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Exploration Indigenous *Bacillus* Bumiaji-Malang Against *Ralstonia solanacearum* Causing Potato Bacterial Wilt

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Abstract

Ralstonia solanacearum is a soil borne pathogen, which has a very wide host range and causes bacterial wilt. The use of biological agents, such as bacterial groups has been tried in several areas of potato plantations in Java. This study aims to obtain *Bacillus* isolates from the potato cropping area, Sumber Brantas, Bumiaji, Batu, which had the potential to suppress growth R. *solanacearum*. *Bacillus* was isolated from the soil rhizosphere of potato plants, while R. *solanacearum* was isolated from the base of potato stems showing bacterial wilt symptoms on tetrazolium chloride (TZC) selective medium. *Bacillus* spp. and R. *solanacearum* isolates were tested for hypersensitivity on the leaves of the KR-15 tobacco plant. Isolates that cause necrosis symptoms in tobacco leaves can be ascertained to be pathogenic. This study succeeded in obtaining 13 *Bacillus* spp. isolates with different colony morphologies. Three isolates of *Bacillus* spp. were selected from the 13 isolates obtained, based on their ability to suppress the growth of R. *solanacearum* and are expected to be potential as biological agents. Based on genetic analysis, the 3 bacterial isolates were identified as *Bacillus mycoides* and *Bacillus weihenstephanensis.*

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Bacillus spp; Bacterial wilt; Pesticide reduction; Potato plants; *Ralstonia solanacearum*

Introduction

One of the factors that can affect plant productivity is pathogen attack. Plant pathogenic microbes (phytopathogens) can be bacteria, fungi or viruses. Some important phytopathogenic bacteria include *Ralstonia solanacearum, Pseudomonas syringae pv.*

glycines, Pseudomonas fluorescens, Erwinia sp., and *Xanthomonas oryzae*. *Ralstonia solanacearum* is a soil borne plant pathogen that is found in many subtropical and tropical areas which naturally infects roots and multiplies in xylem tissue (Hayward, 1991; Yabuuchi *et al*. 1995). *Ralstonia solanacearum* is one of the *phytopathogens* that attack potato plant. *R. solanacearum* causes bacterial wilt on potatoes. This pathogenic infection is reported to cause huge losses in various potato production centers and a threat to development target areas in Indonesia (Kuswinanti, *et al*., 2014).

Sumber Brantas Village, Bumiaji District, Batu, is one of the centers for potato plantations in East Java. The use of chemicals by farmers in Sumber Brantas village to protect crops from pathogens is still being carried out. Control of soil borne pathogens such as *R. solanacearum* using chemicals formulated as pesticides, fungicides, bactericides and others, is still questionable about its safety. Excessive use of chemicals is harmful to both the biotic and abiotic environment. One of the efforts to overcome these problems is by using bacteria as biocontrol agents.

The mechanisms for controlling pathogens by biocontrol agents include antibiosis, space and nutrition competition, microparasitism, cell wall degrading enzymes, resistance inducers, growth promoters, rhizosphere colonization and anti-quorum sensing (Lo, 1998; Yin *et al*, 2010; Junaid *et al*, 2013). Bacteria that have the ability as biocontrol agents can be isolated from soil in the plantation land (Rahman *et al*., 2012; Saha *et al*., 2012; Ali *et al*., 2020). Several groups of bacteria that are known to have potential as biocontrol agents are *Agrobacterium, Pseudomonas* and *Bacillus* (Fravel, 2005).

The genus *Bacillus* has the ability to synthesize several compounds that are useful in agriculture and industry. Several secondary metabolites produced by several species and strains of *Bacillus* show antibacterial and antifungal activity against plant pathogens (Yu *et al*., 2002; Ongena & Jacques, 2008). *Bacillus* also has different enzymatic capabilities in producing enzymes, including in producing amylase, protease and lipase enzymes.

Bacillus sp. can produce phytohormones that have the potential to develop sustainable agricultural systems. The phytohormones produced can affect plant growth, either directly or indirectly. Indirectly, the phytohormones from bacteria inhibit pathogenic activity in plants, while the direct influence of these phytohormones increases plant growth and can act as a facilitator in the absorption of some nutrients from the environment.

