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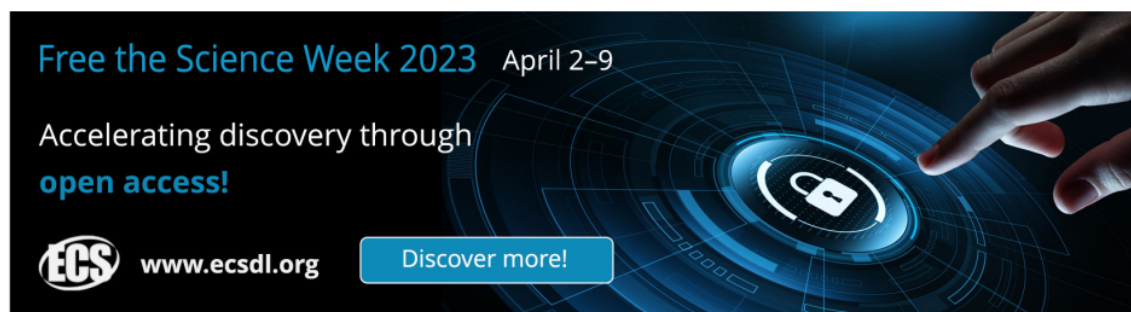
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Activity test of *Bacillus Spp* against bacterial wilt (*R. solanacearum*) on tomatoes by in vitro

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Abstract. *Ralstonia solanacearum* is a pathogenic bacteria that attacks tomatoes and causes wilt disease. Many efforts have been made to control this disease through cultivation, use of chemical pesticides, and development of resistant varieties, but bacterial wilt disease remains a serious problem economically. Nowadays, many biological controls are being developed using microbes. The use of *Bacillus spp* as an unfriendly microbe is very potential to control because it has pathogenic inhibitory activity. This study aims to obtain bacterial isolates of *Bacillus spp* which can suppress the development of bacterial wilt disease in tomato plants. The research was conducted in vitro at the Laboratory of Plant Physiology in the Agriculture Faculty, Unpatti. The results showed that *Bacillus niabensis* strain PT-32-1 and *Bacillus subtilis* strain SW116b could inhibit *Ralstonia solanacearum*, wilt disease in tomato plants in vitro.

1. Introduction

Tomato (*Lycopersicon esculentum* Mill) is a vegetable commodity of fruit that is classified as a seasonal plant (Solanaceae). As a horticultural plant, Tomatoes have a strategic role in daily needs. The content of tomatoes is very useful to meet the body's nutritional needs, in the form of vitamins and minerals. Tomato fruit contains vitamins A, B, and C, while the minerals are contained in the form of iron (Fe), calcium (Ca), and Phosphorus (P) [1]

During its cultivation, tomatoes experienced various obstacles that caused a decrease in quality and production quantity. One of the influential factors is the attack of plant-disturbed organisms. According to [2] harvest failures due to disease attacks are estimated at 14% global. One of the diseases that attack tomato plants is a wilt bacterial disease, that is caused by *Ralstonia solanacearum*. In Indonesia, until now wilt bacterial disease is still one of the common serious diseases in various types of horticultural crops. It was often been reported that this bacterium has many host plants, including tomatoes, potatoes, eggplant, chilies, chickpeas, beans, ginger, and bananas [3]

According to [4] on infested land of *R. solanacearum* naturally, in Germany, this disease caused 75% incidence. Tomato production in Indonesia is constrained by wilt bacterial disease (*R. solanacearum*) where the development of disease increases in the rainy season. This is because tomato production decreased, starting from 30%-60% [5]. This statement is supported by [6] that wilt bacterial disease is a serious disease that attacks tomato crops in Indonesia. Severe attacks can cause cultivated crops to die of crop failure. Wilt bacterial diseases are endemic and quickly develop infected plants to



healthier plants in the surrounding area [7] The initial symptoms it causes are wilt on young leaves and shoots, caused plants to die in a short time. The loss due to this pathogen infection can reach 100% [8].

