Potential of *Bacillus spp* as a biocontrol agent against Ralstonia bacterial wilt in bananas

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**Abstract.** Bacterial wilt disease in banana plants caused by *Ralstonia solanacearum* is an important disease that can reduce banana production. Until now there is no effective method to control this disease. The use of biocontrol agents such as *Bacillus spp* is an alternative method of controlling *R. solanacearum* in bananas. The aim of this study was to determine the activity of *Bacillus spp* against banana wilt disease caused by *R. solanacearum*. In vitro testing was carried out in the Plant Physiology Laboratory, Faculty of Agriculture, Pattimura University. The study used *Bacillus subtilis* strain SW116b and *Bacillus subtilis* strain HPC2-1 isolates. The results showed that the SW116b stain *Bacillus subtilis* has the highest activity against *R. solanacearum*, which is 10.5mm, so it has the potential as a biological control agent in suppressing the development of ralstonia wilt disease in bananas.

1. Introduction

Banana is a plant that is widely cultivated in regions that have hot and humid climates such as Southeast Asia, the Pacific Islands, Papua, South America and Africa. The nutritional content of Bananas for every 100 grams is 89 calories, 22.8g carbohydrates, 75% protein, 2.6g fiber and 8.7mg vitamin c. Based on data from the [1, Indonesia produced 7 million tons of bananas, in 2017 it was 7.16 million tons and in 2018 it increased to 7.26 million tons. Banana production in Maluku Province in the last three years has decreased as follows: in 2017 amounted to 35,196 tons, in 2018 decreased to 33,319 tons and in 2019 amounted to 5.33 tons. One of the factors of decreased production in this area is the presence of disease.

In its growth and development, banana plants often experience disease disorders such as fusarium wilt and bacterial wilt. This disease is caused by two very dangerous types of soil borne pathogens, namely fusarium wilt caused by *Fusarium oxysporum* f. sp. cubense (Foc) [2], [3] and bacterial wilt caused by the bacterium *Ralstonia solanacearum* [4]. The two pathogens can attack one banana stem at the same time [5].

Blood disease is still a major obstacle in banana cultivation in Indonesia. This disease is often found in Kepok bananas but other varieties can also be infected. The symptoms caused are very similar to those of Moko's disease in Latin America which is caused by *Ralstonia solanacearum* race 2 [6]. In adult plants the base of these leaves will break so that the leaves will hang around the stem. The pups show wilting, although the infection is not always systemic. In banana plants that have already produced
fruit, the heart of the banana looks dry and shriveled and blackened. To see other symptoms, if the stem is cut, it appears in the vascular vessels there is necrosis that is brownish red. In addition, from the cut part, white to reddish brown or blackish bacterial mucus will appear.

When viewed from the outside, bananas that are stricken with blood diseases often look healthy. However, if the banana is cut, it will rot and contain bacterial mucus that is reddish yellow or blackish red. Blood disease is spread through several intermediaries, namely from one place to another by humans through plant material or fruit obtained from infected plants. Another method of spread is through pollinating insects stopping at sick flowers and then stopping at healthy flowers. This disease can also occur because through sick seeds, through agricultural means, watercourses, and means of transportation. The way to control blood diseases can be done through preventive methods, controlling the infectious insects / disease vectors and curative methods, namely by eradicating plants.

Bacterial wilt control still uses fungicides and technical cultures, but has not been able to reduce the incidence of disease in the field. To overcome this, it is necessary to develop biocontrol methods that are safer and more environmentally friendly. Biological control is directed as research on antagonist agents develops. *Bacillus spp* is a genus of bacteria that is reported to be able to increase plant resistance [7],[8] Morphological and physiological characters as well as the inhibition mechanism of *Bacillus spp* need to be studied to determine their effect on banana bacterial wilt infection. Recent studies on microbial siderophores in the rhizosphere are associated with their biocontrol activity due to their competitive effects with plant pathogens. Bacteria produce a wide variety of siderophores under iron-limiting conditions: hydroxamates, phenol-catecholates, and carboxylates. In nature, different types of siderophores such as hydroxymate, catecholets and carboxylates, are produced by different bacteria [8]. In addition to siderophore activity, siderophore bacteria have an important role in inducing resistance in plants to pathogenic infections [9]. The purpose of this study was to determine the effectiveness of the strain culture *Bacillus spp* best for its development in suppressing Ralstonia wilt disease in banana plants.

2. Methods
The research was conducted at the Laboratory of Physiology and Disease Pathology, Faculty of Agriculture, Pattimura University.

