BIODIVERSITAS
Volume 23, Number 10, October 2022
Pages: 5149-5158

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231022

Physio-biochemical, molecular characterization, and phage susceptibility of *Ralstonia pseudosolanacearum* associated with tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*)

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Manuscript received: 11 August 2022. Revision accepted: 5 October 2022.

Abstract. *Nurdika AAH, Arwiyanto T, Sulandari S. 2022. Physio-biochemical, molecular characterization, and phage susceptibility of* Ralstonia pseudosolanacearum *associated with tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*). Biodiversitas 23: 5149-5158. Ralstonia solanacearum* is a plant pathogen that causes bacterial wilt diseases in various plant species. The high diversity of these bacteria strains is the basis for their grouping into the *R. solanacearum* species complex (RSSC). RSSC associated with tomato and eggplant in Indonesia are commonly known as *R. solanacearum*. It is necessary to characterize *R. solanacearum* which infects tomatoes and eggplant according to the latest classification. The high diversity of strains also affects their susceptibility to bacteriophages as host-specific biocontrol agents. This research was conducted by characterizing *R. solanacearum* isolates from different locations based on their physio-biochemical properties, biovar, virulence, phylotype, sequevar, and susceptibility to 12 bacteriophage isolates. The phylotype identification was carried out using multiplex polymerase chain reaction with several specific primers for *R. solanacearum.* Amplification and sequencing based on the *egl* gene region were carried out to determine the sequevar of *R. solanacearum* isolates. As a result, isolates RS18, RS19, RS23, and RS24 had morphological, physio-biochemical, and biovar characteristics according to *Ralstonia pseudosolanacearum* phylotype I, biovar 3. The two most virulent isolates, RS19 and RS24 were identified as sequevar 14. RS19 and RS24 isolates were susceptible to 7 of the 12 bacteriophage isolates used in this study. Variations of bacteriophage isolates resulted in different plaque morphology which could be attributed to *R. pseudosolanacearum* susceptibility and bacteriophage virulence.

Keywords: Bacteriophage, *Ralstonia pseudosolanacearum*, *Ralstonia solanacearum* species complex, *Solanum lycopersicum*, *Solanum melongena*

INTRODUCTION

Ralstonia solanacearum is a plant pathogen that causes bacterial wilt disease in the Solanaceae family and other plant species (Saputra et al. 2015). The occurrence of bacterial wilt disease starts from the entry of *R. solanacearum* through wounds or natural openings on plant roots. These bacteria then thrive in the xylem vessels and produce extracellular polysaccharides (EPS) which blocked water supply and cause plant to wilt (Xue et al. 2020). Bacterial wilt can certainly inhibit plant growth and development. This bacterium is also soil-borne and has high survivability in soil, water, crop residues, and weeds, making it difficult to control completely. This condition causes *R. solanacearum* to be one of 10 plant pathogenic bacteria that are considered to have the potential to cause major losses in agriculture (Mansfield et al. 2012).

The wide host range of *R. solanacearum* and the diversity of its strains causes this bacterium to be grouped into *R. solanacearum* species complex (RSSC) (Fegan and Prior 2006). Various methods are used in the process of characterization of bacteria in RSSC. Characterizations that are commonly carried out are based on host range (race), region of origin (phylotype), ability to utilize carbon sources (biovar), and variation of *egl* gene sequences (sequevar). *Ralstonia solanacearum* is divided into 5 races based on their host range (Kelman et al. 1994) and 5 biovars (Hayward 1964; He et al. 1983) based on their ability to utilize or oxidize disaccharides (trehalose, maltose, cellobiose) and hexose alcohols (mannitol, sorbitol, dulcitol). Reclassification based on phylogenetic analysis divides RSSC into *R. pseudosolanacearum* (phylotypes I and III), *R. solanacearum* (phylotypes IIa and IIb), *R. syzygii* subsp. *syzygii*, *R. syzygii* subsp. *Indonesiensis*, and *R. syzygii* subsp. *celebensis* (phylotype IV) (Safni et al. 2014).

