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ISOLATION AND CHARACTERIZATION OF BACTERIA FROM SHALLOTS (*Allium cepa* L.) AS IN-VITRO BIOCONTROL AGENT OF *Fusarium oxysporum* f.sp *cepae*

ISOLASI DAN KARAKTERISASI BAKTERI ASAL BAWANG (*Allium cepa* L.) SEBAGAI AGEN BIOKONTROL IN-VITRO *Fusarium oxysporum* f.sp *cepae*

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Abstract

Shallot is one of the leading vegetable commodities with many benefits such as for seasonings and herbal medicinal ingredients. The demand for shallots continues to increase; however, shallot production is still relatively low. One of the limiting factors causing low shallot production is due to wilt disease caused by *Fusarium oxysporum* f.sp *cepae* (Foc). Bacteria have many roles in suppressing the growth of Foc, and this study aims to obtain potential bacterial isolates from the shallot plant to inhibit the growth of Foc Based on fungal diameter zone inhibition, degree of inhibition, and chitinase test, it was obtained 9 isolates which could suppress the growth of Foc. The results indicated that the AB3, TB2, and UB1 bacterial isolates could inhibit the growth of Foc with a percentage of inhibition of 46.80; 40.24; and 35.11%, respectively. The analysis showed that AB3, TB2, and UB1 isolates were categorized as moderate in suppressing the growth of Foc. The 16S rRNA sequencing results showed that AB3 and TB2 isolate had similarities with *Bacillus subtilis* by 99,75%, and 100%, respectively, while UB1 isolate had similarities with *Pseudomonas nitroreducens* by 89,35%. Based on the result showed that *Bacillus* sp. AB3 and TB2 isolates, and *P. nitroreducens* UB1 isolate have more potential as biological control agents to control the *Fusarium* wilt at in vitro assay. The field efficacy studies on these potential antagonists need to be done in the future.

Keywords: *Fusarium oxysporum* f.sp *cepae*; Percentage of inhibition; *Pseudomonas nitroreducens*; Shallot

Abstrak

Salah satu faktor pembatas yang menyebabkan rendahnya produksi bawang merah adalah penyakit layu yang disebabkan oleh Fusarium oxysporum f.sp cepae (Foc). Bakteri antagonis memiliki banyak peran dalam menekan pertumbuhan Foc, dan penelitian ini bertujuan untuk mendapatkan isolat bakteri antagonis potensial asal tanaman bawang merah untuk menghambat pertumbuhan Foc. Berdasarkan nilai zona hambat diameter jamur, derajat hambat dan uji kitinase, diperoleh 9 isolat yang dapat menekan pertumbuhan Foc. Hasil penelitian menunjukkan bahwa isolat bakteri AB3, TB2, dan UB1 mampu menghambat pertumbuhan Foc dengan persentase penghambatan masing-masing sebesar 46,80; 40,24; dan 35,11% dengan kategori penghambatan pertumbuhan Foc moderat. Hasil sekuensing 16S rRNA, menunjukkan bahwa isolat AB3 dan TB2 memiliki kemiripan dengan Bacillus subtilis masing-masing sebesar 99,75%, dan 100%, sedangkan isolat UB1 memiliki kemiripan dengan Pseudomonas nitroreducens sebesar 89,35%. Berdasarkan hasil penelitian menunjukkan bahwa Bacillus sp. isolat AB3, TB2, dan P.nitroreducens isolat UB1 berpotensi digunakan sebagai agen pengendali hayati untuk mengendalikan penyakit layu Fusarium pada uji in vitro. Studi kemanjuran lapangan terhadap isolat antagonis potensial ini perlu dilakukan di masa depan.

***Kata kunci**: Bawang merah; Fusarium oxysporum f.sp cepae; Pseudomonas nitroreducens; Persentase hambatan*

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INTRODUCTION

Shallot (*Allium cepa* L.) is one of the leading horticultural commodities in Indonesia due to its economic value. Based on data from the Food and Agriculture Organization (FAO) in 2009–2013, Indonesia ranks fourth after New Zealand, France, and the Netherlands as shallots exporters in the world *(Pusat Data & Sistem Informasi Pertanian, 2016)*. The shallot production centers in Indonesia are spread across the provinces of Central Java, East Java, West Java, and West Nusa Tenggara. The growth rate of shallot production in 2010–2019 was 4.10% per year (Adiyoga, 2020).

The low rate of shallots production can be caused by an unfavorable environment such as an unstable climate, as well as attacks by pests and diseases. Diseases often found in shallots are "moler" disease or Fusarium wilt caused by *Fusarium oxysporum* f.sp *cepae* (Foc) (Nugroho, 2013). At the initial attack of Foc infection of the roots or stems bordering the soil surface, and then the transportation of nutrients and water is blocked so that the plants become wilt. The yellowing leaves symptoms tend to be twisted (Sumartini, 2012).