R. solanacearum pathogen interferes with the transport of water and food substances by way of damaging plant cells. Enzymes that play a role in this process are the enzymes cellulase and pectinase. This enzyme destroys the cell walls of plants containing cellulose and pectin. As a result of this attack, there are physiological deviations of plants that are disrupted the process of translocation of water and other nutrients so that plants become wilt and then die [2]

Various efforts have been made in the control of bacterial wilt diseases, including the use of pest and disease-free seeds, planting of non-similar types of crops, non-simultaneous planting on large land, and the use of synthetic pesticides, but this method has not received satisfactory results. Also, the use of pesticides raises concerns about agricultural products that are contaminated due to pesticide residues. Long-term use of synthetic chemical pesticides can kill non-pathogenic microorganisms, poison humans due to improper use of doses, poison animals and pollute the environment, the occurrence of pathogenic resistance, and the emergence of new physiological breeds.

Another control alternative to controlling bacterial wilt diseases is by biological control using microbes. The use of biological agents in the control of plant diseases has several advantages, among others: 1) does not negatively affect the environment, 2) safe for you- such natural certain PESTS, 3) prevent the onset of secondary PEST explosions, 4) produce products that are free of residues synthetic chemical compounds, 5) safe for human health, 6) contained around crops to prevent farmers' exposure to synthetic chemical pesticides, and 7) can lower production costs because APH applications are carried out once or twice in one harvest season [9]

Bacillus sp. is one of the group of gram-positive bacteria that is often used as a biological controller of root diseases. Members of this genus have advantages because bacteria form spores that are easy to store, have a long life expectancy, and are relatively easy to inoculate into the soil. *Bacillus spp.* has been shown to have the potential as a good biological control agent, for example against pathogenic bacteria such as *R. solanacearum* [10] Some *Bacillus spp.*, species among the *B. subtilis* are able to prevent the development of anthracnose disease in chili peppers (*Colletotrichum gloeosporioides*) [11]

The purpose of the study was to determine the ability to isolate *Bacillus spp* in suppressing wilt bacteria (*R. solanacearum*) in tomatoes in vitro.

2. Methods

The research was conducted in the Laboratory of Plant Physiology and Plant Diseases Faculty of Agriculture, Pattimura University took place in May - Juli 2018

2.1. Isolate *Bacillus sp* Bacteria

This study using Bacterial Isolates *Bacillus niabensis* strain PT-32-1 and *Bacillus subtilis* strain SW116b from rhizosphere potato that is a collection of Plant Physiology Laboratory in Faculty of Agriculture, Pattimura University. Multiplication bacillus is isolated by growing it in agar (NA) media and incubated at a temperature of 28 °C for 24 hours. Isolate rejuvenation in the same medium and is done repeatedly to obtain a pure isolated culture.

2.2. Isolation of Pathogenic Bacteria

Pathogenic bacteria (*R. solanacearum*) used from samples of diseased plants (inoculum) namely tomato plants from farmers' gardens in Waimital, West Seram Regency that shows symptoms of the bacterial wilt disease. Wilt symptomatic plants are taken for the insulation. Pathogenic bacteria are isolated from parts of the plant that show symptoms of wilt in the field. The stems or roots of tomato plants with wilt bacteria on their surface are disinfected with 70% alcohol, then cut into small pieces, and put in a test tube that contains sterile water for 10 to 15 minutes. After sterile water in the test tube is dewy, we use sterile Ose needles. The suspension of the bacteria is scratched on the surface of the NA medium and then incubated. After 48 hours of incubation, a single colony was selected and grown on the NA medium again, then tested its properties, among other Gram test with 3% KOH.

