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Isolation and Performance Testing of *Bacillus subtilis* As Biological Agents to Control the Diplodia Disease on Siam Citrus

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Abstract. This study aims to determine the feasibility of the *Bacillus subtilis* isolate as a biological agent and the concentration of bacterial cells capable of controlling Diplodia in the Siamese citrus (*Citrus nobilis*). Isolation and biological agency testing on soil samples taken from horticultural land were carried out at the UMSIDA Microbiology Laboratory. The trial of the biological agency application was carried out in Talok Village, Dlanggu District, Mojokerto Regency. The application experiment was carried out in a randomized block design, with treatment in the form of bacterial cell concentrations in the application volume consisting of no biological agents, 10^{-7}, 10^{-8}, and 10^{-9} CFU.ml^{-1} in 100 ml. The experiment was repeated five times in order to obtain 20 experimental units. The experimental data were analyzed by ANOVA at 5% followed by the 5% HSD test. The results showed that the bacterial isolates of *B. subtilis* were found to be potential biological agents with the white rhizoid-shaped colony and dry surface, gram-positive, and non-pathogenic in nature. Biological agent *B. subtilis* with a cell concentration of 10^{-8} CFU.ml^{-1} in 100 ml of application solution gave the best effect to reduce the intensity of Diplodia disease in *C. nobilis*.

1. Introduction

Various obstacles are encountered in citrus cultivation activities and efforts to increase the productivity of citrus crops; one of them is disease attack disorder. The Diplodia disease in citrus plants that attacks the stems and branches is caused by the fungus *Botryodiplodia theobromae*. This pathogenic fungus in Taiwan attacks Kumquat (*Fortunella margarita* (Lour.) Swingle) citrus orchards with an attack area of up to 80% [1]. In Indonesia, the incidence of disease in citrus plants, including the Siamese orange (Citrus nobilis var. microcarpa Hassk), which is the most widely cultivated citrus cultivar, is able to reach 90% [2]. This diplodia disease causes necrotic symptoms on the twigs, branches and stems, gum exudation, and the peeling of the bark and looks like rotting. In humid and warm conditions and in high rainfall conditions, Diplodia will develop optimally [3].

Many citrus farmers have taken control efforts to make use of synthetic chemical pesticides based on active sulfur ingredients mixed with limestone. However, so far the use of toxic sulfur-based chemical compounds is considered less effective in controlling Diplodia because the disease often recurs. The use of other chemical substances also shows low effectiveness, pesticide resistance, threatens the life of non-target organisms, and environmental pollution [4].

The alternative that is currently being campaigned by many parties is to use materials that are not synthetic chemical in nature. One of them is using the biological agent bacteria Bacillus subtiis.

Bacteria of this type can be obtained in an agricultural environment, but its characteristics as a potential biocontrol agent and its effectiveness need to be tested, considering that the *B. subtilis* species has a variety of strains with a diverse spectrum ranging from strong biocontrol agents to pathogenic ones. Volatile organic compounds (VOCs) produced by plant growth-promoting rhizobacterium (PGPR) have been shown to have the potential to control plant pathogens, stimulate plant growth, and induce systemic disease resistance [5-6], as well as increase the availability of N, P and Fe [7]. PGPR, or products derived from PGPR, usually requires physical contact with plant parts to stimulate plant growth [8]. The bacterium *B. subtilis*, which has strong characteristics as a biological agent, produces a variety of biologically active compounds with a wide spectrum of agronomic and industrial agricultural uses [9-10]. Besides being a biocontrol agent, it can be shown its ability to control several plant diseases. On the other hand, so far it is necessary to test the density of this bacterial cell population when applied in disease control; although the Attachment to the Regulation of the Minister of Agriculture No. 70/ SR.140/10/2011 states that the bacterial population according to the quality standard of *B. subtilis* with the type of liquid carrier is 108 CFU.mL^{-1}.

This study aims to determine the feasibility of the *B. subtilis* isolate as a biological agent and the concentration of bacterial cells capable of controlling Diplodia disease in the Siamese citrus (*Citrus nobilis*).