Foc has a wide host range and can move from one plant to another through soil or air (Semangun, 2004). Foc enters plants through wounds caused by nematodes, insects, or agricultural implements. Plant infection can occur through the roots which is initiated by colonization of the outer part of the roots in secondary root extension. Colonization continues to occur in the intracellular space in the cortex tissue and extends to the parenchyma tissue, then invades the xylem vessels.

Various efforts have been made to control pathogenic bacteria, including using certified seeds, crop rotation, using high-yielding varieties, or stockpiling crop residues after harvest mechanical means i.e., by removing symptomatic plants. However, this method is not optimal in suppressing the development and spread of the disease. Likewise, control by using pesticides, in addition to leaving residues, can also cause environmental pollution (Sumartini, 2012). Thus, other methods that are more effective and environmentally friendly are needed through the use of antagonistic microorganisms as an important alternative in reducing the use of synthetic fungicides.

The use of antagonistic microorganisms is getting more attention because they can be used as environmentally friendly biological control agents. Indonesia has high biodiversity, including microorganisms, so it has the opportunity to obtain antagonistic microorganisms that can suppress the growth of other microorganisms, especially those that are pathogenic. Bacteria are a group of soil microorganisms capable of producing many secondary metabolites and antifungal compounds. These compounds include streptomycin, neomycin, tetracycline, and nystatin (Madigan et al., 2003). The ability of bacteria, especially *Streptomyces* to produce antibacterial compounds, has been used in agriculture to control various pathogens. Hwang et al. (2001) reported that *Streptomyces humidus* was able to inhibit the pathogens *Phytophthora capsici* and *Pseudomonas* sp., by producing phenylacetic acid and sodium phenyl acetate compounds. Cook and Baker (1996) stated that the use of *Streptomyces* would be more effective than the use of chemical compounds in suppressing pathogen attacks. Experiments in the field proved that giving 10^6–10^7 cfu/mL of *Bacillus subtilis* to barley seeds attacked by *Rhizoctonia solani*, *Pythium* spp., and Foc could increase yields by 9% and harvest time 2 weeks earlier. In wheat seeds, *Streptomyces griseus* can also increase yields up to 40%, and in carrot seeds 48%. This increase was due to the ability of *Streptomyces griseus* to inhibit the growth of *Rhizoctonia solani* via the antifungal compounds production.

Some bacteria i.e., *Pseudomonas fluorescens* and *Bacillus* sp. have been used to control *F. oxysporum* f.sp. *gladiolus* on gladiolus plants (Soesanto et al., 2008). According to research by Shu-Mei et al. (2008) the bacteria *Bacillus* sp. Isolated from soybean plant was able to inhibit the growth of *F. oxysporum* fungi on soybean by 80.2–96.7% under in vitro assay. Sajili et al. (2018) stated that *Bacillus* sp. and *Pseudomonas* sp. bacteria were able to inhibit the in vitro growth of *F. oxysporum* fungi of rockmelon. Santoso et al. (2007) stated that *P. fluorescens* P60 bacteria could suppress the intensity of *F. oxysporum* in shallots by 41.96%. Djatnika (2012) also stated that the bacteria *Pseudomonas* spp. able to control wilt on *Phalaenopsis* orchids caused by *F. oxysporum*.

Based on the above-mentioned description, research on bacterial isolation that is effective in suppressing the growth of Foc in shallots as a biological control agent still needs to be done. To date, bacterial isolates from Brebes as the control of moler disease in shallots have not been widely reported. Thus, it is important to screen for bacteria that are antagonistic to bacterial wilt disease in shallots. The research was conducted to isolate the bacteria from Brebes, Central Java. This location was chosen due to the reason that farmers in Brebes have been complaining for a few years about an increase in the severity of the "moler" disease. It has been reported the severity may reach up to 60% and yield losses >40% (Supyani et al., 2021). Hence, the objectives of the study were to screen for bacterial isolates from these shallot fields and to obtain bacteria that were antagonistic to Foc. The research is expected to gain isolates that are effective in inhibiting the growth of Foc fungi. In addition, the data obtained can be used as supporting data for the production of microbial-based biopesticides.

MATERIALS AND METHODS

Materials used in this study were Nutrient Agar (NA) and Potato Dextrose Agar (PDA) medium, colloidal chitin, chlorampenicol, congo red 0.3 %, NaCl, 70% ethanol, agar powder, Schales reagent, PBS pH 7 (10 mM), N-asetil-D-Glukosamin, Promega go taq green master mix, 16S primer pairs (63 forward, 387 reverse), RNase solution, protein precipitation solution, isopropanol, DNA rehydration solution, nuclease-free water, gelred nucleic acid gel stain 10,000x, 100 bp plus DNA ladder, blue juice loading dye, shallot (*Allium cepa* L.) variety "Bima curut" from Brebes, and Foc-fungi culture from diseased shallot plants at ICABIOGRAD. Microscope (Olympus), microcentrifuge Sorvall Legend Micro 21R, UV-Vis spectrophotometer U-2800, ESCO-PCR thermal cycler, and orbital shaker MESH 30.

