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In Vitro Assay of Lytic Bacteriophage to Suppress the Growth of *Ralstonia syzygii* subsp. *indonesiensis,* the Causal Pathogen of Potato Wilt Disease

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Abstract. Bacterial wilt disease, the one of the major diseases of potatoes, caused by *Ralstonia syzygii* subsp. *indonesiensis* (*Rsi*). Many efforts have been made to control bacterial wilt disease, including physical control, chemicals, and the use of bacteriophages. Previous studies have shown that bacteriophage application in controlling plant diseases is a fast-expanding area and has great potential to replace chemical methods. This study aims to determine the potential of lytic bacteriophage in suppressing the growth of *Rsi in vitro*. This study used a Non-Factorial, Completely Randomized Design with 3 replications and 6 treatment levels: R1 (*Rsi* isolate 1 without bacteriophage), R2 (*Rsi* isolate 1 with bacteriophage), R3 (*Rsi* isolate 2 without bacteriophage), R4 (*Rsi* isolate 2 with bacteriophage), R5 (*Rsi* isolate 3 without bacteriophage), and R6 (*Rsi* isolate 3 with bacteriophage). The results showed that bacteriophage could reduce the *Rsi* population at 24 hours by looking at the optical density (OD) value of 600 nm wavelength and growing on NA medium using the spread-plate method. The best treatment was at R6 with a population of 1 x 10^8cfu/ml *Rsi*. This research suggests that bacteriophage has the potential to suppress the growth of *Rsi,* which causes potato bacterial wilt disease, *in vitro*.

Keywords: bacteriophage, bacterial wilt disease, *in vitro* assay, potato, *Ralstonia syzygii* subsp. *indonesiensis*

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1. Introduction

Potato is an important horticultural commodity in Indonesia and worldwide. Compared to other vegetable production, potatoes have a high average yield, but it fluctuates every year. [1] reports total potato production in North Sumatra in 2020 was 1.22 million tons with production centers in Karo Regency which has a production of 703.6 thousand tons. The quality and quantity of potato yields can be reduced by several cultivational challenges, such as pest infestation and plant diseases. Bacterial wilt disease caused by *Ralstonia solanacearum* species complex is a major disease that affects potato plants in tropical countries, such as Indonesia, also sub-tropical and temperate environments. The genus *Ralstonia* is considered to be the most important plant

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pathogenic bacterial species [2]. *R. solanacearum* species complex has undergone taxonomic changes; phylotype IV that infects the family *Solanaceae* is caused by the pathogen *Ralstonia syzygii* subsp. *indonesiensis* [3].

Many efforts have been made to control bacterial wilt caused by the pathogenic bacterium *R. syzygii* subsp. *indonesiensis* (*Rsi*), including land sanitation, balanced fertilization, and using bactericides such as antibiotics and copper-based compounds. However, antibiotics and copper resistance have been identified and characterized in many plant-pathogenic bacteria [4]. Moreover, the use of antibacterial chemicals can affect human health and contaminate agricultural areas because bactericides leave chemical residues. Therefore, several alternative measures are currently being experimented and researched, such as the use of biofumigant [5] and biological agents, including *Trichoderma koningii* [6] and the antagonistic bacteria *Bacillus substilis* [7]. Another alternative currently being extensively developed is the application of bacteriophages, which are viruses that infect and kill bacterial cells directly. Bacteriophages live and reproduce in unicellular organisms, unlike other viruses that reproduce in the bodies of multicellular organisms [8]. Bacteriophages have been used as promising antibacterial agents to manage plant diseases since the early 20th century because of their high specificity, lack of negative impact on humans and animals, and lack of environmental residues and therefore can replace the use of chemical control in agriculture [9].

Studies have shown the effectiveness of using bacteriophage to control several bacterial plant diseases, including the black rot disease of cabbage caused by *Xanthomonas campestris* pv. *campestris* [10] (Mallmann and Hemstreet, 1924), soft rot disease of carrot caused by *Erwinia carotovora* subsp. *carotovora* [11], Stewart's wilt disease of corn by caused *Pantoea stewartii* [12], tumour disease of tomato caused by *Agrobacterium tumefaciens* [13], bacterial leaf blight of rice caused by *Xantomonas oryzae* [14], tobacco bacterial wilt caused by *Ralstonia solanacaerum* [15], bacterial wilt disease in tomato plants [16], and banana blood disease (*Ralstonia syzygii* subsp. *celebesensis*) [16] [17]. Another advantage of using bacteriophages, besides being environmentally friendly, bacteriophages are viruses that only infect bacteria, where viruses are obligate microbes that have very high tolerance for the environment Viruses can crystallize themselves under unfavourable conditions and revert to their normal state when environmental conditions become favourable. Specific bacteriophages target specific pathogens; for example, bacteriophages that infect bacteria *R.solanacearum* cannot infect the bacteria *Xanthomonas campestris* [16]. Based on these backgrounds, we conducted a preliminary study of bacteriophage to control the growth of *Rsi in vitro*.