2.1. Sources Bacteria *Ralstonia solanocearum*
Samples of banana plants suspected of being infected with *Ralstonia solanocearum* were obtained from banana plantations owned by farmers in Taeno village, Ambon city. Planting the sample was carried out by pour plate on the media (NA). identification is done macroscopically and microscopically.

2.2. Sources bacteria *Bacillus spp*
Bacteria *Bacillus subtilis* and *Bacillus subtilis Strain SWI16b HPC2-1* origin rhizosphere potato used in this study were obtained from the collection of the Laboratory of Plant Physiology Faculty of Agriculture, Pattimura University. Bacterial isolates were propagated using nutrient agar medium (NA) and incubated at 28°C for 24 hours. The purity of the isolates was then rejuvenated using the quadrant method. Test the effectiveness of the culture of *Bacillus sp.* against *R. solanocarum*.

2.3. The Implementation of The Test
Media used in this test uses two media to confirm the inhibitory ability of the pathogen through the suppression activity that is formed. The media used in this test were: NA (meat extract, 10 g; peptone, 10 g; NaCl, 1.5 g; Agar, 15 g; Aquadest, 1000 ml); King’s B Agar 10% (Proteose peptone No.3, 2 g; glycerol, 1 g; K2HPO4, 0.15 g; MgSO4.7H2O, 0.15 g; Agar, 15 g; Aquadest, 1000 ml). In vitro antagonism test against *R. solanacearum* was carried out using a modified technique developed by [10,11]. Pure cultures of isolates *B. subtilis* aged 48 hours were used. Sterile filter paper pieces with a diameter of 5mm were put in the bacterial suspension for ± 1 minute and drained. After incubation, the plates were turned over and added 1 mL of chloroform, then left for 2 hours at room temperature. After
all the chloroform vapor had evaporated, the petri dishes were reversed to their original position, then 200 µL of suspension was poured *R. solanacearum* in 4 mL to 0.6% water. The cultures were incubated for 24 hours at 29°C to observe the resistance zone. After the incubation period, isolates that produced inhibitory compounds were recorded as indicated by the presence of an inhibition zone around the antagonistic bacterial colony.

2.4. Observation Parameters
Parameters observed were colony morphology on NA and King’s B media. Functional test of bacteria *Bacillus spp* and their inhibitory activity against isolates *R. solanacearum* in both media. Qualitative analysis was used on the growth of *Bacillus subtilis* stain HPC2-1 and *Bacillus subtilis* stain SW116b on different growth media.

3. Results and Discussion
From the results of colony morphological observations, the isolates of *Bacillus subtilis* strain SW116b and *Bacillus subtilis* HPC2-1 showed the same morphological characters in shape, color, edges and colony elevation. Colonies of Bacillus isolates that grew on nutrient media were generally round in shape, smooth edges, raised elevations and colony colors were white to brownish. (Figure 1). Bacillus colonies can have different shapes and structures because colony growth is influenced by environmental conditions and the character of each species and specific strains [12]. The results of the population count of the two Bacillus strains after incubation for 1 day can be seen in Table 1.

![Figure 1. a.isolate R. solanocearum from banana; b.isolates B. subtilis](image)

| Bacteria isolates | Total population | form colonies | Bank elevation | Color colony |
|-------------------|------------------|---------------|----------------|-------------|
| *Bacillus subtilis* strain SW116b | 3.5 x 10⁵ | Round | Whole | Average | White |
| *Bacillus subtilis* HPC2-1 | 3.8 x 10⁵ | Round-Up | Whole | average | White |

In the table above is known to the average population in the total population of each Bacillus strain is a strain of *Bacillus subtilis* SW116b total population of 3.5 x 10⁵ lower than the *Bacillus subtilis* HPC2-1, 3.8 x 10⁵.

Gram staining of detected Bacillus isolates was carried out to ensure that all of these species were Bacillus. Morphological observations were made to ensure that all the isolates were Bacillus.
3.1. In Vitro Activity Test Against Banana Bacterial Wilt

The antagonistic test showed that Bacillus spp. had inhibitory activity against wilt disease pathogens in banana plants. Inhibition ability is indicated by the clear zone. The differences in inhibition by each strain are Bacillus spp. shown in Table 1. The Bacillus spp. showed same order of biocontrol determinants production [13] including the wilt disease pathogen in banana plants.

| Table 2. Inhibition Zone Formation by Biocontrol Agents on Media Type |
|---------------------------------------------------------------|
| Bacillus spp | Inhibition zone diameter (mm) |
|--------------|-------------------------------|
|              | Nutrient Agar | King, s B |
| Bacillus subtilis Strain SWI16b | 7.5 | 10.5 |
| Bacillus subtilis strain HPC21 | 7.5 | 9.5 |

The formation of the inhibition zone by isolates was Bacillus spp. different from one another. Based on the results of the research, the isolates of Bacillus subtilis strain SWI16b and Bacillus subtilis strain HPC 2-1 had the tapping activity of inhibition zone in King, B media better than. The formation of the inhibition zone indicates the presence of an antibiotic mechanism and the ability to produce antibiotics in Bacillus spp. which can be used as a biocontrol agent. The compounds and sugars contained in the media have an effect on the formation of the inhibition zone. 