2.3. Bland Zone Testing

This test uses several media to confirm the inhibitory ability of the pathogen through the formed bland zone. The media used in this test are Nutrien Agar (NA) and Yeast Peptone Dextrose Agar (YPDA). Tested using the modified [12-13] methods. Isolate *Bacillus* spp which is a pure breed cultured in NA media and aged 48 hours taken and then suspended up to a density of 10^9 CFU / ml in sterile aqua dest. Then a sterile piece of filter paper of 5 mm diameter is inserted into the bacterial suspension (*Bacillus*) for \pm 1 minute and dried. Then the piece of filter paper is planted in the middle of the surface of the NA media on a petri dish with 9 cm diameter and incubated for 2 days (48 hours). Once incubated, the petri dish is turned over and on the sterile lid of the petri dish, chloroform 1 ml is given and left for 2 hours. After that, the Petri dish was turned back to its original position. Further, there is a medium surface is poured suspension *R. solanacearum* (0.2 ml in 4 ml for water 0.6%). Breeds are incubated for 24 hours at a temperature of 300C. The same is done in Yeast Peptone Dextrose agar (YPDA) media. As a control, pieces of filter paper are dipped in sterile aqua dest. Each biocontrol agent was tested three times and the diameter of the resistance zone was measured after incubation at room temperature for 24 hours. The inhibitory zone will be visible around the bad bacteria, are measured and expressed in millimeters

3. Results and discussion

3.1. Isolation of Pathogenic Bacteria

Inoculum pathogen was obtained during observation of symptoms of bacterial wilt disease in tomato plants in Waimital Village, West Seram Regency. Many of the tomato plants show wilt symptoms. When it's taken, this wilt tomato plant, the stem is cut longitude and looks brown. The infected tissue secretes a fine strand of milky white consisting of a mass of bacteria (ooze), which seeps out of the cut-off boundary of an infected plant is pressed. Characteristics of tissues that are brown and if it's pressed there is a mass of this bacteria that distinguishes the wilt symptoms because the wilt bacteria is caused by the other causes/pathogens. This disturbance may be caused by the blockage of cells so that the translocation of water from the bottom (root) to the top of the plant becomes inhibited, as a result of which the plant becomes wilt. The tomato plant is taken and isolated.

The result of isolation from tomato plants obtained bacteria *R. solanacearum* has grown on nutrient media to be as follows: colonies are milky white, rather gloomy, irregularly shaped until round. This is in line with the results of [14] research, that the *R. solanacearum* bacterial colony is milky white, and irregularly shaped when grown on YPGA media. The bacterial properties of the obtained isolates are Gram-negative after being tested with KOH.

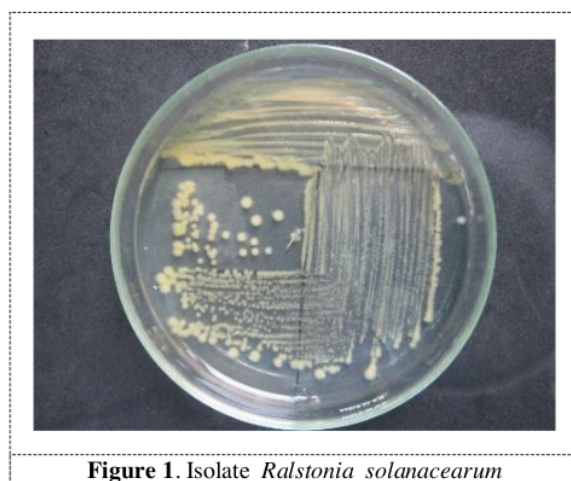


Figure 1. Isolate *Ralstonia solanacearum*

3.2. Reisolation, Rejuvenation and Purification of *Bacillus SPP* Bacteria

Bacteria that are successfully isolated is done again to multiply, isolate, and later purified. The results of bacterial isolation show that bacteria stored at low temperatures of 4°C after isolation can grow well with colony populations as presented in Figure 2.

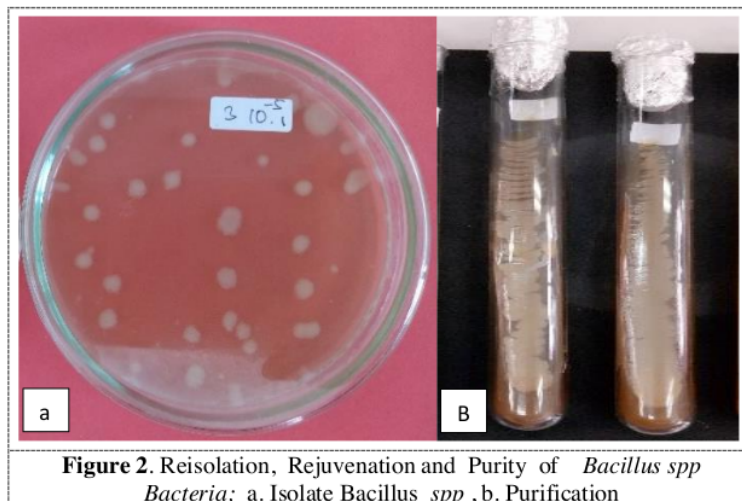


Figure 2. Reisolation, Rejuvenation and Purity of *Bacillus spp* Bacteria; a. Isolate *Bacillus spp* , b. Purification