2. Materials and Methods

2.1. Isolation of the Bacterial Pathogen from Potato Plant

Bacteria were isolated from potato plant samples exhibiting symptoms of complete plant wilt by adapting method of [18]. Isolation was carried out by extracting the ooze (bacterial mass) from infected potato plant stems. The infected stems were cleaned using running water, and then the surface was sterilized by spraying 70% alcohol. Infected plant stems were cut and dipped into sterile water until the ooze came out and became visible. The ooze was streaked with nutrient agar (NA) (beef extract 10 g, peptone 10 g, NaCl 5 g, agar 15 g/L) medium in a Petri dish and incubated at 28 ºC for 24-28 hours. The bacterial colonies were transferred into the semi-selective media, casein peptone glucose-tetrazolium chloride (CPG-TZC) (casein hydrolysate 1 g, peptone 10 g, glucose 5 g, agar 15 g, 1% tetrazolium/L), and incubated for 4 days at 28 ºC.

2.2. Identification of Bacterial Pathogen

The bacterial pathogens isolated from the potatoes from 3 locations, Karo district, North Sumatra, Indonesia (Barus Julu, Simpang Inala and Sukanalu villages), were identified by their morphological, physiological, and biochemical characteristics. The streaming test of observing the bacterial ooze in the vascular plant tissue was performed as per method of [19]. This streaming test is recorded as a positive reaction when the ooze excretes from the vascular tissue [19]. The bacterial colonies were observed based on their color, shape, size, surface, elevation and margin. Gram staining was performed, and the growth was observed at 27 ºC and 40 ºC. The potassium hydroxide (KOH) test was performed according to method of [20]. The colony of tested strains was stirred into the solution of two to three drops of 3% KOH using a clean loop for 5 s. If the KOH solution became viscous after 5 seconds, as evidenced by a thread-like slime following the loop, the test was recorded as positive. The starch hydrolysis test was performed using an agar medium as described by [21] to determine the ability of the bacteria to produce certain exoenzymes, including α-amylase and oligo-1,6-glucosidase, to hydrolyse starch. The agar medium contained one dose of CPG medium (casein hydrolysate 1 g, peptone 10 g, glucose 5 g, agar 15 g/L including 0.2% soluble starch). The tested strains were streaked onto the medium and incubated at 28 °C until heavy growth occurred. The plates were flooded with iodine solution (iodine 1 g, potassium iodide 2g, distilled water, 100 ml). The utilization of carbohydrate tests was based on method of [22]. The Hayward's basal medium contained peptone, 1 g; ammonium dihydrogen phosphate (anhydrous), 1 g; potassium chloride, 0.2 g; magnesium sulfate heptahydrate (anhydrous), 0.2 g; agar, 3 g; bromothymol blue, 0.08 g; and distilled water 1 L; pH 7.0-7.1. Several loops of freshly grown cultures were inoculated aseptically into the sugar media plates, including lactose, fructose and maltose. The plates were incubated at 28 °C for four days. The colour change from green to yellow was recorded as a positive reaction.

2.3. Enrichment of Bacteriophage

The bacteriophage isolate ϕBTF1 was collected from the Laboratory of Plant Disease, Faculty of Agriculture at University of Sumatera Utara from study of [17] . The isolate ϕBTF1 was identified by [23] as Ordo Caudovirales and Family Myoviridae. Bacteriophage enrichment was performed according to method of [24]. The single colony of bacterial pathogen *Rsi* was inoculated into the nutrient broth (NB) medium and incubated at 37 °C for 24 hours until it reached OD_{600nm}=1. One hundred microliters of *Rsi* cultures were mixed with the 100 µl of phage supernatant in a sterile tube and incubated at 37 °C for 30 min. Then 5 ml of soft agar (0,2% agar mixed with NB medium) at 47 °C was added into the mixtures, poured into the NA medium plates and incubated at 37 °C for 24 hours.