The application of bacteria as a biocontrol agent, although it is environmentally friendly, but it's not widely used. Potato farmers in Sumber Brantas Village are also not interested in using it, due to its effects that do not meet their expectations, such as slow response effect. Thus, exploration of bacteria as biocontrol agents must be carried out to obtain potential isolates to suppress growth of *R. solanacearum* which causes bacterial wilt on potato plants in Sumber Brantas Village.

The purpose of this study was to obtain *Bacillus* spp. isolates from potato plantation in Sumber Brantas Village, Bumiaji District, Batu, which were able to suppress the growth of the *R. solanacearum* which causes bacterial wilt on potato plants, and also to identify *Bacillus* spp. isolates that were the most capable to suppress the growth of *R. solanacearum*.

Materials and Methods

Isolation of *Ralstonia solanacearum*

Diseased plant samples were taken from the potato planting area in the Sumber

Brantas area, Batu, Malang. The base of the potato stems showing wilting symptoms, washed with sterile water then cut into pieces 5 mm x 5 mm in size then crushed with a mortar until smooth and added 1 ml of sterile distilled water. The bacterial suspension in the mortar was taken as much as 0.5 ml and then put in a test tube containing 4.5 ml of $MgSO_4.7H_2O$. Furthermore, it was diluted to reach 10-5 dilution based on the Kelman (1953) method. A total of 0.1 ml of the diluted suspension was cultured in a Petri dish containing sterile Triphenyl Tetrazolium Cloride (TTC / TZC) medium, and leveled with a sterile L-shaped glass rod and incubated at 30°C for 2 x 24 hours. The growing bacterial colonies were separated between virulent and non-virulent colonies on new sterile Kelman TZC solid media, and re-incubated according to the previous work step.

Isolation of *Bacillus* spp.

Soil samples from the rhizosphere of potato plantation were weighed 10 grams and put in an Erlenmeyer, were added 90 ml of sterile distilled water and shaken for 30 minutes at a speed of 150 rpm. Furthermore, the sample was heated in a water bath at 80 °C for 15 minutes, then dilutions were carried out in series until 10-5. Dilutions of 10-3, 10-4 and 10-5 were taken as much as 100 µl grown on Petri dishes containing Tryptic Soy Agar (TSA) medium (Singh *et al*., 2008). Petri dishes were incubated at a temperature of approximately 28 ^oC for 24 hours.

Hypersensitivity Test

Bacillus spp. isolates were propagated in TSA media, *R. solanacearum* isolates in TZC media for 48 hours. All colonies were harvested, each isolate was added in a tube of 2 ml of sterile distilled water. The suspension was then infiltrated into tobacco K-15 on the lower surface, incubated for 48 hours and observed for leaf necrosis. *Bacillus* spp. isolates that showed negative reactions (no leaf necrosis were observed) were used for further testing (Klement *et al*, 1990). Meanwhile, *R. solanacearum* isolates used for further research were those that showed a positive reaction (showing symptoms of necrosis).

Antagonist Test

The antagonist test was carried out in vitro. *R. solanacearum* was cultured on TZC medium, made a suspension at λ_{600nm} = 2 or reached a population of about $2.0x10^8$ CFU / mL, added to Yeast Pepton Glucose Agar (YPGA) medium and poured on a Petri dish. Ten µl of cells (OD_{600} = 3) *Bacillus* spp. was dropped on sterile filter paper with a diameter of 0.8 cm, and placed in a Petri dish that already contained *R. solanacearum*. Furthermore, the Petri dishes were incubated for 48 hours at 28 ^oC and observed for the formation of clear zones and measured the diameter. As a control filter paper was dripped with sterile water and each treatment was repeated three times.

In Vivo Selection of Antagonistic Bacteria Isolates

The in vivo test used the KR-15 variety of tobacco as a test plant. Prepare 24-hour-old *Bacillus* spp. isolates, 48-hour-old *R. solanacearum* isolates, and 2-month-old tobacco plants in polybags. Tobacco plants were placed in a plastic cover in the green house, the plants that were ready are sprayed with *Bacillus* spp. isolate, respectively. According to the treatment, there were three tobacco leaves in each treatment and left for 3 days. Furthermore, the tobacco plants, inoculated with *R. solanacearum*, were placed on the lower surface of the leaf veins. Plants remained in plastic cover, observed daily starting from

two days after treatment until three weeks after inoculation of *R. solanacearum*. The percentage of inhibition was calculated by the formula:

$$\frac{B}{A} \times 100\%$$

A : Leaf area (cm^2)
B : Necrosis area (cm^2)