The morphology of the colony from isolate *Bacillus* obtained can be seen in Table 1 where the colony is round with intact to jagged edges, has a flat elevation and the colony is white.

Table 1. Morphology Colony of *Bacillus spp* and total population of Reisolation and Rejuvenation Results

Bacteria Active Ingredients	Total Initial Population	Colony Shape	Edge	Elevation	Colony Color
<i>Bacillus niabensis</i> Strain PT-32-1	3.7×10^5	Round	Jagged	Flat	White
<i>Bacillus subtilis</i> Strain SWI16b	3.5×10^5	Round	Intact	Flat	White

To ensure the functional ability of bacterium, furthermore carried out bacterial purity test through various tests, such as Gram Reaction, Dissolution of phosphate, Nitrogen Fixation. The results obtained are presented in Table 2.

Table 2. Results Test of Qualitativity Functional Of Bacterial Isolate *Bacillus spp* Qualitative

Bacteria	Gram Reaction	Dissolution of Phosphate	Nitrogen Fixation
<i>Bacillus Niabensis</i> Strain PT-32-1	+	+	+
<i>Bacillus subtilis</i> Strain SWI16b	+	+	+

3.3. *Bacillus SPP* Antagonist Test Against wilt Tomato Bacteria *in vitro*

Antagonistic test results showed the presence of inhibition activity by *Bacillus spp* against pathogens wilt diseases in tomato plants. Inhibition ability is indicated by the formation of around clear zone. The size of the inhibition zone in each media can be seen in Table 3.

Table 3. Diameter of Halo Zone On Some Types of Media

<i>Bacillus spp</i>	Diameter of the Halo zone (mm)	
	Na	YPDA
<i>Bacillus niabensis</i> Strain PT-32-1	4.0	6.0
<i>Bacillus subtilis</i> Strain SW116b	4.0	6.5

Bacillus spp is a bad thing to various plant pathogens including pathogens of wilt diseases of tomato plants. After incubation is seen a clear zone and indicating that the isolate *Bacillus spp* used produces secondary metabolites capable of inhibiting or shutting down pathogens. The formation of halo zones indicates the presence of antibiosis mechanisms and the ability to produce antibiotics in *Bacillus spp* that can be used as bio controller agents. According to [15], *B. subtilis* can produce antibiotics as antibiosis mechanisms such as *Bacillomycin D* and iturin produced by *B. subtilis* AU195 and QST713.

One of the suppression mechanisms by strains of members of the genus *Bacillus* is antibiosis indicated by the formation of resistance zones in *Bacillus spp.* cultures that are grown on the medium in layers with pathogenic bacteria. Antibiosis is an antagonistic mechanism by producing secondary metabolites in the form of antibiotics or antibiotic-like compounds such as pelysis enzymes, volatile compounds, siderophores, and other toxic substances [16]

In Table 3. It seems to be a difference in the extent of the halo zones that are formed. It turns out that in the YPDA media inhibitory zone is greater than NA media. Differences in resistance zones in *Bacillus sp.* isolates are thought due to the condition and nutritional content of the media used. The compound and sugar content is contained in the media, that affects the formation of the halo zone. [17] explained that antibiosis compounds are strongly influenced by the composition of the medium, both quantitatively and qualitatively. But in principle, both isolates way show the ability to inhibit the growth of wilt pathogenic bacteria (*R. solanacearum*) in tomatoes.

4. Conclusion

Based on the results, *Bacillus spp* can suppress the development of *R. solanacerum* on tomatoes, so that it can be used as a biological agents.

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