Isolation of Bacteria from Shallot (*Allium cepa* L.) and Purification of Bacteria

Bacterial isolation was taken from the leaves, tubers, roots, and soil of shallot plants from Brebes. The bacterial isolation procedure refers to Mostert et al. (2001). In brief, the leaves, bulbs, and roots from representative shallot plants were taken. Samples from the leaves, bulbs, and roots of shallots were cleaned using running water and then sterilized using 70% ethanol for 1 min, after that the samples were immersed in 3% NaOCl solution for 2 minutes, then soaked in 70% ethanol solution for 20 sec and rinsed. using sterile distilled water three times. The clean samples were dried using sterile filter paper for 1–3 h. The sample was cut into 4 parts measuring 1 x 1 cm^2 and then placed on the surface of the NA medium. The culture was incubated at room temperature for 7 days.

Bacterial isolation was also carried out on the soil of the shallot plant which had been taken 10 g and put into a glass beaker then given sterile distilled water and homogenized using a vortex for 15 minutes to obtain soil extract. The results of the homogenized soil extract were then diluted 10^{-2} to 10^{-6}. The extract from each soil sample at the 10^{-4}, 10^{-5}, and 10^{-6} dilutions was taken 1000 µL using a micropipette and dripped into a petri dish that already contained NA media and then flattened using a triangular stirring rod to dry.

The petri dish was placed upside down and incubated at room temperature for 7 days. Colonies growing with different morphological forms were taken and purified on slanted NA media. The culture was incubated at room temperature until the colonies were colored and slimy.

In Vitro Antagonistic Assay of Bacterial Isolates on PDA Media

The antagonist activity test in vitro was carried out using the dual culture test method. Bacterial isolates were grown simultaneously with pathogenic fungi on PDA media in the same petri dish. The culture was incubated at room temperature and the inhibitory activity was determined based on the size of the inhibition zone formed around the colony. The inhibition zone was observed daily (Suryanto et al., 2011). The parameters observed and the fungi inhibition test (inhibition zone diameter) in this study refer to Venkataramanamma et al. (2022).

The inhibition test of the fungi was carried out by observing the growth of the fungi. The zone of inhibition will be seen as a clear area around the area containing antimicrobial substances. The diameter of the fungal growth inhibition zone indicates the sensitivity of the fungus to anti-fungi

substances produced by bacteria. The percentage of Foc fungi inhibition by antagonistic bacteria can be calculated using the formula Melysa et al. (2013), PRIG= R1-R2/R1 x 100%. Where PIRG= percentage inhibition of radial growth (% inhibition), R1= mycelium diameter of the control Foc fungi (cm), R2= diameter of mycelium of fungi Foc on antagonist test (cm). Based on the results of the percentage inhibition of the diameter of the fungi inhibition zone obtained, the classification of anti-fungi activity can be determined by following Mori et al. (1997), percent inhibition (PI) 75= very strong ; 75 PI <50= strong ; 50 PI <25= medium ; 25 PI <0= weak ; 0=inactive.

Chitinase Assay

A simultaneous study was done to determine chitinase. The quantitative chitinase assay is useful by measuring the absorbance value of chitinase in each bacterial isolate using a spectrophotometer. The assay was carried out in duplicate. The assay was started by inserting 10 mL of liquid chitin media into a screw tube, then one loopful of bacterial isolate from the inclined NA medium was transferred into the screw tube and incubated in an orbital shaker (MESH 30) for 48 hours at a speed of 120 rpm until cloudy. The culture was centrifuged at 10,000 rpm for 10 minutes at 4 °C. The resulting supernatant was taken 150 µL as crude extract and then 150 µL of PBS pH 7 (10 mM) and 300 µL of 0.3% colloidal chitin homogenized using a vortex and incubated in a water bath for 30 minutes at 37 °C (Suryadi et al., 2013). After incubation, the mixture was centrifuged (Sorvall legend micro 21R) at 5,000 rpm for 5 minutes. The 500 µL of supernatant was taken and then put into a test tube of 500 µL of distilled water and 1,000 µL of Schales reagent. The mixture and the standard solution were boiled at 100 °C for 10 minutes and then cooled to test the chitinase activity using the Spindler (1997) method. One unit of chitinase activity is the number of enzymes that produce 1 mol of reducing sugar which is equivalent to GlcNAc per minute (Green et al., 2005). Cuvettes and spectrophotometry were prepared to measure absorbance values. The first step is to make an auto zero value using distilled water, then measure the blank using 1,000 µL of distilled water and 1,000 µL of Schales reagent at a wavelength of 620 nm (UV-Vis spectrophotometer U-2800). The standard solution and all samples were measured for absorbance using a wavelength of 620 nm.

Identification of Antagonistic Bacteria

Morphological Identification

Bacterial isolates whose ability to suppress the growth of Foc fungus had been shown in in vitro tests were characterized by colony morphology including color, shape, edge, elevation, and gram staining.