The bacteriophage purification was conducted based on the method of [25]. Plaque was removed using a Pasteur *pipette; the* plaque was then mixed with 2-3 ml of 25% Ringers solvent (CaCl2 .02 g, KCl 0.03 g, NaCl 0.6 g, sodium lactate 0,31 g/L) or salt of magnesium (SM). Phage suspension was vortexed and left for 5-10 min at room temperature. The suspension was then centrifuged at 4°C for 25 minutes and repeated twice. The supernatant was filtered using a 0.22 µm porous filter, and then stored as stock.

2.4. Plaque Assay

Plaque assay was performed using the drip method (spot test) to determine the presence of bacteriophages, which is indicated by the appearance of the boundary lysis ring of bacteriophage infection, and obtain pure colonies of bacteriophages from *Rsi* isolates. Fifty microliters of bacterial cultures of *Rsi* (10^8 CFU/mL) were spread on the NA and CPG-TZC media using a triangle glass rod until dry. The bacteriophage lysate was dripped onto the surface of NA and TTC media by 2 µl using a micropipette and incubated for 24-48 hours at 28 °C [26]. The experiment was repeated six times.

2.5. Bacteriophage Density

A phage plague is a clear zone formed in a lawn of cells due to lysis by phage. At a low multiplicity of infection (MOI), a cell is infected with a single phage and lysed, releasing progeny phage which can diffuse to neighboring cells and infect them; This process is repeated until ultimately resulting in a circular area of cell lysis in a turbid lawn of cells [27]. Plaque assay is performed by calculating the number of infected viruses by the appearance of a lysis zone or clear zone in the soft agar media from different dilution series of bacteriophage. The bacteriophage density was calculated using the multilevel dilution method by [28]. A total of 0.1 ml of bacteriophage stock was added to 0.9 ml of physiological saline (up to 10^{-6} dilution). From each dilution (10^{-1} to 10^{-6}), 200 µl of bacteriophage was taken to be added to 6 ml of NB containing *Rsi* culture and homogenized. The suspension was then poured into the NA medium and replicated

6 times and incubated at 37 °C for 24 hrs. The Plaques formed on the Petri dish were observed, and the number of bacteriophage populations was calculated with the following formula:

$$\text{Number of bacteriophages} = \text{number of plaques x dilution level [29]} \quad (1)$$

2.6. *In Vitro* Assay of Population Reduction of Bacterial Pathogen

The fresh cultures of the bacterial pathogen were added into a new nutrient broth (NB) medium supplemented with 5 mM $CaCl_2$ and $MgCl_2$, it was incubated in a shaker incubator at 200 rpm at 37°C until it reached an OD of 600 = 1 ($\leq 1x10^8$cfu/mL). One hundred bacterial cultures in each dilution were added to 72 ml of the new NB medium. The phage stock was diluted using SM buffer (NaCl5 8g, MgSO4.7H2O 2g, Tris-ClpH7 550ml, gelatin 5ml/L) up to 10^8 pfu/mL. The bacteriophage suspension was added to the bacterial cultures and incubated in the shaker incubator at 20-30 rpm at 37 °C. Observation of the OD600 was carried out on the culture every hour until lysis occurred (a decrease in the OD of each host bacteria) using a spectrophotometer with NB medium. The bacterial cultures were treated the same in all treatments and became a control treatment. The results were observed every 1 hr for 24 hrs [17].

2.7. Bacteriophage Infection Cycle

Observation of the bacteriophage infection cycle against the bacterial pathogen was carried out to determine the lysis or lysogenic cycle between treatments, which was performed using the absorbance value from turbidimetric calculations. If the growth of the bacterial pathogen continues to increase after the application of bacteriophages, then phage multiplication is assessed to occur lysogenically, and if it is vice versa, the phages are said to have lytic multiplication [23].

3. Results and Discussion

3.1. Identification of Bacterial Pathogen

The plant from which the bacterial pathogen was isolated had/exhibited wilt symptoms in the whole plant but the leaves were still green in colour (Fig 1A). It was observed that three tuber samples showed positive oozing and browning in the vascular bundle and tuber vessel. The infected potato tuber had a brown-black coloured ring in the tuber vessel when cut vertically (Fig 1b). Cream to grey mass also appeared in the tuber vessel, and the potato became rot (Fig 1b). The infected stem base of the plant was cut and dipped in clean water in a clear Beaker glass until the milky white ooze excreted (Fig 1c). In the streaming test, three potato plant samples showed positive bacterial ooze streaming in clear water.

