unable to produced inhibition zone.

3.5. **Antibiosis test**

*In vitro* tested against *A. solani* showed that AKS and S2 isolates were able to inhibit the growth of *A. solani* (Table 5). Inhibition of AKS bacteria isolate against *A. solani* was known from clear inhibition zone between colony of bacteria isolate and *A. solani*. Antibiosis mechanism is the ability of bacteria to produce antifungal compounds, so that it caused growth of hyphae of *A. solani* became abnormal or malformation, such as swelling and shrinking of hyphae [14].

3.6. **Molecular identification**

Sequence analysis revealed that the 16S rRNA gen of C71 bacteria isolate possessed 99.8% similarity with the sequences of *Lysinibacillus fusiformis* (Accession Number: KY910256.1). *L. fusiformis* are Gram positive, rod-shaped bacilli that form endospores, belongs to family Bacillaceae. *L. fusiformis* had been known to produce catalase enzyme, cytochrome oxidase, and metabolite utilization to reduction nitrate [15].

4. **Conclusion**

All tested PGPR isolates from Gunung Ciremai National Park forest had potential to delay incubation period and inhibit the development of Alternaria blight disease. AKBR and C71 bacteria isolates had the highest effectiveness in suppressing *A. solani* infection with effectiveness rate 68.08% and 62.01%, respectively. All tested PGPR isolates increased seed germination and tomato seedling growth. Molecular identification using PCR indicated that C71 bacteria isolate has 99.8% homology with *Lysinibacillus fusiformis.*

**References**

[1] Melly N, Satriana, Etria H 2015 *J. Teknol. Indus. Per. Indo.* 7 35-9
[2] Elis K, Zulfahri G, Diki K 2013 *Tomato response to organic and inorganic fertilizer combination preprint* gr-qc/0203013
[3] Reni C and Roeland E V 2006 *J. Gen. Plant. Pathol.* 72 335-47
[4] Sientje M S 1999 *Bul. HPT.* 11 67-72
[5] Anupama N P, Sudisha J, Shin I I, Amrutesh K N, Lam S 2015 *J. Plant. Sci.* 231 67-73
[6] Erica B F, Lara D S, Launa S F, Luiz H R, Servio P R, Leandro M M 2017 *Front. Microbiol.* FM08(2017)172
[7] Iis A 2006 *J. Analys. Keb. Kehutanan* 3 87-94
[8] Orryani L and Magfirahtul M 2017 *J. Natur. Sci.* 6 73-82
[9] Prasetyo A 2018 The effectiveness of plant growth promoting rhizobacteria to control downy nematode.
mildew disease of cucumber in the field \textit{Preprint} gr-qc/1810018

[10] Amalya R O 2013 The effectiveness of plant growth promoting rhizobacteria to suppress downy mildew (\textit{Peronosclerospora maydis} (Rac.) Shaw) of sweet corn \textit{Preprint} gr-qc/1012013

[11] Rida I 2012 \textit{J. Agro. Tekno. Trop. JATT01}(2012)004

[12] Ashifa C T, Muh I P, Hasrullah, Ersyan, Tita A S, Abdul M J 2017 \textit{J. Hasanud. Stud.} 1 16-21

[13] Imanda N S and Suharjono 2015 \textit{J. Biotrop.} 3 95-8

[14] Eliza, Abdul M, Djaatnika I, Widodo 2007 \textit{J. Hort.} 17 150-60

[15] Eric W, Kamal K, Joan M 2015 \textit{Int. J. Infec. Diseases} 35 93-5