Characterization and Identification of Bacteria

Three bacterial isolates were selected to be identified, of the 13 isolates that showed the worst symptoms on the in planta test. Characterization was carried out through colony macroscopic, cell microscopic, physiological, and genetic observations. Colony macroscopic characterization was performed by culturing bacteria on nutrient agar for 24 hours. Microscopic characterization was conducted by Gram staining, while physiological characters were performed using KIT A, which include, catalase, oxidase, oxidative-fermentation requirements, hydrolysis of gelatin, starch, levan formation, Proskauer's Voges test, arginine, dehydrolase, motility, tolerance for bacterial growth at several temperatures, pH and HCl concentration, use and overhaul of carbon, citrate and nitrogen compounds (Lelliot and Stead, 1987; William *et al*., 1989, Chun and Vidaver, 2001).

Identification was continued molecularly, by sequencing the 16S rRNA gene. Preparation before the sequencing process refers to the protocol kit (Genomic DNA Mini Kit, Geneaid), 16S rRNA was amplified by Polymerase Chain Reaction (PCR) technique using universal primers (27F 5 "-AGA GTT TGA TCC TGG CTCAG-3" and 1429R 5 "-GGT TAC CTT GTT ACG ACTT-3"). The PCR mixture was prepared in a 25 µl volume containing 1 µl F primer, 1 µl R primer, 12.5 µl mix (KAPA Taq Ready Mix, Kapa Biosystems, United States), 2.5 µl DNA extract, and 8 µl dH_2O. Then the DNA amplification was carried out on a PCR (Swift Maxi Thermal Cyber) machine with an initial denaturation for 1 minute at a temperature of 95 oC. Then followed by 30 denaturation cycles at 95 ^oC for 1 minute, annealing at 55 ^oC for 1 minute and extension at 72^oC for 1.5 minutes. Followed by an additional extension step at 72^oC for 5 minutes. 2 for 5 minutes. PCR products were visualized by electrophoresis on 1% agarose gel which had been added with ethidium bromide and TBE buffer.

The sequencing of PCR results was carried out by a sequencing service provider company. DNA sequence homology searches were executed using a DNA database (GenBank) using the BLAST program from the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results and Discussion

Isolation of Bacteria

The results of the isolation of *R. solanacearum* from potato plants, obtained 6 different isolates based on colony morphology. *R. solanacearum* belongs to a group of Gram-negative bacteria, the morphology of short rod-shaped cells, single cells. Colony morphology of *R. solanacearum* was irregularly rounded, milky white, the texture of the colony was slimy / shiny, the edges of the colony were uneven, the elevation was convex. According to Ray *et al* (2013) *Ralstonia* sp. can grow on Sucrose Peptone Agar (SPA) media, with irregularly shaped colonies, slightly convex elevation, slimy, creamy milk color, shiny surface, and in terms of physiology *Ralstonia* sp. in the form of a prosthetic with a size of

0.5-0.7 µm. Based on these characteristics which were confirmed by the characteristics listed in Bergey's Manual of Determinative Bacteriology and as reported by Ray *et al* (2013), the bacteria isolated in this study were identified as the *Ralstonia* genus. Meanwhile, *Bacillus* spp., based on differences in colony morphology, there were 13 isolates of *Bacillus* spp. on Nutrient Agar medium. In general, the colonies have a characteristic whitish cream color and a round and irregular colony. The edges of the isolates were flat and some were irregular. This characterization showed 13 isolates including the genus *Bacillus* sp.

Hypersensitivity Test

Hypersensitivity test results on tobacco plant leaves showed that three isolates of *R. solanacearum* were able to cause necrose symptoms, namely isolates Rs-3, Rs-4 and Rs-1, each of which had an incubation period of 4 days after incubation (DAI), 8 DAI and 11 DAI (Figure 1). From these results selected Rs-3 which has a faster incubation period. According to Agrios (2005), environmental conditions that support the growth of pathogens will accelerate the incubation period of the disease, so it will be faster to infect plants. Meanwhile, *Bacillus* spp. none of the 13 isolates showed symptoms of necrosis, therefore all of them have the opportunity to be used for further research.

