Isolation and selection specific bacteriophage from banana in North Sumatera to biologically control *Ralstonia syzygii* sub sp. *celebesensis* in vitro

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**Abstract.** Blood disease caused by *Ralstonia syzygii* sub sp. *celebesensis (Rsc)* has been an important issue affecting the production of banana plants in Indonesia. Various controls used such as bactericides, sanitation, biological agents and male flower transfer have not been able to prevent the development of this disease. Specific bacteriophages that control this pathogen have been developed, however, the study of using bacteriophage isolated and selection from banana in North Sumatera has not been explored. The purpose of this study was to isolate and to select specific bacteriophage from banana in North Sumatera to biologically control *Rsc*. This research was conducted at the Laboratory of Plant Diseases, Universitas Sumatera Utara from January to June 2020. This experiment was conducted with nonfactorial Complete Randomized Design (CRD) by applying 4 selected bacteriophages and 1 control treatment (Control, φBTF1, φBTF2, φBTF3, BTF4 with four replications. The results showed that three bacteriophages including φBTF1, φBTF2, and φBTF4 replicated using the lytic cycle and decreased the population of *Rsc* for 24 hours observation. On the contrary, φBTF3 had lysogenic replication and kept *Rsc* alive but changing the pathogen became avirulent.

1. Introduction

Blood disease caused by the pathogen *Ralstonia syzygii* subsp. *celebesensis (Rsc)* has spread throughout Indonesia and causes a 35% yield loss in banana plantations [1]. Symptoms of *Rsc* was characterized by male flower buds and peduncles blackening and contracting, the fruit shows reddish dry rot and plant vascular tissue shows a reddish color change, which secretes reddish-brown bacterial fluid when the fruit was cut off [2,3]. *RSC* is transmitted by the insect vectors including *Trigona Minangkabau* and *Eriopota thrax* [4,5]. The Life cycle of the pathogen can be divided into five steps: root invasion, passing through the root cortex, proliferation in xylem vessels, phenotypic switch induces exopolysaccharide (EPS) production and withering of air organs leading to plant death and allowing transmission of pathogens to the soil [6].

Various attempts have been made to control *Rsc* such as: tph the lanting of non-contaminated bananas, the use of sterile knives when harvesting bananas, the use of agents such as endophytic bacteria species *Bacillus subtilis* [7,8] but these approaches have not shown significant results. Addy *et al.* reported that two bacteriophages isolated from banana in Lumajang, East Java, Indonesia, i.e.
\( \phi \text{RSSKD1} \) and \( \phi \text{RSSKD2} \), could infect nine isolates of bacterial wilt pathogen of banana in a study conducted in vitro [9]. Another study of bacteriophage showed that inoculation of bacterial cells infected with \( \phi \text{SMRSM3} \) (a bacteriophage infecting \textit{Ralstonia solanacearum} into tomato plants did not cause bacterial wilt symptom, while \( \phi \text{RSM3} \) infected cells and increased the expression of genes associated with pathogenesis-related proteins (PR), including PR-1a, PR-2b, and PR7, in tomato plants. To be able to invade pathogenic bacteria, bacteriophages replicate both lytic and lysogenic. Each bacteriophage has either a lysogenic or lytic replication method, but there is also a bacteriophage that has both cycles [10]. With some reports on bacteriophages invention bacterial wilt pathogen, therefore the specific bacteriophage information of \( Rsc \) of banana blood diseases has become a very important topic to investigate. The purpose of this study for isolation and selection of specific bacteriophage from banana in North Sumatera to biologically control \( Rsc \).

2. Materials and methods

2.1. Methodology

This research was conducted at the Laboratory of Plant Diseases, Universitas Sumatera Utara from January to June 2020. This research was conducted with nonfactorial Complete Randomized Design (CRD) by applying 4 selected bacteriophages and 1 control treatment (Control, \( \phi \text{BTF1} \), \( \phi \text{BTF2} \), \( \phi \text{BTF3} \), \( \phi \text{BTF 4} \) with four replications and 20 units in total).