Molecular Identification

Bacterial DNA Isolation, DNA Amplification with Polymerase Chain Reaction (PCR), Electrophoresis, and 16S rRNA Sequencing

The three isolates of the higher antagonistic bacteria in inhibiting the growth of Foc fungi were tested for 16S rRNA sequencing. Bacterial DNA isolation procedure refers to Kepel and Fatimawali (2015). Bacterial isolates were grown on Nutrient Broth (NB) media in screw tubes for 24 hours, then transferred to sterile Eppendorf tubes and centrifuged at 13.000 rpm for 5 minutes. The supernatant produced from the centrifuge process was removed without disturbing the white pellet and then centrifuged again at 13,000 rpm for 5 minutes. The supernatant obtained was discarded and added with 600 µL of nuclei lysis solution and then homogenized using a micropipette. The isolates were incubated in the freezer at -25 °C for 5 minutes. The DNA isolate was added with 3 µL of RNase solution homogenized using a micropipette, and incubated at 37 °C for 30 minutes.

The DNA solution was added with 200 µL of protein precipitation solution, then mixed and incubated in a freezer for 5 minutes. After incubation, the isolates were centrifuged at 13,000 rpm for 5 minutes. The supernatant produced from the centrifuge was transferred into a new sterile Eppendorf tube and 600 µL of isopropanol was added, then vortexed and centrifuged at 13,000 rpm

for 5 minutes. The supernatant resulting from the centrifuge process was discarded and the tube was dried for one hour. After the drying process, 50 µL of DNA rehydration solution was added to the DNA isolate. DNA isolates were stored in the freezer at -25 °C.

PCR mix solution containing primer 63 forward, primer 1387 reverse (Wardani et al., 2017), Promega go taq green master mix, nuclease-free water, and DNA template. The material was mixed into a sterile Eppendorf tube, after which 23 µL of the mixture was put into a PCR tube, and 2 µL of template DNA was added. The PCR follows the program protocol of the ESCO-PCR thermal cycler. PCR samples from the electrophoresis process that showed positive results were sequenced 16S rRNA at 1st Base (PT. Genetika Science Indonesia).

Data Analysis

The data from the observation of bacterial identification, observation of the zone of inhibition, and their percentage in the antagonist test were determined. The data obtained from the calculation of the inhibition zone were analyzed using the Statistical Package for the Social Science (SPSS) program ver 20 by conducting an independent t-test with a 5% significant level.

The analysis of the sequencing results was carried out by BLAST nucleotide sequences from the sequencing results with the database available on the website www.ncbi.nlm.nih.gov which was used to find the similarity of a nucleotide sequence with the database sequence (Sjafarenan et al., 2018)

RESULTS

Antagonistic Activity of Isolates Against Foc In Vitro

Effort to suppress the biological suppression of Foc fungi growth in this study was made by conducting an in vitro antagonism test between Foc fungi and antagonistic bacteria produced from healthy shallot plants as shown in Figure 1.

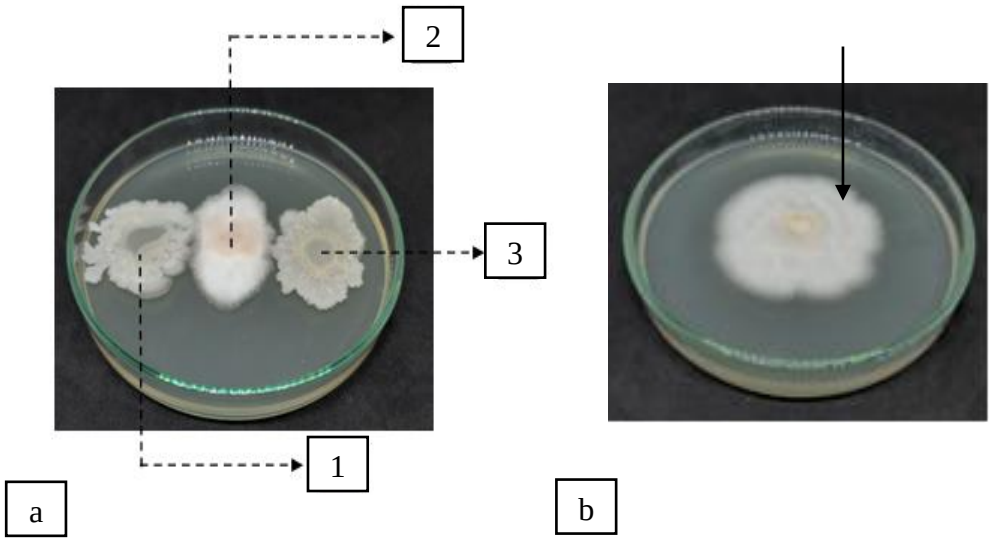

Figure 1. Representative bacterial isolates of DB4 (isolated from shallot leaves) at in vitro antagonistic assay against *Fusarium oxysporum* f.sp *cepae* (a); DB4 antagonist bacteria (1 & 3); *Fusarium oxyporum* f.sp *cepae* (2); and control *(Fusarium oxyporum* f.sp *cepae)*