Antagonist Test

The in vitro antagonist test results of 13 *Bacillus* spp. isolates against *R. solanacearum* showed similar results, namely that no isolate was able to produce an inhibition zone. The absence of a zone of inhibition from *Bacillus* spp. isolates does not mean that the bacteria were unable to suppress the development of *R. solanacearum*. The inability of *Bacillus* spp. isolates to form a zone of inhibition in vitro, presumably due to the condition and nutritional content of the media used, allows bacteria to not produce antibiotic compounds. Saputra (2015) stated that *Bacillus* did not show bactericidal against *R. solanacearum*. These results are also in line with research by Zicca *et al*., (2020*) who reported that B. oleronius, B. licheniformis, and B. megaterium* did not show a zone of inhibition when grown together with *Xylella* fastidiosa in the same media. The mechanism of inhibition of antagonistic bacteria can also be by competition for space and nutrition, releasing degrading enzymes or through induced resistance mechanisms (Lo, 1998).

Figure 1. Hypersensitivity test results for *R. solanacearum* bacteria isolates in Tobacco KR-15, symptoms of necrosis caused by isolate (A) Rs-6, (B) Rs-4, (C) Rs-1.

Solanacearum suppression testing in greenhouse

The results showed that the ability of *Bacillus* spp. to suppress the growth of *R. solanacearum* was different. This is indicated by the percentage area of necrosis symptoms that appear varies (Figure 2). This difference was due to the different abilities of the thirteen *Bacillus* spp. isolates in suppressing *R. solanacearum*. According to Choudhary and Johri (2008) biological control of *Bacillus* spp. through the mechanism of antibiosis, secretion of lysing enzymes and inducers of systemic resistance.

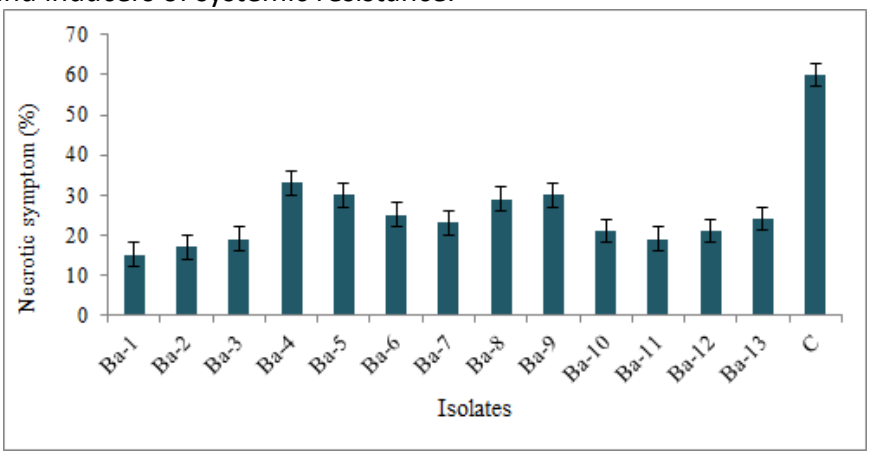


Figure 2. Potential of *Bacillus* isolates in suppressing necrotic symptoms in plants caused by *R. solanacearum*

Identification of *Bacillus* spp.

Three *Bacillus* spp. isolates which had the strongest ability to suppress the *R. solanacearum* growth, Ba-1, Ba-2 and Ba-11, were selected for further identification.

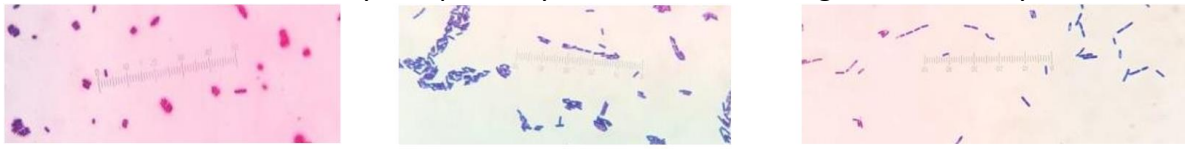
Table 1. Macroscopic colony and microscopic characters of selected bacteria

Isolate Codes	Microscopic and Macroscopic Characteristics of *Bacillus* Isolates					
	Surface	Edge	Shape	Color	Gram	Endospores
Isolate Ba-1 (figures 3a and 4a)	Flat	Flat	Round	White	+	+
Isolate Ba-2 (figures 3b and 4b)	Flat	Irregular	Round	White	+	+
Isolate Ba-11 (figures 3c and 4 d)	Slimy	Flat	Round	White	+	+