The morphological characteristics of the *Rsi* cultures from three locations are shown in Table 1 and Fig 1. The white-cream-coloured colonies that grew from the bacterial ooze that was isolated and streaked onto the CPG medium were irregular, round, fluidal and opaque (Fig 1d). When

grown on CPG-TZC medium, the colonies were white-cream with pink centres (Fig 1e). The pure cultures from three different locations in the Karo district (R1, R2, R3) were obtained.

Table 1. Colony Morphological Characteristics of *Rsi*

Isolates of *Rsi*	Colony morphology			
	Colony color on CPG medium	Colony color on CPG-TZC medium	Shape	Elevation
Rsi 1	White-cream	White-cream with pink centre	Irregular round	Raised
Rsi 2	White-cream	White-cream with pink centre	Irregular round	Raised
Rsi 3	White-cream	White-cream with pink centre	Irregular round	Raised

Table 2. Phenolypic of *Rsi* by Conventional Phenotypic Assays

Phenotypic profiles	*Rsi* 1	*Rsi* 2	*Rsi* 3
Gram reaction	-ve	-ve	-ve
Bacterial shape	rod	rod	rod
Growth at 27 °C	+ve	+ve	+ve
Growth at 40 °C	-ve	-ve	-ve
Potassium hydroxide test	+ve	+ve	+ve
Starch hydrolysis	-ve	-ve	-ve
Oxidative acid from:	+ve	+ve	+ve
Lactose	+ve	+ve	+ve
Maltose	+ve	+ve	+ve
Glucose	+ve	+ve	+ve

Additional information:
rod = bacteria are classified as bacilli groups
+ve = positive test result
-ve = negative test result

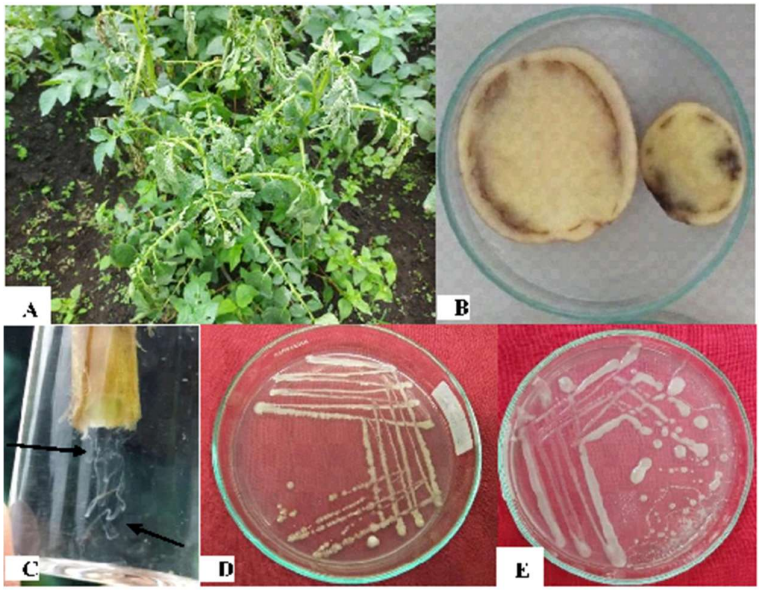

Figure 1. Bacterial Isolation from Infected Potato Plant in Three Locations in Karo District, North Sumatera, Indonesia. A) Visual Symptoms of Infected Potato Plant by *Rsi,* B) Brownish Discoloration of the Vascular Ring, C) Bacterial Ooze Coming Out in Clear Water from Cut End of A Wilt Infected Potato Stem, D) White-Cream Color Colonies of *R. Syzygii* subsp. *indonesiensis* on CPG medium, E) White-Cream with Pink Centers Colonies of *R. Syzygii* subsp. *indonesiensis* on CPG-TZC medium

All three *Rsi* isolates showed a positive reaction to the Gram staining test, potassium hydroxide test, carbohydrate production from lactose, maltose, and glucose, and showed a negative reaction in starch hydrolysis (Table 2). In the temperature sensitivity test, the test isolates showed positive and typical growth at 27˚C but did not show any potential growth at 40˚C (Table 2).