There were 13 bacterial isolates in the antagonist test resulting from the bacterial isolation process on shallot plants. Three bacterial isolates came from the roots (AB1, AB2, and AB3), 4 isolates from the leaves (DB1, DB2, DB3, and DB4), 3 isolates from the tubers (UB1, UB2, and UB3), and 3 other isolates from the soil of shallot plants (TB1, TB2, and TB3). Foc diameter measurement data in the antagonist test showed that of the 13 bacterial isolates tested, only 9 bacterial isolates namely AB1, AB3, DB2, DB3, DB4, UB1, TB1, TB2, TB3 could suppress the growth of Foc. The average Foc diameter produced by the 9 bacterial isolates is presented in Figure

2. The lower average Foc diameter resulting from the antagonistic test was AB3 bacterial isolates of 1.27 cm, followed by TB2, and UB1 of 1.45 cm, and 1.56 cm, respectively.

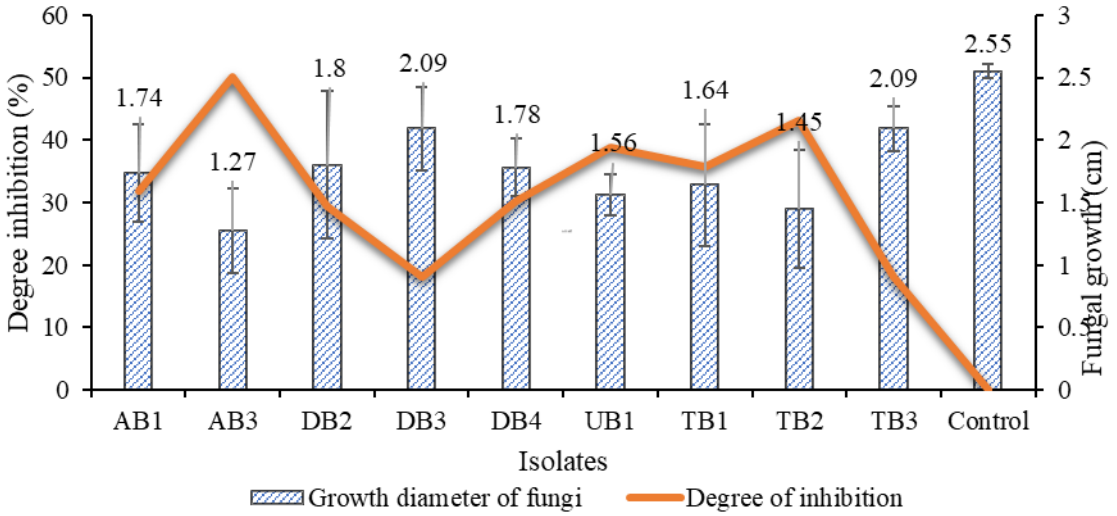

Figure 2. The growth of 9 isolates of bacteria against Foc in dual culture assay and degree of fungal inhibition percentage value at invitro test was calculated by formula DI= (T-C)/C x 100%, T= isolates treatment; C= control treatment

Nine isolates of antagonistic bacteria were able to inhibit the growth of Foc fungi as evidenced by the graph of the percentage of inhibition (Figure 2). The bacterial isolates had a range of inhibition percentages of 13.49–46.80%. The best percentage of Foc inhibition was produced from bacteria originating from the roots of the shallot plant, namely AB3 with an average percentage value of 46.80%, followed by bacteria from soil (TB2) and tubers (UB1) with average inhibition percentage values of 40.24% and 35.11%, respectively.

The results of the independent t-test test showed that there was a significant difference between the Foc diameter in the antagonist test and the control Foc diameter because it had a 2-tailed significance value <0.05 (Table 1).

The best 2-tailed significance value of 0.001 was produced by the Foc diameter in the antagonist test with soil bacterial isolates. The best 2-tailed significance value was then followed by Foc diameter in the antagonist test with bacterial isolates from roots, leaves, and tubers which had a 2-tailed significance value of 0.004; 0.013; and 0.041. The results of the 2-tailed significance value on antagonist bacterial isolates were in accordance with the average value of the percentage of inhibition Foc which showed that the average value of the highest percentage of inhibition Foc was found in isolates of antagonistic bacteria from soil with a percentage of 29.64%.

Table 1. Independent t-test

Origin of isolates from plant parts	Isolates	Independent sample t-test			
		Mean	St.dev	Sig 2-tailed	signif
Root	AB1, AB2, AB3	2.030	0.909	0.004	*
Leaf	DB1, DB2, DB3, DB4	1.970	0.861	0.013	*
Bulb	UB1, UB2, UB3	2.030	0.909	0.041	*
Soil	TB1, TB2, TB3	1.731	0.850	0.001	*

Note: *= significant at P <0.05

Morphology Characteristics Antagonist Bacteria

The morphological characters of antagonistic bacteria from roots, leaves, tubers, and soil on shallots showed varied characteristics. This can be seen in the macroscopic characterization of bacteria such as different colors, shapes, edges, and elevations (Table 2).