2.2. Isolation and identification of \textit{Ralstonia syzygii} sub sp. \textit{celebesensis} \( Rsc \) isolate was obtained from a variety of “Kepok” (\textit{Musa acuminate balbisiana}) from a banana farm in Dusun I Aman Damai, Sei Semayang Village, Sunggal District, Deli Serdang Regency, North Sumatra, Indonesia at the coordinates: 30°35`55`` LU and 98°03`22`` BT. \( Rsc \) isolation was carried out using the method of Imas et al. [11] by isolating the pathogen from the stalk of the infected banana and then it was cultured on casamino acid peptone glucose medium (CPG composition (g/L): casein hydrolysate 1g, peptone 10g, glucose 5g, agar 15g) incubated at 28°C for 4 days. The growing colonies were then cultured on triphenyl tetrazolium chloride medium (TTC composition equal to CPG with add 5ml tetrazolium added 1% concentration [11]. The colony morphology of \( Rsc \) such as color, shape, size, surface, elevation, and margin was observed. Physiological and biochemical characteristics were carried out including Gram test, KOH 3%, Oxidation/Fermentation (O/F) reaction, pigment fluorescent production, growth on 3.3 Diindolymethane (DIM) medium, hydrolyze Arginine, growth on Yeast-Extract Dextrose CaCO\(_3\) (YDC) medium, growth at the temperature of 40°C [12,13] and molecular identification was carried out using a polymerase chain reaction (PCR). Specific fragments of bacterial genome DNA were amplified using a pair of primers, namely 121F (5'-CGT ATTTGGA TGC CGT AAT GGA-3') and 121R (5'-AAG TTC ATT GGT GCC GAA TCA-3') [14]. Furthermore, amplification was carried out on a PCR machine with an initial denaturation program of 96°C for 5 minutes, followed by 30 cycles which included a second denaturation at a temperature of 94°C for 15 seconds, annealing a temperature of 59°C for 30 seconds, extension 72°C for 30 seconds and continued with the final extension at 72°C for 10 minutes then ended with a final hold at 11°C for 4 minutes. The amplification product was analyzed using 2% agarose gel electrophoresis at a voltage of 100 volts for 30 minutes and visualized with a UV transilluminator lamp [15].

2.3. Isolation, purification and bioassay tests of bacteriophage specific \textit{Ralstonia syzygii} sub sp. \textit{celebesensis} Bacteriophages were isolated from soils that were the same location as \( Rsc \) isolation by the method of Cross et al. [16]. Bacteriophages were purified according to the method of Goodridge et al. [17]. Bioassay test of bacteriophage specific-\( Rsc \) was tested according to the method of Attenbury et al. [18] by calculating the area of the plaque assay produced by a bacteriophage specific- \( Rsc \) on CPG medium. Bacteriophage detection was carried out by double-layer agar technique and observed by the
formation of the resulting clear zone. This method has been extensively researched and developed by Fascino et al. [19] who used plaque assay to detect phage excitement in bacterial samples tested.

2.4. Observation of bacteriophage specific- Rsc replication
Lytic replication is done by measuring the population of Rsc bacteria (initial population $1 \times 10^8$ CFU) that have been infested with bacteriophages (population $1 \times 10^5$ plaque-forming unit (pfu/ml)) at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 hours after application. Population decline is calculated by the method total plate count (TPC) to observe the population of bacteria that are still alive against the phage infestation by taking 0.1 ml volume of bacterial culture that was infused with phage and plating on the CPG medium. The bacterial population was calculated by formulae [20]:

\[
\text{Bacteria population (CFU/ml)} = \text{colonies bacteria} \times \text{the amount taken} \times \text{the dilution factor} \quad (1)
\]

The lysogenic cycle was observed by the same method of phage culture and infestation in the lytic detection treatment. In the lysogenic cycle, the change of Rsc virulence was characterized by observing the growth of bacterial colonies on the CPG-TZC medium. Virulent Rsc colony was identified as slimy surface, milky white edge, middle of the colony had pink spot was a virulent colony (result -) and flat surface, not slimy and center of the colony had dark red color (result +) indicted was avirulent Rsc [21,22].

2.5. Statistical analysis
Data were statistically analyzed according to the standart analysis of variance by oneway ANOVA with The Minitab 21 software. The comparison between means was carried out by Duncan's Multiple Range Test with level 5% [23].

3. Results and discussion

3.1. Isolation and identification RSC
The pathogen isolated was identified as bacteria *R. syzygii* sub sp. *celebesensis*. This was observed by observing visual symptoms of the plant, i.e. fruit showing reddish dry rot and plant vascular tissue showing reddish color changes, which emit reddish-brown bacterial fluid when cut and one that comes out like a thread (ooze) [2,3]. The bacterial colony showed a milky white surface to cream with a flat colony edge and the colony showed the edges of the cream color colony with the center of the colony being pink and very slimy on CPG-TZC medium [11-14] (figure 1).

![Figure 1. Symptoms of an *R. syzygii* sub sp. *celebesensis* infestation. (a). Healthy banana fruit and (b). banana infected by *R. syzygii* sub sp. *celebesensis*. (c). Culture of *R. syzygii* sub sp. *celebesensis* on CPG-TZC medium.](image)

The results of physiological and biochemical tests also showed that the pathogen isolate was identified as *Rsc* (table 1).
Table 1. Physiology and biochemical tests on R. syzygii sub sp. celebesensis isolate

| Physiological and biochemical tests | Results | Schaad et al. [13] |
|------------------------------------|---------|-------------------|
| KOH 3%                             | +       | +                 |
| Garam reaction                     | -       | -                 |
| Oksidata /Fermentatif reaction     | -/-     | -/-              |
| Pigmen fluorescents production     | -       | -                 |
| Grown DIM medium                   | -       | -                 |
| Hidrolisa Arginin                  | -       | -                 |
| Grown in YDC medium                | -       | -                 |
| Grown at temperature 40°C          | -       | -                 |

From the molecular assay, the isolate was amplified at a band size of 317 bp indicating the isolate tested was R.syzygii sub sp. celebesensis [14] (figure 2.)