The bacterial pathogen that was isolated from the potato plant exhibiting wilt symptoms was confirmed as *Rsi*, particularly on the appearance of oozing and browning in the vascular bundle and tuber vessel. *R. solanacearum* species complex is the only wilt-inducing pathogen that produces ooze [30]. These specific characteristics were supported by morphological and biochemical characteristics.

3.2. Bacterial Density

The highest concentration of bacteriophage population was found in dilution 10^{-6} with a total of 8.65 x 10^{7}pfu/ml, and the lowest bacteriophage population was found in 10^{-1} dilution with a total population of 2.78 x 10^{2}pfu/ml (Table 3). The calculation was based on visual observation, i.e., calculating the number of plaques that appeared on the soft agar media. The higher the dilution level, the higher the concentration of the bacterial plaques.

Table 3. Number of Bacteriophage Population on Different Dilution Levels

Level of dilution series	Number of phage plaques (pfu)	Population number of phage (pfu/ml)
10^{-1}	27.8	2.78 x 10^{2}
10^{-2}	57.0	5.7 x 10^{3}
10^{-3}	70.5	7.05 x 10^{4}
10^{-4}	71.3	7.13 x 10^{5}
10^{-5}	61.0	6.10 x 10^{6}
10^{-6}	86.5	8.65 x 10^{7}

3.3. Population Reduction of *Ralstonia syzygii* subsp. *indonesiensis in Vitro*

The curve trend in treatment without bacteriophage application (R1, R3, and R5) showed that bacterial growth continued to increase up to 24 hours of observation (Fig. 2). R5 treatment (*Rsi* isolate 3) showed the highest number of *Rsi* population at the end of observation (24 hours) (149.33 x 10^{8}cfu/ml) followed by R3 treatment (*Rsi* isolate 2) (151.3 x 10^{8}cfu/ml) and R1 treatment (*Rs*i isolate 1) (120.33 x 10^{8}cfu/ml), respectively. However, all treatments with bacteriophage application (R2, R4, and R6) showed a reduction in *Rsi* population. R6 treatment (*Rsi* isolate 3) began to show a population decrease at the 20th hour of observation and reached the lowest number of *Rsi* population (1 x 10^{8}cfu/ml) at 24 hours of observation. Other bacteriophage treatments (*Rsi* isolate 1/ R1 and isolate 2/R2) also showed a population decrease at the end of observation (24 hours), which were 3.33 x 10^{8}cfu/ml.

3.4. The Infection Cycle of Bacteriophage

The application of bacteriophage ϕBTF1 could reduce the population of *Rsi* as evidenced by optical density values in the wavelength of 600 nm (Fig 2 & Fig 3). The decrease in the population of *Rsi* determined that the bacteriophage isolate ϕBTF1 is a lytic bacteriophage that had undergone the lytic life cycle. In the initial observation period, the *Rsi* grew gradually till 16 hours, but the growth of the *Rsi* in the treatments of R2 (isolate 1), R4 (isolate 2) and R6 (isolate 3) slowly decreased from the OD values between 1-1.5 to below 1 at the end of the observation period (24 hours).

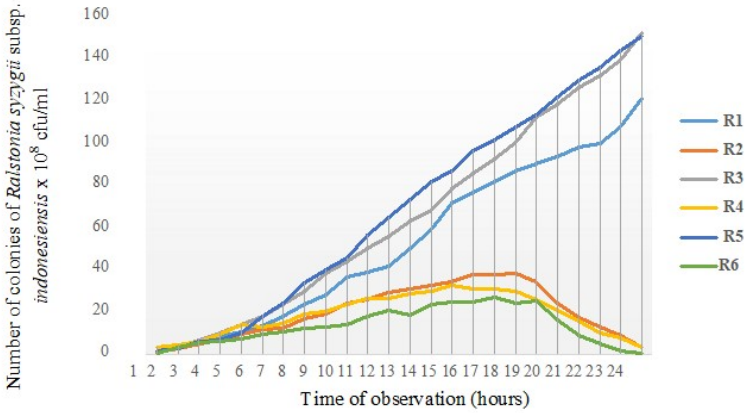

Figure 2. The Response of Bacteriophage Application to the Population of *Rsi* observed Hourly Up to 24 Hours at A Dilution Level of 10^8.