Isolates of antagonistic bacteria were taken randomly by taking into account the different morphology during the process of isolation of bacteria on NA media. Each isolate showed a different type of colony based on its macroscopic and microscopic characteristics. These differences

may be due to environmental factors and the origin of the isolates. Seven isolates of bacteria had circular colonies, and 2 isolates of bacteria with irregular shapes where 5 isolates had lobed edges, 3 isolates had rounded edges (entire), and 1 isolate had curled edges with colony elevation shaped like a crater (flat, raised margin), convex, flat, raised, curved (plateau), and hilly (umbonate). There were 5 isolates of antagonistic bacteria which were classified as gram-positive bacteria, namely AB3, DB2, DB4, TB2, and TB3 while the other 4 isolates were classified as gram-negative bacteria, namely AB1, DB3, UB1, and TB1.

Table 2. Morphology characteristic of bacterial antagonist

Isolates	Morphology				
	Color	Shape	Margin	Elevation	Gram reaction
AB1	White	Circular	Lobate	Flat, raised margin	−
AB3,DB4	White	Circular	Entire	Convex	+
DB2	White	Circular	Curled	Flat	+
DB3,TB1	White	Irregular	Lobate	Raised	−
UB1	White	Circular	Lobate	Plateau	−
TB2	White	Circular	Entire	Umbonate	+
TB3	White	Circular	Lobate	Convex	+

Quantitative Chitinase

Chitinase activity data on quantitative chitinase testing of 9 isolates of antagonistic bacteria showed mixed results. Chitinase activity was obtained from the measurement of absorbance values in quantitative chitinase testing using a spectrophotometer with 2 repetitions. The chitinase activity values obtained from the 9 bacterial isolates ranged from 0.628 U/mL to 3.291 U/mL (Figure 3). Four isolates namely UB1, AB3, TB1, and TB3 showed higher chitinase activity than other isolates, with activities of 3.291 ppm, 2.901 ppm, 1.326 ppm, and 1.023 ppm, respectively.

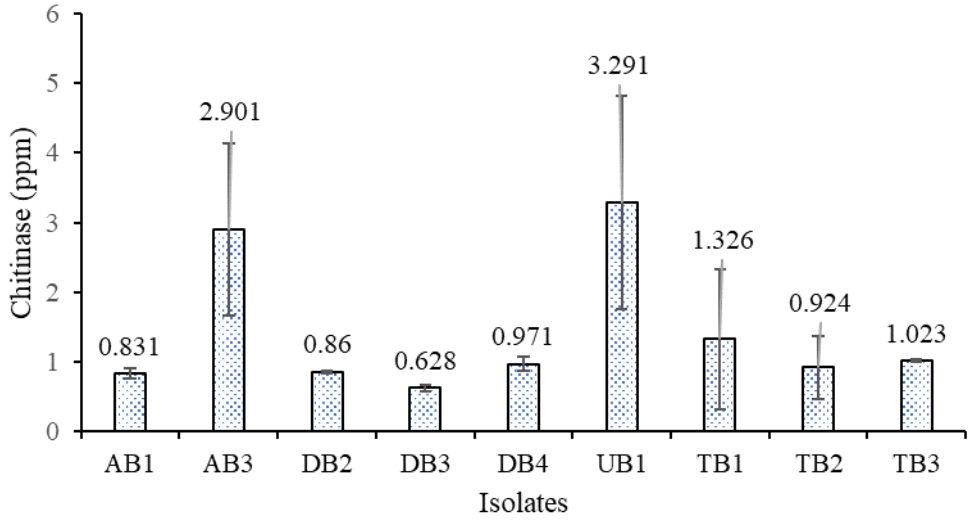

Figure 3. Chitinase activity of bacterial isolates. The mean value was not significant according to DMRT (P= 0,05)

Molecular Identification of Bacteria

DNA isolation was carried out on 3 isolates of the best antagonist bacteria in inhibiting the growth of Foc fungi, namely isolates of bacteria AB3, TB2, and UB1. After the DNA isolation process, the three bacterial isolates were subjected to PCR and electrophoresis testing. The complementary pair resulted in an elongation by the polymerase enzyme during the elongation stage so that during visualization by electrophoresis, DNA bands of samples AB3, TB2, and UB1 appeared with a size of approximately 900–1,000 bp (data not shown). The size of the DNA band in the electrophoresis process is directly proportional to the blast results in the BLAST analysis which shows that the AB3 sample has a base length of 1,459 while TB2 and UB1 have a base length of 1,267 and 1,419.