2. Methods

Isolation and Testing of Bacteria. The research activity began with exploration to find soil-borne bacterial isolates, which focused on bacteria that morphologically appear as *B. subtilis*. The sampling location was located in a horticultural planting area in Sumberbrantas Village, Batu, East Java at an altitude of 1,600 m above sea level. A total of 250 g of sample soil was put in a 1000 ml Erlenmeyer containing 500 ml of distilled water, then stirred evenly using a magnetic stirrer for five minutes and let stand 30 minutes. A total of 1 ml of suspension has been allowed to stand and dissolved in a test tube filled with 9 ml of sterile distilled water and vortexed; this condition is called the first dilution (10^-1). Furthermore, from the 10^-1 dilution, 1 ml of suspension was taken and put into a test tube containing 9 ml of sterile distilled water and stirred evenly with vortex, then a 10^-2 dilution was produced. A similar step is carried out until the final dilution is 10^-4. For the bacteria growing medium, Nutrient Agar (NA) was prepared made with ingredients consisting of 5 g of sodium chloride, 5 g of peptone, 3 g of yeast extract, 20 g of dry agar, and 1000 ml of distilled water. All ingredients are put in a beaker glass and added with distilled water up to 1000 ml and stirred evenly and heated for 30 minutes, then put in a media bottle and sterilized using an autoclave (1 atm, 121ºC) for 30 minutes. For the growth of bacteria from the sample suspension, a growth medium was prepared by pouring liquid NA medium aseptically into the petri dish in the incase. The next day, 0.5 ml of soil suspension resulted from the last dilution, which was taken using a disposable syringe, was sprayed onto the surface of the NA media in a petri dish, and leveled. After being incubated for 24-48 hours, observations were made of the emerging colonies.

All isolation processes and testing of potential bacterial isolates found as biological agents for disease control were carried out at the Laboratory of Biological Agents at the UPT Food Plants and Horticulture, Department of Agriculture and Food Security, Surabaya, East Java. The examination and determination of Siamese citrus plants showing symptoms of illness and the pathogen causing the disease were carried out at the UMSIDA Microbiology and Biotechnology Laboratory. All research activities are carried out in December 2019 - March 2020.

Identification of *B. subtilis*. Bacterial identification was carried out by observing the morphological characteristics of the colony and by testing the physiological and biochemical properties. Some of the test steps carried out were: (i) selective media test, namely, the purified bacterial isolates were grown on selective media (NA media) and incubated for 24-48 hours. Next, determine the shape, color, and texture of the colony; (ii) the KOH test is performed to differentiate between the tested bacteria, including gram-positive or gram-negative bacteria. Bacterial suspension placed on the preparation if it is not attached to the loop needle when removed shows the characteristics of Gram-positive Bacillus

subtilis; and (iii) Soft Rot test, conducted to differentiate pathogenic and non-pathogenic bacteria. Bacteria will be smeared on the potato and incubated for 24-48 hours to determine the presence or absence of rotten potato which will indicate the characteristics of the bacteria whether it is a biocontrol agent or a pathogen.

Biological Agensia Application Test. The determination of the test plants was carried out by observing the planting of Siamese oranges in the citrus plantation area of the farming community in Talok village, Dlanggu district, Mojokerto regency, East Java province. The sick Siamese citrus plant has been confirmed to have been attacked by the Diplodia stem rot disease caused by *Botryodiplodia theobromae*. through a series of Koch Postulates [11] tests with moderate attack symptoms.

For the application test, a propagule of the biological agent isolates *B. subtilis* was prepared which was formulated in a liquid medium to make it more durable. The storage of bacteria in liquid media is carried out by cutting into small pieces the isolate on the media so that it is then put in a small bottle containing sterile distilled water then closed tightly and shaken so that the suspension is evenly distributed. Storage is carried out at a temperature of 10-15°C. For the purposes of the application experiment, *B. subtilis* was carried out using a growing medium for sugar soybean extract (SSE). To make an SSE as much as one liter requires 200 grams of washed soybeans, boiled with a liter of distilled water, after boiling the soybeans is drained, then add 20 grams of sugar to the soy boiled water and heat it until it dissolves using low heat for five minutes. Then the soybean extract can be transferred to a storage bottle and ready to be sterilized in an autoclave for 30 minutes (1 atm., 121°C). After being sterilized, put the liquid bacterial isolate on the SSE as much as two bottles for three liters of the SSE. Then aerated for three days to reproduce aerobically. Furthermore, the liquid formulation suspension formed is ready to be used for experiments.

To determine the standard population, a suspension growth containing bacterial cells was carried out with multiples of 10 dilutions starting from 10^7 to 10^{11}. The colonies that emerged from each dilution were counted to determine the average population of bacteria stored as stock. Growth in culture using NA media resulted in a bacterial population with an average of 10^9 CFU.ml^{-1}. Determination of the bacterial cell population to suit the treatment was carried out by diluting it by adding a sterile aqua dest.

