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The Potential of *Bacillus subtilis* subsp. *subtilis* RJ09 as a Biological Control Agent Against Leaf Spot Diseases on Clove

Potensi *Bacillus subtilis* subsp. *subtilis* RJ09 sebagai Agens Pengendali Hayati Penyakit Bercak Daun Cengkih

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ABSTRACT

Cloves is one of the main plantation commodities and is native to Indonesia. Leaf spot disease with necrosis symptom is often found in clove plants. Interferences to photosynthesis caused by leaf spot disease can cause plant death. Bacillus is known to inhibit the development of pathogens through nutrient competition and antibiosis mechanism. This study aims to identify *Bacillus* sp. RJ09 through molecular approach and determine its potential in suppressing the development of *Pestalotiopsis* sp., the cause of clove leaf spot by *in vitro* examination. Identification of the fungus was carried out by observing the morphology which included the color of the colony, the shape and size of the conidium of the isolated fungus. Identification of *Bacillus* sp. RJ09 was performed by polymerase chain reaction (PCR) using a pair of primers that amplified gyrB gene region of ±1400 bp, followed by sequence analysis. The ability of *Bacillus* sp. RJ09 as a biological agent against fungi associated with leaf spot was determined using an *in vitro* double culture method. *Pestalotiopsis* sp. was found as the fungus associated with leaf spot disease and can cause black necrotic spots on the leaves. Molecular identification of *Bacillus* sp. RJ09 showed the highest similarity value of 99.40% with *B. subtilis* subsp. *subtitles*. *In vitro* dual culture showed *B. subtilis* subsp. *subtilis* RJ09 can inhibit the growth of *Pestalotiopsis* sp. colonies by 75%.

Keywords: antagonism, *gyrB, Pestalotiopsis,* sequencing

ABSTRAK

Cengkih merupakan tanaman asli Indonesia yang menjadi salah satu komoditas utama sektor perkebunan. Penyakit bercak daun berupa nekrosis sering ditemukan pada tanaman cengkih. Gangguan terhadap fotosintesis yang disebabkan oleh penyakit bercak daun dapat menyebabkan kematian pada tanaman. *Bacillus* diketahui dapat menghambat perkembangan patogen melalui mekanisme persaingan nutrisi dan antibiosis. Penelitian ini bertujuan mengidentifikasi *Bacillus* sp. RJ09 secara molekuler dan mengetahui potensinya dalam menekan perkembangan *Pestalotiopsis* sp., penyebab bercak daun tanaman cengkih secara *in vitro*. Identifikasi cendawan dilakukan dengan mengamati morfologi yang meliputi warna koloni, bentuk, dan ukuran konidium cendawan hasil isolasi. Identifikasi *Bacillus* sp. RJ09 dilakukan dengan *polymerase chain reaction* (PCR) menggunakan sepasang primer yang mengamplifikasi wilayah gen *gyrB* sebesar ±1400 pasang basa (pb) yang dilanjutkan dengan analisis sikuen. Pengujian kemampuan *Bacillus* sp. RJ09 sebagai agens hayati terhadap cendawan yang berasosiasi dengan bercak daun dilakukan dengan metode kultur ganda *in vitro*. Hasil identifikasi cendawan bercak daun tanaman cengkih diperoleh *Pestalotiopsis* sp. yang dapat menyebabkan bercak nekrosis berwarna kehitaman pada daun. Hasil identifikasi molekuler *Bacillus* sp. RJ09 menunjukkan nilai kemiripan tertinggi 99.40%

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dengan *B. subtilis* subsp. *subtilis*. Pengujian *in vitro* menunjukkan *B. subtilis* subsp. *subtilis* RJ09 dapat menghambat pertumbuhan koloni *Pestalotiopsis* sp. sebesar 75%.