The results of the analysis of DNA fragments listed in Table 3, show that the bacterial isolates AB3 and TB2 have similarities with the same bacterial species, namely *Bacillus subtilis*. Isolates AB3 and TB2, which were similar to *B. subtilis* bacteria. Isolate *Bacillus* AB3 had macroscopic and microscopic characteristics, gram-positive bacteria, the colonies were white, circular with entire edges, and convex elevation. The *Bacillus* TB2 isolate was also a gram-positive bacterium that formed white colonies and had a circular shape with entire edges, and an umbonate elevation.

Table 3. Blast analysis to three bacterial isolates

Isolates code	BLAST Analysis					
	Bacteria	Total score	Query cover	Per. identify	E value	Accession
UB1	*Pseudomonas nitroreducens* strain R5-760-1	1016	99%	89.35%	0	JQ659788.1
TB2	*Bacillus subtilis* EPS 35_NOU	1933	99%	100.00%	0	MT013387.1
AB3	*B. subtilis* strain JX-1	1495	99%	99.75%	0	KX708698.1

DISCUSSION

Foc fungus attack on shallot plants is one of the obstacles in cultivation that causes "moler" disease or *Fusarium* wilt. The antagonistic bacteria were able to suppress the growth of pathogenic fungi under in vitro tests. Weller (1998) stated that bacteria that can stimulate their growth and inhibit the growth of harmful Foc fungi are considered capable of acting as biological control agents. Safitri et al. (2019) stated that one of the factors that influence the growth of pathogenic fungi *Fusarium* sp. is very dependent on environmental conditions, i.e., temperature, humidity, and nutrients. Based on the classification of antifungals (Mori et al., 1997), the percentage of inhibition of the three antagonist bacteria was included in the moderate (25.12–45.77%) level category in inhibiting the growth of *F. oxysporum* fungi.

The results of the average percentage of Foc fungi inhibition were found in isolates of antagonistic bacteria from roots, leaves, and tubers with percentages of 27.35%, 18.90%, and 17.67%, respectively. These results indicate that the average soil bacteria can inhibit the growth of pathogenic fungi. This is in line with the research of Herdyastuti et al. (2009) which stated that *Serratia plymuthica* is a soil-derived bacterium capable of suppressing the growth of the fungi *Verticillium dahlia* and has the potential as a biological control agent.

The bacterial isolates obtained were observed for gram reaction. Gram-positive bacteria are characterized by the formation of a purple color due to the ribonucleic acids in the cytoplasm of gram-positive cells forming a stronger bond with the crystal violet purple complex so that the chemical bond is not easily broken by alcohol solutions, while gram-negative bacteria are characterized by the formation of a red color. The complex dissolves upon administration of alcohol so that it takes on a red color of safranin (Cappucino & Sherman, 2011). The difference in color between positive and negative bacteria indicates that there are differences in cell wall structure between the two types of bacteria. The cell wall in gram-positive bacteria consists of thick peptidoglycan while the cell wall in gram-negative bacteria consists of peptidoglycan which is thinner than gram-positive bacteria, and contains lipids, and fats in a higher percentage than gram-positive bacteria (Syauqi, 2015).

Quantitative chitinase testing was carried out to determine the formation of the final product of reducing sugar GlcNAc released from chitin during the hydrolysis reaction by obtaining absorbance values for each bacterial isolate using a spectrophotometer. The chitinase activity of microorganisms varies greatly and depends on several factors. Several studies state that the factors that cause variation are enzymatic reaction time, substrate and enzyme concentrations, incubation time, media type, and environmental factors such as temperature and pH (Setia & Suharjono, 2015). The substrate used in the chitinase test is chitin obtained from shrimp shells. Susi (2002) stated that the presence of a substrate can stimulate a microorganism to secrete its cell metabolites and enzymes will react when there is a substrate. The interaction that occurs between the enzyme and

the substrate will produce the final product which is a combination of the enzyme-substrate reaction that affects the catalytic molecular structure of the enzyme. Different bacterial isolates will have different regulatory systems in the synthesis of enzymes in them. The complexity of these mechanisms differs from the simplest, easy-to-understand induction and repression systems to those involving complex mechanisms (Maggadani et al, 2017).

According to Adam (2004) the cell wall of fungi is a complex consisting of chitin, glucans, and other polymers so the presence of the chitinase enzyme in this test will degrade the chitin contained in the cell walls of Foc fungi so that the cell walls will undergo lysis. This is in line with Octriana (2011) that the presence of chitinase activity can inhibit the growth of Foc fungi. Mukarlina et al. (2010) also stated that chitinase activity can occur due to the presence of secondary metabolites produced by bacteria which are naturally a microbial defense mechanism to survive or compete.

Based on the nature of its growth, *B. subtilis* is mesophilic. These bacteria can produce protease, amylase, lipase, and chitinase enzymes as enzymes to decompose pathogenic cell walls. *B. subtilis* is also a group of antagonistic bacteria that are widely used to control plant pathogens. *B. subtilis* bacteria utilize root exudates in the soil and dead plant material as a source of nutrients. The mechanism of inhibition of antagonistic bacteria *B. subtilis* is through antibiosis, competition, and growth promoters (Elad & Freeman, 2002).