In Indonesia, the RSSC that infect horticultural plants from the Solanaceae family such as tomatoes, chilies, and eggplant are better known as *R. solanacearum*. In fact, there are at least several phylotypes with different species names that are capable of infecting the Solanaceae family. Species of *R. solanacearum* phylotypes I, IIa, IIb, and III are known to be associated with the Solanaceae family (Genin and Denny 2012). There is still quite a lack of studies on the characterization of local isolates of this bacterium based on physio-biochemical and molecular properties along with the reclassification of RSSC that

occurred. Thus, it is necessary to characterize the species that infect horticultural plants from the Solanaceae family. Isolates RS18, RS19, RS23, and RS24 are pathogens that cause bacterial wilt isolated from tomatoes and eggplant rhizosphere. These four isolates were confirmed to be virulent to tomato and eggplant, so they can be used as representations for characterization in this study.

The variety of strains in the RSSC certainly affects the control techniques used. Currently, *R. solanacearum* control techniques have been widely studied, one of which is by using bacteriophages as biocontrol agents. Bacteriophages are viruses that specifically infect bacteria and interfere with the metabolism of their hosts. This is done by injecting its genetic material and utilizing the host cell for the viral replication process. Bacteriophages have the advantage of high specificity, so they only infect the target bacteria without affecting other microbes (Loc-Carrillo and Abedon 2011). Bhunchoth et al. (2018) in their research identified 30 bacteriophage isolates from 4 different families (*Myoviridae*, *Podoviridae*, *Siphoviridae*, and *Inoviridae*) that were able to cause lysis of *R. solanacearum* cells.

However, due to the very high specificity of bacteriophages, differences in bacterial strains can affect the infectivity of bacteriophages. The various strains in the RSSC have different susceptibility to various bacteriophage isolates. Fujiwara et al. (2011) reported that the variety of isolates, either single or in combination, affected the lytic activity of bacteriophages against *R. solanacearum*. This study aims to characterize *R. solanacearum* that infects tomatoes and eggplants, its suitability with the classification of *R. pseudosolanacearum*, and its susceptibility to several bacteriophage isolates. As a result, the pathogens causing bacterial wilt disease in tomatoes and eggplants were identified as *R. pseudosolanacearum* phylotype I, biovar 3, sequevar 14. *Ralstonia pseudosolanacearum* tested in this study had different susceptibility to 12 bacteriophage isolates.

MATERIALS AND METHODS

Ralstonia pseudosolanacearum isolates

The *Ralstonia pseudosolanacearum* isolates used were a collection from the Plant Pathology Laboratory, Gadjah Mada University. One loop of bacteria suspension from the stock culture in sterile water streaked on casamino acid peptone glucose (CPG) medium. Colonies of *R. pseudosolanacearum* that grew after 48 hrs of incubation were characterized by cloudy white, fluid, convex, irregular, and opaque characteristics.

A total of 10 different isolates were used in this study (Table 1). All isolates were tested for their ability to infect Servo Tomatoes to confirm virulent isolates (data not shown). Based on the preliminary test, four virulent isolates RS18, RS19, RS23, and RS24 were selected for further testing. The remaining six isolates were used as a comparison in the phylotype characterization.

Physiological and biochemical characterization of *Ralstonia pseudosolanacearum*

Physiological and biochemical tests were carried out including Gram test, catalase enzyme production, growth under anaerobic conditions, nitrate reduction, and arginine dihydrolase activity. All tests were carried out as method describe by Schaad et al. (2000). The Gram test was carried out by mixing one loop of bacterial colonies with 3 % KOH in an object glass. The formation of a mucoid string is an indication of Gram-negative bacteria according to the characteristics of *R. pseudosolanacearum*. The production of catalase enzyme was carried out by mixing one loop of bacteria colony with hydrogen peroxide (H_2O_2) in an object glass. A positive reaction is indicated by the formation of air bubbles.

An anaerobic growth test was carried out on an agar medium containing bromothymol blue (BTB) in a test tube. One loop of bacterial colonies was inoculated into the BTB medium and liquid paraffin was poured on it to create conditions without oxygen. As a comparison, one loop of bacterial colonies was also inoculated into the BTB medium without paraffin given for available oxygen conditions. Changes in the color of the media from green to yellow in both treatments showed a positive response in the anaerobic growth test.

The nitrate reduction test was carried out using a nutrient agar medium containing 0.1% KNO_3 in a test tube. One loop of bacterial colonies was inoculated into the medium, then incubated for 24 and 48 hours. After incubation, 1 mL of reagent a (1 g α-naphthylamine in 200 mL 30% acetic acid) and 1 mL of reagent b (0.5 g sulfanilic acid in 150 mL 30% acetic acid) were dropped onto the media. The reduction of nitrate to nitrite is indicated by a change in the color of the medium to pink or red after 1 hr.