Kata kunci: antagonisme, *gyrB, Pestalotiopsis,* sikuensing

INTRODUCTION

Clove (*Syzygium aromaticum*) is an important spice plant that is widely grown as a plantation commodity in Indonesia. Cloves are widely used by Indonesian people as an important spice for various purposes such as food, cosmetics, and other industries (Shofiana *et al*. 2015). Almost 95% of clove in Indonesia is cultivated in almost all provinces on a smallholder plantation scale. According to agricultural R&D data in 2015, the development of clove plantation area has experienced ups and downs over the last 20 years (Setiawan and Rosman 2015). One of the factors causing the decline in clove production is leaf spot disease caused by *Pestalotiopsis* sp. which was first detected in North Sumatra in 2016. The disease was airborne and spreads very quickly (Syamsafitri *et al.* 2021). This disease will cause necrotic spots on clove leaves which result in damage of leaf structure. Leaf necrosis can cause photosynthesis to be hampered so that the leaves wilt and trigger plant death. This problem causes a decrease in the amount of clove production (Wahyuno and Martini 2015). The decline in clove production caused by pathogen infection has a linear impact on decreasing farmer income. In turn, this causes losses for clove plantations in Indonesia (Diyasti and Amalia 2021).

Disease control commonly practiced today is the application of chemical pesticides. Continuous and unwise use of pesticides can have a negative impact on the environment, therefore better alternatives are needed to control biotic diseases in cultivated plants. Utilization of antagonistic microbes, e.g. *Bacillus* sp. is an environmentally friendly control option. *Bacillus* sp. as a biocontrol agent is known to have the ability to control biotic diseases directly and promote plant growth and resistance against the disease (Dwimartina *et al*. 2021).

Bacillus can inhibit the development of pathogens through the mechanism of competition for nutrients and antibiosis. The effectiveness of *Bacillus* in controlling diseases in various plants showed significant results. Sevirasari *et al*. (2022) reported that the application of *B. velezensis* together with *Trichoderma harzianum* and arbuscular mycorrhizal fungi in hot pepper showed more tolerant to Geminivirus infection. Likewise, Wulan *et al*. (2022) revealed that treatment with *B. velezensis* and *B. cereus* was able to suppress twisted disease on shallot by 72.2% to 100%. Kuswinanti *et al*. (2014), stated that antagonistic bacteria can function as pathogen control agents through competition, antibiosis, parasitism, or induced resistance mechanisms. The use of antagonistic bacteria to increase yields and protect plants from plant pathogens is a promising approach in modern agricultural systems.

The study aimed to identify *Bacillus* sp. RJ09 using *gyrB* sequence analysis and to determine its potential as biocontrol agent against *Pestalotiopsis* sp.

MATERIALS AND METHODS

Isolation of Fungi Associated with Leaf Spot Disease

Leaf samples with necrosis symptoms (dry brown and blackish brown) were collected from Samigaluh District, Kulon Progo Regency, Special Region of Yogyakarta. The leaves were surface sterilized using 96% ethanol for 1 minute, followed by 10% NaOCl for 10 minutes, and rinsed in sterile water for 10 minutes (Hutabarat *et al*. 2022). Leaf samples were then cut to a size of 1 × 1 cm before placed onto potato dextrose agar (PDA) medium. Incubation was carried out at room

temperature for 5 days. The growing fungal colonies were then made into pure cultures. Fungal characterization was carried out based on morphological observation which includes the color of colonies, the shape and size of conidia (Wahyuno and Martini 2015). After successful isolation, Koch's postulate was carried out using clove leaves to confirm that the isolated pathogen was the causative agent of leaf spot disease. The pure fungal isolate was inoculated directly by attaching the culture disk to clove leaves. The leaves were kept in homogeneous conditions and observations were made for 20 days.

Identification of *Bacillus* sp. RJ09

Bacillus sp. RJ09 isolate used in this study is provided from previous studies (Wisanggeni 2018). The isolate was cultured on casamino acid-peptone-glucose (CPG) medium, and incubated for 48 hours.

DNA extraction was carried out to obtain total genomic DNA of *Bacillus* sp. RJ09 using PROMEGA kit according to the protocol (Abdulla 2014; Abed 2013). DNA was amplified using *gyrB* specific primer, i.e. forward primer 5'-CCCAAGCTTAACTGCACTGGGAAATYGTHGAYAAYAG-3' and reverse primer 5'-CGGAATTCGGATCCACRTCGGCRTCBGTCATRAT-3' (Joko *et al*. 2019). PCR was run with the composition: 1 µL forward primer, 1 µL reverse primer, 5 µL master mix green, and 3 µL DNA *Bacillus* sp. RJ09. PCR was set at 95 °C for 3 minutes for initial denaturation, then continued for 34 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 minute. PCR was finished with the final temperature at 72 °C for 5 minutes and hold at 12 °C.