Bacillus subtilis produces antibiotics that are toxic to other microbes. The antibiotics produced were surfactin, fengisin, iturin A, polymyxin, difisidin, subtilin, subtilosin, protein, and bacitracin. Bacitracin is a polypeptide that is effective against gram-positive bacteria and works to inhibit the formation of pathogenic cell walls (Soesanto et al., 2008). Rao (1994) stated that *B. subtilis* can produce antibiotics aterimin and bacitracin which are highly toxic to pathogens. *B. subtilis* bacteria have been proven to be good biological control agents. It was also reported by Djaenuddin et al. (2014) that as many as 8 isolates of the best antagonist bacteria that had been identified, namely *B. subtilis* were able to inhibit the development of cob rot disease *Fusarium moniliforme* in maize in vitro and in vivo. The results of Aini et al. (2013) stated that *B. subtilis* can produce macromolecular degradative enzymes that can destroy fungi cell walls such as proteases and several enzymes secreted in the medium such as amylase, glucanase, xylanase, chitinase, and proteases.

DNA samples AB3, TB2, and UB1 were well amplified, indicated by the presence of DNA bands that were visible. This shows that the DNA of isolates AB3, TB2, and UB1 contains nucleotides that are complementary to the primer sequence and work specifically with the DNA template. The 16S rRNA sequencing process was carried out after the formation of DNA bands which were visible during electrophoresis testing. This sequencing test is one way to identify a gene and is the final stage in determining the nucleotide sequence of the amplified fragment by PCR. The identity of a gene whose sequence is known can be determined by comparing the sequence data contained in Genbank. Chen et al. (2015) stated that if the level of similarity of bacterial isolates was 97%, then the isolates were considered as species of the same genus. The results of the gene sequencing analysis through the National Center for Biotechnology Information/NCBI BLAST-N 2.0. showed that there was a similarity between AB3 isolate samples and *Bacillus subtilis* with a similarity level of 99.75%. The TB2 isolate also had a similarity with *B. subtilis* with a similarity level of 100%, while the bacterial isolate UB1 had a similarity with *Pseudomonas nitroreducens* with a similarity level of 89.35% (Table 3). These three samples of bacterial isolates were the best isolates in inhibiting the growth of Foc.

Most of the clinical bacterial isolates with sequences of 500 bp were able to provide adequate differentiation because the region showed more diversity. However, sequences with a length of about 1500 bp are recommended for establishing species-level identification (Clarridge, 2004). The results of DNA fragment analysis on bacterial isolate UB1 showed that the isolate had a base length of 1419 bp and had a similarity with *P. nitroreducens* (Table 3). Based on macroscopic and microscopic characteristic tests on UB1 isolate, this isolate is a gram-negative bacterium, with white colonies, circular shape with lobate edges, and plateau elevation. The group of *Pseudomonas*

bacteria has a cocobasil shape, flat edges, convex, shiny, semi-translucent, 1–2 mm in diameter, has 2–3 flagella, and is a gram-negative bacterium. Specific characters in *Pseudomonas* are straight rod-shaped, motile, and do not ferment (Buchanan & Gibbon, 1974). Madigan et al. (2003) stated that the *Pseudomonas* group was able to produce compounds that inhibit the growth of pathogens. Pseudomonas bacteria are widely used to control soil-borne pathogens. This group of bacteria can produce pseudobactin siderophore which can inhibit the development of pathogens (Thomashow & Weller, 1990). These antagonistic bacteria can also produce fungicidal compounds and compete with pathogens for Fe utilization (Singh et al., 1999). Fe competition is one of the factors in biological control activities, the amount of Fe in the media greatly affects the effectiveness of antagonistic microbes in suppressing the growth of Foc fungus (Segara et al., 2010).

Singh et al. (1999) also explained that *Pseudomonas* bacteria were the most influential in suppressing the growth of Foc fungi because these bacteria were able to produce several types of fungicidal metabolites. Several compounds produced by *Pseudomonas* bacteria are siderophore (pyoverdine), 2,4-diacetylphoroglucinol, pyoluteorin, monoacetylphloroglucinol, and salicylic acid (Haas & Defago, 2005). Bacteria are also endophytic which will induce plant resistance to pathogenic infections (Kavino et al., 2007).

CONCLUSION

Bacterial isolates that had the potential to inhibit the growth of Foc fungus were isolates AB3, TB2, and UB1. Isolates AB3, TB2, and UBI had inhibition percentage values of 46.80, 40.24, and 35.11%. Isolates AB3 and TB2 had similarities with *Bacillus subtilis* bacteria with a similarity level of 99.75 and 100%, while UB1 isolates had similarities with *Pseudomonas nitroreducens* bacteria with a similarity level of 89.35%. The three potential isolates in this study may be used as biological control agents, however further research is needed on the ability of the three effective antagonist bacterial isolates to inhibit Foc under in vivo test.

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