A B C

Figure 3. Morphological colony of *Bacillus* spp. which had the strongest ability to suppress the growth of *R. solanacearum*. Isolate colonies (A) Ba-1, (B) Ba-2, (C) Ba-11

The round colony shape and white colony color generally indicate that the bacteria belong to the genus *Bacillus* sp. According to Corbin (2004), the colony of *Bacillus* sp. has the general characteristics of having a whitish cream color and a round and irregular colony shape, flat and uneven colony edges. According to Hatmanti (2000), the bacteria *Bacillus* spp. colony has various kinds of flat and uneven edges, the surface is rough and not slimy, there are even tend to be dry and powdery, the colonies are large and not shiny.



A B C

Figure 4. Morphology cell of *Bacillus* spp. (A) Ba-1, (B) Ba-2, (C) Ba-11.

The three isolates showed a rod-shaped form of bacterial cells, Gram positive, forming endospores. According to Sofyan *et al*. (2009), *Bacillus* sp. is a Gram-positive bacteria with a short rod to a single rod with a single arrangement.

Table 2. Physiological and Biochemical Test with Kit A.

No.	Characteristics	*Bacillus* Isolates Code		
		1	2	11
1.	Oxidase	+	+	+
2.	Motilitas	+	+	+
3.	Nitrate	+	+	+
4.	Lysine	-	-	-
5.	Ornithine	-	-	-
6.	H2S	-	-	-
7.	Glucose	-	-	-
8.	Mannitol	-	-	-
9.	Xylose	-	-	-
10.	ONPG	-	-	-
11.	Indole	-	-	-
12.	Urease	-	-	-
13.	VP	-	-	-
14.	Citrate	-	-	-
15.	TDA	-	-	-
16.	Gram Test	Positif	Positif	Positif
17.	Shape	Rod	Rod	Rod
18.	Endospore	Present	Present	Present

Table 3. Molecular identification of bacterial isolates

No	Code Isolates	Results	Identification (%)
1	Ba-1	*Bacillus weihenstephanensis*	99%
2	Ba-2	*Bacillus weihenstephanensis*	99%
3	Ba-11	*Bacillus mycoides*	99%

The homology of Ba-1 and Ba-2 isolates based on the 16S rRNA gene from the BLAST results showed that these bacteria were related to several *Bacillus weihenstephanensis*. Meanwhile, Ba-11 isolate is related to *Bacillus mycoides* (Table 3). Based on the 16 s rRNA sequence of *Bacillus weihenstephanensis*, one group with *B. cereus, B. mycoides, B. anthracis, B. pseudomycoides and B. cytotoxicus*, because they both have the cspA, glpF, gmk, purH, and tpA genes (Habazar *et al*., 2018). In a further development between B. *mycoides* and B. *weihenstephanensis* it is stated that they are not different species. B. *mycoides* grows in the temperature range 10-15 °C to 35-40 °C, while B. *weihenstephanensis* grows in a temperature range of 7 - 43 °C (Soufiane *et al*., 2013).

According to Habazar (2018), research on B. *weihenstephanensis* as a biological agent is still very limited. B. *weihenstephanensis* is known to have the potential to inhibit the phytopathogenic growth of *Verticillium* (Hollensteiner *et al*., 2017) and as an insecticidal agent in *Schistocerca gregaria* (Mashtoly *et al*., 2019). Genomic analysis conducted by Hollensteiner *et al*. (2017) revealed that B. *weihenstephanensis* has genes encoding anti-fungal substances and secondary metabolite gene clusters including non-ribosomal polypeptides. While, B *mycoides*, able to synthesize protease and cellulase enzymes According to Sofiane *et al*, 2013, it is strongly suspected that the *Bacillus mycoides* and B. *weihenstephanensis* group's has ability as biological controllers to plant pathogens is associated with induced resistance mechanisms, because they show the high ability to control plant disease.

Conclusions

There were 13 isolates of *Bacillus* spp. with different characteristics which have potential biocontrol agents. Among these isolates, there were three best candidates for biocontrol agents, namely Ba-1, Ba-2, and Ba-11. Based on the 16S rRNA gene sequence analysis, Ba-1 and Ba-2 isolates have a 99% similarity identification with *Bacillus weihenstephanensis*, while Ba-11 shows 99% similarity with *Bacillus mycoides*.

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