PCR products were analyzed using electrophoresis on 2% agarose gels which were prepared by adding 24 mL 1x TBE and 0.24 g agarose gels (Aksoy *et al*. 2021). DNA marker of 1 kb ladder was used for the measurement of amplified DNA at a target of 1400 bp. Electrophoresis was carried out at 70 volts for 50 minutes and DNA bands obtained were visualized under trans-ultraviolet illuminator (Trianom *et al*. 2019).

The DNA amplicon of *Bacillus* sp. RJ09 was then sent to First Base, Malaysia for nucleotide sequencing. Identification was carried out using *gyrB* primer-based molecular analysis (Hidayat *et al*. 2019; Rahma *et al*. 2020). Sequencing data were then edited using BioEdit software and analyzed using the Blast-N program from the *National Center for Biotechnology Information* website (https://www.ncbi.nlm.nih.gov/). Identification of molecular sequencing was carried out to determine the similarity of *Bacillus* sp. RJ09 with data on GenBank (Jayanti and Joko 2020). Alignment and phylogenetic analysis were performed using MEGA 10.1.8 software. The phylogenetic tree was constructed using neighbor-joining method with bootstrap analysis of the 2-parameter Kimura method and 1000 times replication.

***In vitro*-Antagonism Assay**

The assay was conducted to determine the ability of *Bacillus* sp. RJ09 to inhibit the growth of pathogen. The inhibition test was carried out using dual culture method (Sakunyarak and Satithorn 2014). *Bacillus* sp. RJ09 was grown simultaneously with isolated pathogenic fungi in PDA medium. Antagonist inhibition rate was measured using the formula:

$$I = \frac{(r1 - r2)}{r1} \times 100\%, \text{ with}$$

I, Antagonist inhibition rate (%); r1, radius of the fungal pathogen colony opposite to the center of the antagonist colony; and r2, radius of the fungal pathogen colony towards the center of the antagonist colony (Figure 1).

RESULTS

Identity of Fungal Pathogen Causing Clove Leaf Spot

Blackish-brown necrosis symptoms were observed on clove plants (Figure 2a). Necrotic spots can spread over the entire leaf and cause the leaves to wilt and fall prematurely.

Fungal colonies were obtained on CPG media after 13 days-incubation period with white morphology and diameter reached ±85 mm (Figure 2b). Microscopic observation shows the presence of conidia which are oblong in shape and rather blunt at the ends, the conidia consist of 5 cells separated by septum and have 3 whips at the ends of the conidia. The spores of *Pestalotiopsis* sp. have a size of 17.19 µm from apical to basal cell and have brown color in the median cell (Figure 2c).

The results of Koch's postulates showed the presence of typical symptoms caused by the fungus *Pestalotiopsis* sp. (Figure 3). Black-brown spots began to appear on the 14th day

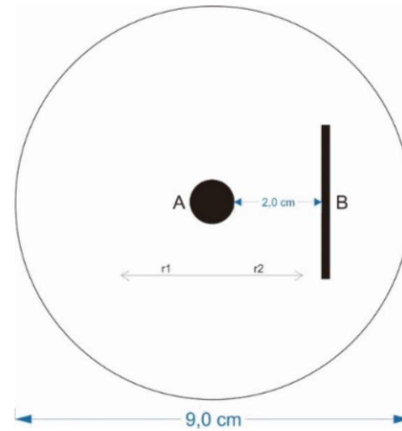

Figure 1 Illustration of antagonist test by *in vitro* dual culture. a, fungal pathogen isolate and b, bacterial antagonist.