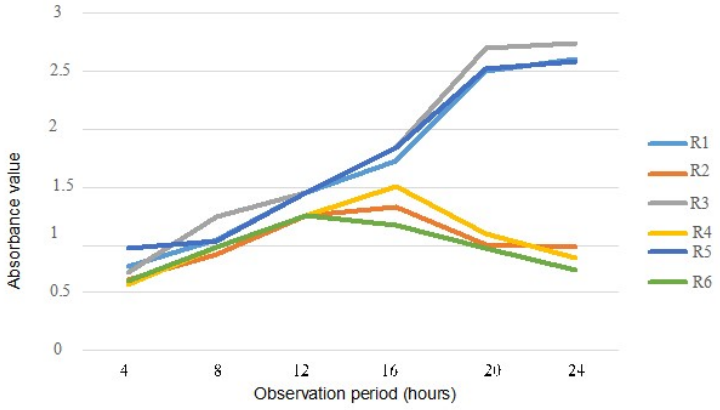

Figure 3. The Response of Bacteriophage Application to the Population of *Rsi in Vitro* on the Basis of Absorbance Values.

This study shows that the application of bacteriophage isolate ϕBTF1 could reduce the growth of the *Rsi* population under *in vitro* condition by the increase of bacteriophage density and the decrease of *Rsi* population. This preliminary *in vitro* study suggests that bacteriophage can be a promising alternative biological agent to manage bacterial wilt disease of potato. In a previous study, bacteriophage isolate ϕBTF1, which was used in this study, was found to suppress the infection of *R.syzygii* subsp. *celebesensis,* which cause blood disease in banana [23]. The current

study under *in vitro* condition also approved the potency of this phage isolate in suppressing the population growth of bacterial wilt pathogen of potato (*Rsi*). In isolates that were not applied with bacteriophages, the graph shown an increase in the *Rsi* populations because the bacteria grew in the suitable medium and there were no agents that inhibited their growth.

Phage isolate ϕBTF1 is a lytic bacteriophage that involves the reproduction of viruses using a host cell to manufacture more viruses; they then burst out and lyse the host cell. Strong lytic activity is required to practically control the bacterial wilt pathogen *Ralstonia solanacearum* [31]. During the infection cycle of the lytic phage, enzymes, mainly holins and lysins, are produced [32]. Lysin enzymes are very effective chemicals that attack one of the four key peptidoglycan linkages to target the integrity of the bacterial cell wall [33]. Lysin builds up in the cytoplasm during phage development in the infected bacteria in preparation for phage maturation. When holin molecules are injected into the cytoplasmic membrane at a genetically predetermined moment, patches are formed that eventually cause generalized membrane breakdown, allowing the cytoplasmic lysin to access the peptidoglycan, leading to cell lysis and the release of progeny phage [34] [35]. Small RNA and DNA phages use phage-encoded proteins to disrupt bacterial host enzymes involved in peptidoglycan production, leading to an incorrect assembly of cell walls and eventual lysis [36] - [37].

Bacteriophages are prokaryotic viruses present abundantly in all environments and can be found wherever the potential host-bacteria are present [38]. A total number of 10^{32} virions of bacterial viruses are present in the environment, and this number is 100 times more than the entire number of currently characterized bacteria [39]. This widespread occurrence of bacteriophages presents the great advantages of isolating them in nature for plant disease control purposes.

A previous study using lytic bacteriophages to control bacterial wilt disease proved that bacteriophages are highly host specific, and lytic bacteriophages could reduce the pathogenic wilting activity of *R. solanacearum* in tomato seedlings as well as potato tuber [40]. In greenhouse and field tests, tomato plants treated with various phage combinations further reduced the occurrence of the bacterial wilt *Ralstonia* disease by 80% [41]. These studies suggested that single-phage applications to bacterial wilt disease were highly stable with strong lytic activity for effective management of phage resistance.

4. Conclusion and Recommendation

The application of bacteriophage was able to reduce the population of *Rsi,* growing on NA medium using the spread plate method, at 24 hours of observation by looking at the optical density (OD) value of 600 nm wavelength. By the application of lytic bacteriophage ϕBTF1, *Rsi* isolate, 3 isolate had the least population (1 x 10^8cfu/ml). This research suggests that lytic bacteriophage has great potential to suppress the growth of potato bacterial wilt pathogen *Rsi in vitro* and can

be continued with *in vivo* testing to determine there is an effect of bacteriophages application to control *Rsi*. So the results of the research can be used as basic information to control bacterial wilt disease in potato plants.

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