The affected part of the stem is cleaned first with a thin scrape with a knife (about one mm deep) to remove dead tissue on the surface of the stem bark. Furthermore, the bacterial cell suspension according to the treatment placed in a one-liter capacity hand sprayer is sprayed onto the surface of the stems which are sore until the entire surface of the stem is evenly wet. Then observed.

Experimental Design and Statistical Analysis. The experiment was arranged in a randomized block design (RBD) with treatment in the form of the application of the biological agent Bacillus subtilis 10-7 CFU.ml-1 with a bacterial cell concentration of 10^7 CFU.ml^{-1} (Bs1), 10^8 CFU.ml^{-1} (Bs2), 10^9 CFU.ml^{-1} (Bs3), and 10^0 CFU.ml^{-1} or control (Bs0). With six repetitions, 24 experimental units were obtained or 24 Siamese orange trees were symptomatic with Diplodia stem rot in the moderate attack intensity.

Observation data from the experimental application of biological agents obtained were analyzed using analysis of variance (ANOVA) and if the results of the analysis of variance were significantly and significantly different, the analysis was continued with the 5% BNJ test to determine the differences between treatments.

Symptom Recovery Observation. At the beginning of the observation, the measurement of the wound area was carried out by comparing the weight of the paper which is a model or image of a stem surface wound with the weight of standard paper, and multiplied by the area of the standard paper as stated in formula (1):

$$Ain = \frac{ainB}{b} \qquad \ldots\ldots\ldots (1)$$

with the following conditions: A = the area of the wound surface of the plant stem of the sample *i*; a*i* = the weight of the paper which is a printed image of the wound surface of the plant sample *i*; *n* = is the time of observation, namely 0, 1, 2, and 3; *b* = weight of a sheet of paper with standard size, B = area of a sheet of paper with a standard size.

The time to observe the initial wound area coverage was carried out six hours before the inoculation or spraying of the *B. subtilis* suspension according to the treatment and indicated by the symbol t0. The next observation time is seven, 14, and 21 days after application which is indicated by the symbols t1, t2, and t3.

Wound healing is characterized by drying the wound at the edges toward the center of the wound. In this case, the wound on the edge is relatively new, so that recovery occurs immediately at the edge, and recovery of the wound is characterized by the widening of the edge that dries inward or the center of the wound.

Furthermore, the percentage of recovery is determined compared to the initial wound area (t0) which is calculated by the formula (2):

$$\Delta P = [(Ai_{nt} - Ai_{n_0})(Ai_{n_0})^{-1}] \times 100\% \quad \text{............. (2)}$$

with the following conditions: ΔP = percentage of wound recovery, Ai = plant sample *i*, *nt* = observation week t, *n0* = initial observation.

3. Results and Discussion

Potential Biocontrol Agent.   The results of the exploration for biological agents obtained two isolates on the NA media. Based on observations, it was obtained the morphological characteristics of *B. subtilis*. The colonies are rhizoid-shaped, white in color, and flat dry surface. The KOH test results showed that the bacteria *B. subtilis* was gram-positive. The bacterial suspension was placed on the preparation then stirred using a loop needle, when it was removed it was not attached to the loop needle showing the characteristics of gram-positive *B. subtilis*. The soft rot test results showed that *B.subtilis* is a non-pathogenic bacterium because the bacterial propagules smeared on the surface do not rot the potatoes.

The whole test showed that the isolate of these findings was *B. subtilis* which has the potential as a biocontrol agent.

The results of the application of the biological agent *B. subtilis* to citrus plants that were attacked by diplodia, *B. theobromae*, showed a decrease in the intensity of the attacks which indicated that there was the relief of symptoms. The results of the analysis of variance showed that the application of *B. subtilis* at various concentrations of bacterial cell populations had a very significant effect ($p < 0.01$) on the recovery of symptoms of Diplodia stem rot on Siamese citrus. The mean recovery index 1-3 weeks after inoculation is shown in Figure 1. Meanwhile, the ability to heal wounds in the three types of treatment with the concentration of *B. subtilis* bacteria cells compared with without bacteria (B0) or self-recovery in plant tissue is shown in Table 1.
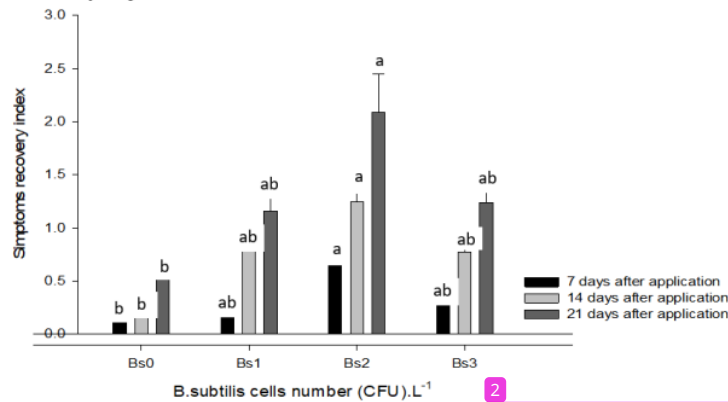