Table 1. *Ralstonia pseudosolanacearum* isolates used in this study

Isolate code	Plant host	Sample origin	Tested characteristics in this study
RS8	Tomato	Magelang, Central Java	Phylotipe
RS9	Tomato	Magelang, Central Java	Phylotipe
RS11	Tobacco	Klaten, Central Java	Phylotipe
RS16	Tomato	Bandung, West Java	Phylotipe
RS18	Tomato	Marga Mekar, West Java	Physio-biochemical, virulence, phylotype
RS19	Tomato	Marga Mukti, West Java	Physio-biochemical, virulence, phylotype, sequevar, susceptibility to phage
RS21	Tomato	Magelang, Central Java	Phylotipe
RS23	Eggplant	Temanggung, Central Java	Physio-biochemical, virulence, phylotype
RS24	Eggplant	Magelang, Central Java	Physio-biochemical, virulence, phylotype, sequevar, susceptibility to phage
RS25	Eggplant	Magelang Central Java	Phylotipe

Test of arginine dihydrolase activity using Thornley's medium in a test tube. The medium was inoculated with one loop of *R. pseudosolanacearum* colonies. A total of 1 mL of sterile paraffin was poured into the medium, then incubated for 24 hrs at 28ºC. The positive reaction for the degradation of arginine to ornithine + CO_2 + NH_3 was indicated by a change in the color of the medium from pink to red.

Tobacco hypersensitivity reaction and virulence test of *Ralstonia pseudosolanacearum*

Tobacco hypersensitivity reaction test

Bacteria colonies (48 hrs old) were suspended in 100 mL of sterile water. The bacterial suspension was then measured with a spectrophotometer with an OD_{600} (Optical density) value of 0.1 which is equivalent to a density of 10^8 CFU/mL. The bacterial suspension was taken as much as 1 mL with a syringe, then the needle was removed. The suspension was inoculated on the underside of tobacco leaves (Ji et al. 2007). The formation of necrosis on the leaves after 24 - 48 hrs of incubation was an indication that the isolate was capable of triggering a hypersensitivity reaction.

Virulence test of Ralstonia pseudosolanacearum *on tomato and eggplant*

Suspension of *R. pseudosolanacearum* was inoculated on tomato varieties Servo, Kaliurang, and eggplant EG203 varieties to determine the virulence of each isolate. *Ralstonia pseudosolanacearum* with an OD_{600} (Optical density) value of 0.1 was inoculated when the plant was 28 days old. A total of 10 mL of *R. pseudosolanacearum* suspension was poured on the roots of the test plants that had been injured using a sterile scalpel blade (Navitasari et al. 2020). Virulence was observed based on wilting symptoms, incubation period, disease incidence, and severity for 21 days after inoculation. The resistance levels of Servo, Kaliurang tomatoes and EG203 eggplant against bacterial wilt disease were categorized based on Laeshita and Arwiyanto (2017). Based on the results of the test, the two most virulent isolates were selected to be used for the identification of sequevar and susceptibility to bacteriophages.

Testing of *Ralstonia pseudosolanacearum* Biovar

Testing of *R. pseudosolanacearum* biovar was carried out according to the method by Hayward (1964) to classify *R. solanacearum* into 5 biovars based on their ability to utilize monosaccharides (dextrose), 4 disaccharides (lactose, trehalose, maltose, cellobiose), and 3 hexose alcohols (mannitol, sorbitol, dulcitol). The basal medium used as the basis for the test consisted of 1 gram of $NH_4H_2PO_4$, 0.2 gram of KCl, 0.2 gram of $MgSO_47H_2O$, 1 gram of peptone, 0.08 gram of bromothymol blue, 1.5 gram of agar, and 1 liter of sterile distilled water. The pH of the basal medium was mantained to 7.0. Carbon sources in the form of monosaccharides, disaccharides, and hexose alcohols were sterilized separately and added 5 mL of each (10% concentration) for every 45 mL of basal medium. Colonies of *R. pseudosolanacearum* were suspended in sterile water and measured OD_{600} value of 0.1. A total of 20 µL of bacterial suspension was inoculated on the surface of the medium in a test tube. The tube containing the media was then incubated at a temperature of 28-32ºC and observed for color changes at 3, 7, 14, 21, and 28 days after inoculation.