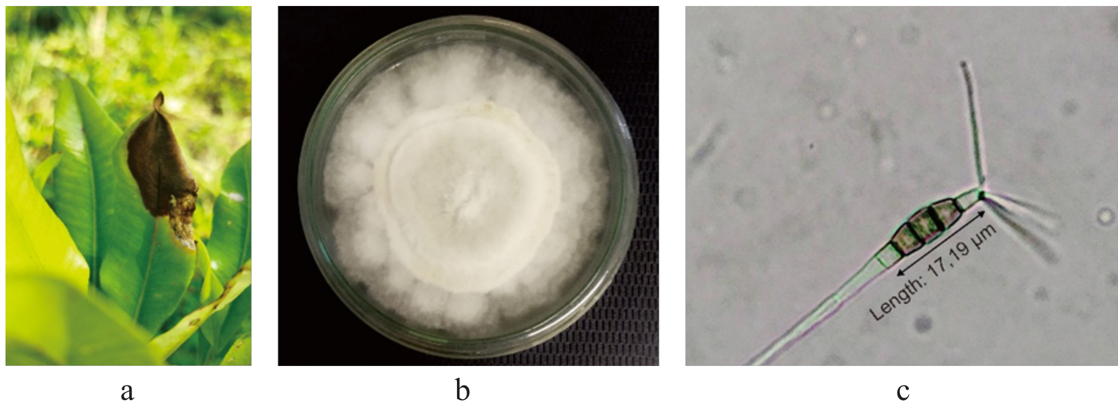

Figure 2 Symptoms and morphological characteristics of *Pestalotiopsis* sp. a, dark brown spots on clove leaves caused by *Pestalotiopsis* sp.; b, colony of *Pestalotiopsis* sp.; and c, conidia of *Pestalotiopsis* sp.

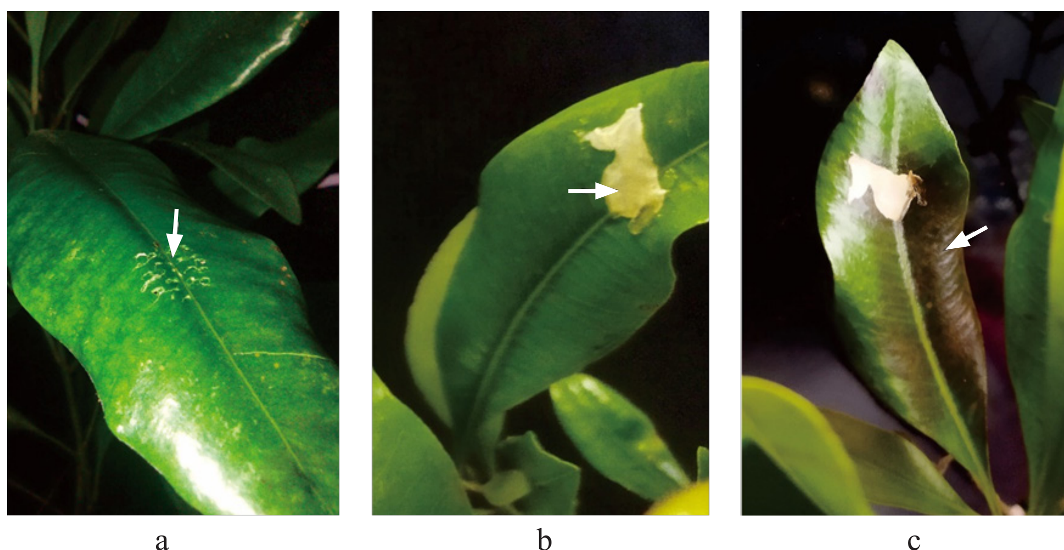

Figure 3 Koch's postulate showed that *Pestalotiopsis* sp. can cause infection on clove leaves. a, needle puncture (control) after 20 days; b, 0 days after inoculation; c, 20 days after inoculation. White disk on leaf showed application of the pure isolate of *Pestalotiopsis* sp., while brown lesions showed the presence of typical symptoms caused by the fungus *Pestalotiopsis* sp.

after inoculation (Figure 3b). Enlarge leaf spots was observed on day 20 after inoculation (Figure 3c); while in negative control treatment no symptoms was developed on leaf samples (Figure 3a).

Identity of *Bacillus* sp. RJ09

Amplification using *gyrB* specific primer was successfully amplified a ±1400 bp DNA band (Figure 4). Sequence analysis using BLAST-N program showed a similarity of up to 99.40% with *B. subtilis* subsp. *subtilis* strains: UCMB5021, N2-2, and SSJ-1 in the Genbank data. *Pseudomonas fluorescens* is involved as a comparison of the outgroup of *Bacillus* spp. This shows that *Bacillus* sp. RJ09 belongs to species group *B. subtilis* subsp. *subtilis* (Figure 5).