The difference in the zone of inhibition in the Bacillus strain tested is thought to be due to the conditions and nutrient content of the media used. [14] explained that antibiosis compounds are strongly influenced by the composition of the medium, both quantitatively and qualitatively. The results of the in vitro antagonism showed that the two Bacillus isolates were able to inhibit pathogen growth. The highest percentage of inhibition was produced by Bacillus subtilis Strain SWI16b in King’s B media while the lowest was produced by Bacillus subtilis Strains HPC2-1 in NA. This shows that Bacillus isolates tested in vitro have the potential to inhibit the growth of pathogens in culture media. Bacillus spp. has been known as a bacterium capable of producing various antibiosis compounds. According to [15] that Bacillus spp. can produce polypeptide-subtilin antibiotics, gramicidine, bacitracin, polymyxin, phytoactin and bulbiformin. This is clearly seen from the results of the test for Bacillus subtilis in-vitro. According to [16] Bacillus subtilis on NA and King, s, B medium can suppress the growth of Ralstonia solanacearum. Bacillus isolates can produce antibiotic compounds capable of suppressing colony growth which are toxic to other microbes, one of which is bacitracin which can inhibit the formation of pathogenic cell walls [17, 18]. Furthermore, the Bacillus subtilis strains SWI16b and Bacillus subtilis strains HPC2-1 in addition to producing antibiosis also have a high ability to colonize, so that these strains are able to compete in space and nutrition with pathogenic bacteria, including soil borne pathogens such as R. solanacearum. This is because of Bacillus subtilis. able to use various substrates as a source of nutrition, and have a much faster growth than pathogenic bacteria, so that they can maintain the population optimally [19]. Space competition between Bacillus subtilis strain SWI16b and Bacillus subtilis strain HPC 2-1 with pathogenic bacteria occurs through restriction of secondary development and spread of pathogenic bacteria by Bacillus subtilis is thus widely distributed [20]. In addition, nutritional competition also occurs as a result of a high population increase of Bacillus subtilis, especially in using carbon, nitrogen, and Fe3+ sources for growth and activity which can result in limited nutrient sources available for pathogen needs [21]. In this case, the effectiveness of Bacillus subtilis in suppressing pathogens is determined by its ability to produce antibiotics, induce resistance, competition, and colonize the root system over a long period of time and environmental factors and the
spread of bacteria in the soil [21]. This shows that the Bacillus isolates tested in vitro have the potential to inhibit the growth of pathogens in culture media.

3.2. *Bacillus* spp. Functional Test

| Bacteria Active Ingredients | Gram reaction | Solvent phosphate |
|-----------------------------|---------------|-------------------|
| *Bacillus subtilis* strain SW116b | +             | +                 |
| *Bacillus subtilis* HPC2-1    | +             | +                 |

Table 3. Results of the functional test for Bacillus spp.

The ability of Bacillus isolate to dissolve phosphate qualitatively was seen from the ability of Ca$_3$(PO$_4$)$_2$ as a source of phosphate bound to the media. The clear zone that forms around the colony indicates that the bacteria are able to dissolve phosphate. The two Bacillus isolates had different clear zone diameters, namely on King medium, s B 9.5 to 10 mm, while on NA medium only 7.5 mm. This is thought to be influenced by the organic acids produced by these bacteria. These organic acids released by bacteria then react with Ca$_3$(PO$_4$)$_2$ and form chelates (stable complexes) with P-binding cations (Ca$^4+$) accompanied by the release of HPO$_4$ [23].

4. Conclusion

Based on the research results, it can be concluded that the isolate *bacillus subtilis* strains SW116b and HPC2-1 can inhibit the growth of *R solanocearum*. Isolate *bacillus subtilis* strain SW116b in King; s B medium had the highest inhibitory activity, which was 10.5mm. Both isolates have the potential as biological agents to suppress the development of disease *Ralstonia solanocearum* in bananas.

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