3.2. Isolation and bioassay test of bacteriophage specific-RSC
The results of isolation from soil samples extracted from healthy banana plants located near Rsc sampling showed bacterial infestations that had the potential to control Rsc. This can be observed by the formation of a clear zone (plaque) on the double layer CPG medium after a 24-hour incubation period and screening of the 4 most extensive clear zones to be used as the selected candidates (ϕBTF1, ϕBTF2, ϕBTF3, ϕBTF4) [16] (figure 3). The ability to form clear zones proves the presence of bacteriophage colonization that infects the bacteria.

3.3. Specific Rsc - bacteriophage replication
The results of the research show that the four bacteriophages (ϕBTF1, ϕBTF2, ϕBTF3, ϕBTF4) had different replication patterns and replication abilities so that the different forms of bacterial population decline. The curve in the control treatment (without the application of bacteriophage) showed that bacterial growth continued to increase for up to 24 hours of observation. ϕBTF1 had the fastest population reduction of 0 bacterial populations at 22 hours of observation followed by ϕBTF2 of 0 population at 24-hour observation. While the ϕBTF4 treatment had a pattern of population increase at 10 hours of observation and 16 hours later the trend curve decreases but does not touch 0 population and when compared with BTF 3 has a very different pattern of population decline that decreases at 14 and 20 hours but rises sharply thereafter (Figure 4). Bacteriophage replication takes place both lytic and lysogenic or has both at once. In this case, ϕBTF1, ϕBTF2, ϕBTF4 have lytic replication because...
they are able to produce pathogenic bacterial cells within 24 hours and even reach a population of zero live bacterial cells ($\phi$BTF1 and $\phi$BTF2). Similarly, [21] stated that bacteriophages replicate with lytic and lysogenic or have both. With Lytic replication, the bacterial cells break open and are destroyed after virion replication.

Figure 3. Phage detection of soil with healthy banana plants (a). Exploration of bacteriophage on CPG and (b) double-layer medium. Four phage bioassay screening ($\phi$BTF1, $\phi$BTF2, $\phi$BTF3, $\phi$BTF4) on CPG double-layer medium for 24-hour observation.

Figure 4. Curve response of bacteriophage (1x10$^5$pfu/ml) to the decline in the population of *R. syzygii* sub sp. *celebesensis* (initial population 1x10$^8$cfu/ml) on observations every two hours to 24 hours with the pouring method observed at 5x dilution factor.

The results showed that only $\phi$BTF3 had a lysogenic ability to change the genetic pathogenic bacteria without killing it, but in this study, the changes occurred in the virulence power of *Rsc* as evidenced by a bacterial correction in CPG-TZC medium (table 2).

This finding is in accordance with Mason *et al.* [22] which stated that the lysogenic cycle does not produce direct host cell lysis. Phage entering lysogen is said to be of moderate virulence. Their viral genomes will integrate with host DNA and replicate together and are harmless if spun into plasmids. This virus is not active but can change certain DNA sequence systems so that it affects the behavior of the host. This change can be attached to any nature depending on the nucleotide base it attaches when
it becomes profaning. Genetic changes observed in this research are pathogen virulence power by culturing it on the CPG-TZC medium which can distinguish virulence by observing colony color and shape. Virulent pathogenic bacteria will show a pink colony with creamy and slimy edges while avirulent bacteria will show a non-slimy colony surface and a dark red colony [24] (figure 5.)

Table 2. Morphology of infected Ralstonia syzygii sub sp. celebesensis on CPG-TZC media and lysogenic replication of selected bacteriophage

| Bacteriophage | Lysogenic replication | Morphology of infected Rsc on CPG-TZC Medium |
|---------------|------------------------|---------------------------------------------|
| Control       | -                      | pink colonies, cream margin and slimy surfaces |
| φBTF1         | -                      | pink colonies, cream margin and slimy surfaces |
| φBTF2         | -                      | pink colonies, cream margin and slimy surfaces |
| φBTF3         | +                      | dark red colonies, dry and flat surfaces |
| φBTF4         | -                      | pink colonies, cream margin and slimy surfaces |

Figure 5. Morphological changes in Rsc colonies due to lytic replication that changes genetically so that the virulence power changes with the detection method on the CPG-TZC medium. (a). Rsc with φBTF1 application, (b). Rsc with the φBTF2 application, (c). Rsc with φBTF3 application, (d). Rsc with φBTF4 and (e) applications Rsc without bacteriophage application (24 hours culture age).

4. Conclusions
The selected bacteriophages isolated from soils with healthy banana plants could control Rsc from banana plantations in vitro. The four selected bacteriophages tested showed that φBTF1, φBTF2, and φBTF4 did lytic replication by reducing the population of Rsc for 24 hours of observation while φBTF3 had lysogenic replication which kept Rsc remained alive but changing the bacterial pathogenic became avirulent.

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