Figure 1. Results observed at 7, 14, and 21 days after planting. Different lowercase letters above the bars of the same color (same time of observation) showed a significant difference between treatments for the concentration of *B. subtilis* cells according to the HSD test ($p<0.05$).

Table 1. Power of symptom recovery compared to control (self-recovery) (%)

Bacterial cell concentration	Observation time		
	7 DAA	14 DAA	21 DAA
10^7 CFU.ml^{-1} (Bs1)	14.9	224.0	145.4
10^8 CFU.ml^{-1} (Bs2)	121.7	346.3	282.0
10^9 CFU.ml^{-1} (Bs3)	61.7	162.1	96.4

Discussion. Based on testing, especially by growing it on potato slices that show no decay, it can be ascertained that this *B. subtlis* isolate is a gram-positive bacterium that is not pathogenic bacteria. This non-pathogenic characteristic is thought to be related to the role of extracellular vesicles, which in gram-positive bacteria is associated with very low pathogenesis and antibiotic resistance, nutrient uptake, and transfer of nucleic acids [12].

The results of the test of pathogen control showed that the application of *B. subtilis* with a population density of 10^8 CFU.ml^{-1} showed the highest response of plants in the form of wound recovery at all observation times, namely $8.72 \pm 2.35\%$ (7 DAA), $19.97 \pm 3.07\%$ 14 DAA), and 36.30 (21 DAA) (Figure 1). The wound healing power in the treatment using this population density was 282.0% compared to the control (Table 1). From these experiments, it appears that the population density of *B. subtilis* that gives maximum results is at 108 CFU.mL-1. It is necessary to further examine whether the population with higher levels of competition between bacteria occurs in the *B. subtilis*-fungus interaction between plant pathogens. However, in the same niche, these bacteria have also developed a competitive mechanism for obtaining nutrient sources and space to control pathogens [13-14]. In the interaction system that occurs in plant tissue that is injured due to pathogen attack, it appears that the treatment of *B. subtilis* shows a high recovery power. This is inseparable from the role of these bacteria in producing volatile PGPR compounds which can help plants increase the rate of photosynthesis, the activity of auxin, cytokinins, and gibberellin but reduce ethylene [6; 8]; and helps increase the activity of catalase enzymes, peroxidase, peroxidase, and total sugar even under stressful environmental conditions [15]. Isolate *B. subtlis* in this experiment is able to suppress pathogens considering its ability to produce compounds that can kill pathogenic pathogens [16] including non-ribosomal cyclic lipopeptides [17] which are volatile organic compounds (VOCs). The characteristic of this compound is its inhibitory activity against target pathogens without direct physical contact [18].

The ability of *B. subtilis* to survive in the same niche in the wound tissue proves that this bacterium is able to develop mechanisms to adapt to changes that take advantage of the availability of food sources or protect itself from extreme environments [19].

With its ability to control pathogenic fungi well. hence the biocontrol agent *B. subtilis* can be used as an alternative chemical fungicide to control plant pathogens [20] including the pathogen that causes the Diplodia stem rot in this citrus crop. The pathogen of Diplodia is also soilborne, therefore combining its capabilities as a biofertilizer and biocontrol agent, isolate B. subtilis is also suitable to be used to improve soil quality [21].

4. Conclusion

The bacterial isolates of *B. subtilis* were found to be potential biological agents with the white rhizoid-shaped colony and dry surface, gram-positive, and non-pathogenic in nature. Biological agent *B. subtilis* with a cell concentration of 10^{-8} CFU.ml^{-1} in 100 ml of application solution gave the best effect to reduce the intensity of diplodia disease in *C. nobilis*.

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