Inhibition of Fungal Pathogens by *Bacillus subtilis* subsp. *subtilis* RJ09

Dual culture test showed the inhibition of *B. subtilis* subsp. *subtilis* RJ09 against the pathogenic *Pestalotiopsis* sp. Observation on day 12 showed less growth of *Pestalotiopsis* sp. with an antagonist inhibition rate of 75% compared to that of control treatments (Figure 6).

DISCUSSION

The observation of conidia followed by Koch's postulate reveals that these symptoms are caused by *Pestalotiopsis* sp. i.e., characterized by the presence of light brown to dark brown spots, and spots that are round or irregular in shape (Suharti and Kurniaty 2013). Initial infection on the leaves is shown by small spots with yellow, brown, or black colors that develop to become gray with black circular edges. Semangun (1991)

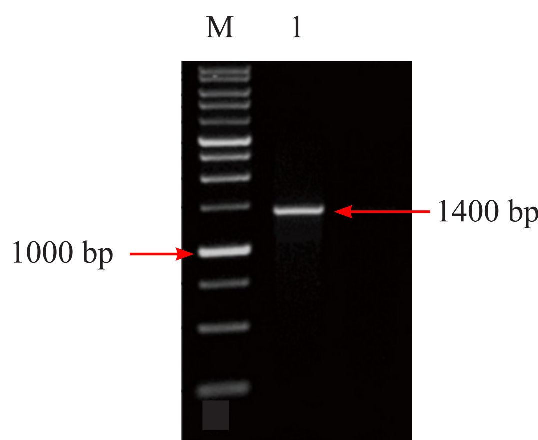

Figure 4 Visualization of PCR amplification using *gyrB* specific primers. M, DNA marker 1000 bp; 1, sample DNA of *Bacillus* sp. RJ09.

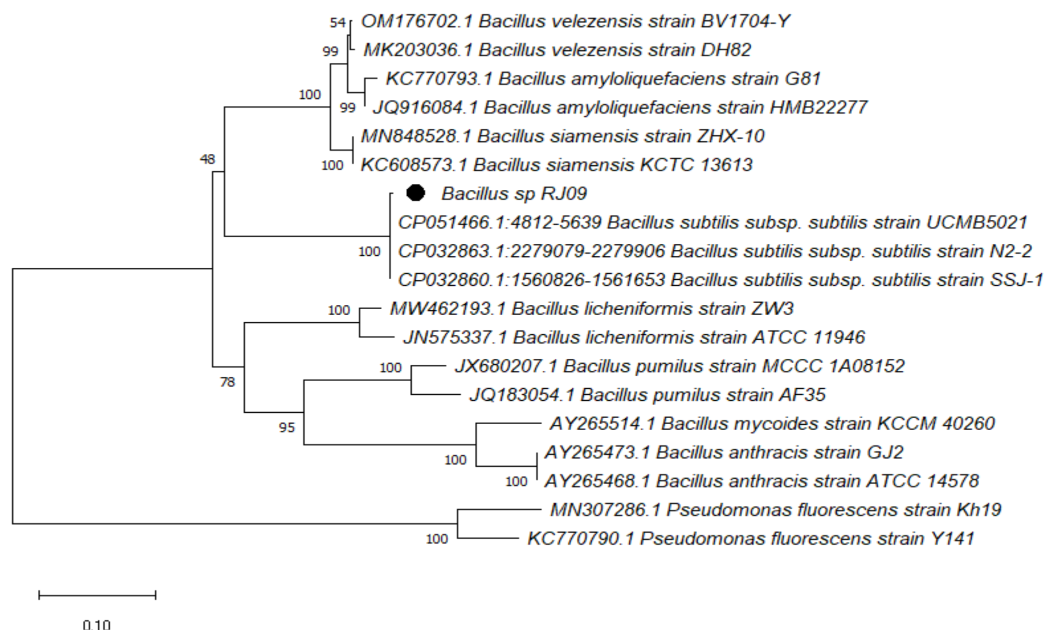

Figure 5 Phylogenetic tree constructed using the neighbor-joining method showed the relationship among *Bacillus* sp. RJ09 with other *Bacillus* spp. from GenBank data based on the *gyrB* gene.

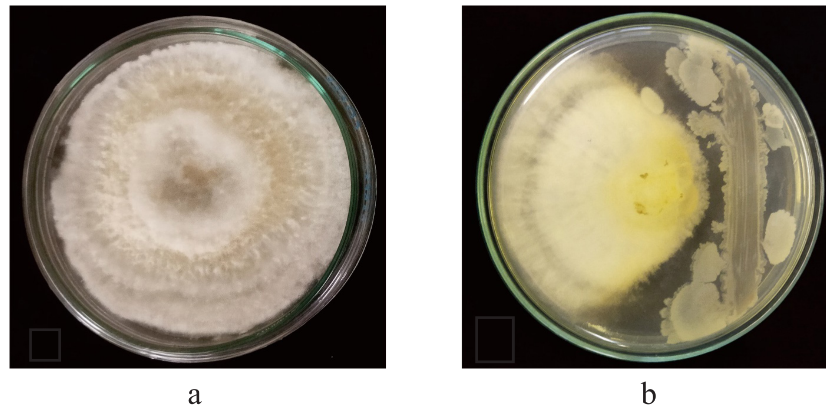

Figure 6 Dual culture assay between *Bacillus subtilis* subsp. *subtilis* RJ09 and *Pestalotiopsis* sp. were observed 12 days after inoculation. a, control of *Pestalotiopsis* sp.; b, *Bacillus subtilis* subsp. *subtilis* (right) and *Pestalotiopsis* sp (left).

reported symptom caused by infection of *Pestalotiopsis* sp. on clove plants as small brown spots on the tips of old leaves. Hadiwijaya (1981) explained that *Pestalotiopsis* sp. can infect clove leaves that have been previously infected by *Cylindrocladium* sp. and live as a secondary pathogen in the center of the leaf spot. This is supported by Ivanova (2016) which stated that spores of *Pestalotiopsis* sp. have a size of 16.8–20 µm. The three central cells are dark brown with short blunt, hyaline, thin-walled basal cells. The identification of *Pestalotiopsis* species is mainly based on the pigment color of the conidia cells (Jeewon *et al*. 2003). Semangun (1991) explained that *Pestalotiopsis* sp. has a 5-celled conidium, with 3 cells in the middle, dark and thick-walled, and the tip of the cell has 2–3 long setae. According to Hu *et al*. (2007), pigmented cells are pale brown; while Steyaert (1949) reported that conidia are usually straight or slightly curved. Pitt and Hocking (1997) explained that mycelium of *Pestalotiopsis* sp. are white and sometimes grayish-white to pale brown.

Bacillus is known to have *gyr*B gene which plays a role in encoding the B subunit protein of DNA gyrase (*topoisomerase type* II) which regulates the supercoiling of DNA double strands. The *gyrB* gene is indispensable for DNA replication where this gene plays a role in the formation of a protein that encodes the *gyrase* enzyme, and the enzyme is distributed universally among bacterial species (Kadriah *et al*. 2013). In our study the phylogenetic tree analysis was carried out by providing several species from the genus *Bacillus* (Wahida *et al*. 2016) which include *B. pumilus*, *B. anthracis*, *B. mycoides*, *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. siamensis*, *B. velezensis*, and *B. amyloliquefaciens*.

The observations of the inhibition of *B. subtilis* subsp. *subtilis* RJ09 against the *Pestalotiopsis* sp. shows 75%, which might be due to the antibiosis from *B. subtilis* subsp. *subtilis* RJ09. This suggested that *B. subtilis* subsp. *subtilis* RJ09 is thought to be able to produce certain antifungal metabolites that can inhibit the growth of *Pestalotiopsis* sp. It is suggested that *B. subtilis* subsp. *subtilis* RJ09 can be categorized as antagonist bacteria that potentially control *Pestalotiopsis* sp. on clove plants. According to Arwiyanto *et al*. (2007), apart from antifungal compounds, *Bacillus* sp. can also produce compounds that are bacteriostatic, which can inhibit the growth of pathogenic bacteria. Vespermann *et al*. (2007) suggested that groups of bacteria antagonistic to plant pathogens are capable of producing volatile compounds. Volatile compounds function as antibiotics that can inhibit the growth of pathogenic fungi. Compant *et al*. (2005) explained that *Bacillus* sp. produces antimicrobial substances such as *bacteriocins*. *Bacteriocins* are polypeptide or protein antimicrobial substances produced by microorganisms that are bactericidal. The *bacteriocins* kill target cells by inserting into the target membrane so that the function of the

cell membrane becomes unstable and the cell undergoes lysis.

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