Molecular characterization of *Ralstonia pseudosolanacearum*

DNA extraction

DNA extraction was performed using the cetyltrimethylammonium bromide (CTAB) method (Ausubel et al. 2010) with adjustments. A total of 3 loops of *R. pseudosolanacearum* colonies were suspended with 540 µL TE buffer and 30 µL 10% SDS in a 1.5 mL tube. The suspension was then incubated at 37ºC for 1 hr. The suspension was then mixed with 100 µL of 5M NaCl and 80 µL of CTAB/NaCl. The suspension was incubated at 65ºC for 10 min in a water bath. Then 750 µL of chloroform:isoamyl alcohol (CIAA) was added to the suspension, shaken until well mixed, and centrifuged at 12000 rpm for 5 min. The supernatant formed was transferred to a new tube, added with 600 µL of phenol:chloroform:isoamyl alcohol (PCIAA), and centrifuged at 12000 rpm for 5 min. The supernatant was transferred again to a new tube and added 96% ethanol (1 M) as much as 0.6 x the volume of the supernatant and incubated for 1 hr at -20ºC. The suspension was then centrifuged at 12000 rpm for 5 min and the resulting pellet was washed using 300 µL of 70% ethanol by vortexing. The pellets were then centrifuged at 12000 rpm for 5 min. The supernatant was discarded, the pellet was dried and 30 µL of TE buffer was added for storage at -20ºC.

Phylotype identification by Multiplex PCR

Ralstonia pseudosolanacearum phylotypes were identified based on the method of Sagar et al. (2014) and Cho et al. (2018) with multiplex PCR using different primers (Table 2). Phylotype-specific multiplex PCR was carried out with the final volume of the reaction mixture as much as 25 µL. The mixture contains 12.5 µL of 2x MyTaq Red Mix (Bioline), 2 µL DNA Template, 3.5 µL nuclease free water, 1 µL of each primers (Nmult:21:1 F, Nmult:21:2 F, Nmult:22:InF, Nmult:23:AF, Nmult:22:RR, 759 R and 760 F). The PCR program used was pre-denaturation at 96ºC for 5 min, then 30 cycles of denaturation at 94ºC for 15 sec, annealing at 59 ºC for 30 sec, and extension at 72ºC for 30 sec, followed by a final extension for 10 min at 72ºC. A total of 5 µL PCR products were visualized using the electrophoresis method on 1% agarose gel stained with ethidium bromide. Observation of the DNA bands formed was carried out on a UV-transilluminator.

Identification of sequevar

Sequevar identification was carried out with ENDO-F and ENDO-R primers (Ji et al. 2007) targeting the *egl* gene region (Table 2) with a 50 µL reaction mixture consisting of 25 µL 2x MyTaq Red Mix (Bioline), 1 µL of each primer, 3 µL DNA template, and 20 µL nuclease free

water. PCR program used was pre-denaturation at 96ºC for 3 min, then 30 cycles of denaturation at 95ºC for 30 sec, annealing at 69ºC for 45 sec, and extension at 72ºC for 2 min, followed by final extension for 10 min at 72ºC (Ji et al. 2007; Siregar et al. 2021). PCR product visualized using electrophoresis method on 1 % agarose gel stained with ethidium bromide. The PCR product with 750 bp size was then sequenced with the services of 1st BASE Sanger sequencing. Phylogenetic analysis was then performed on the sequences using MEGA X software compared to the *R. solanacearum* species complex sequences from GenBank (http://www.ncbi.nlm.nih.gov).

Ralstonia pseudosolanacearum susceptibility to bacteriophage

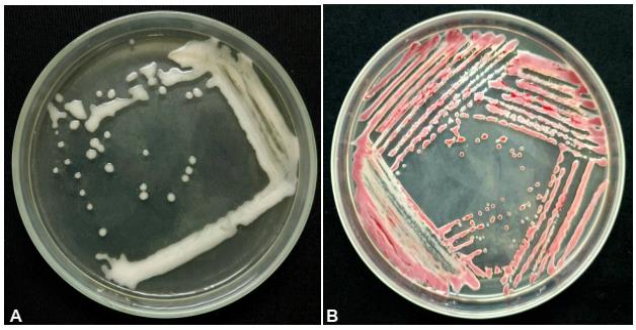
The test was performed using the double layer agar plaque assay method (Bae et al. 2012) to determine the susceptibility of *R. pseudosolanacearum* to 12 different bacteriophage isolates (Table 3). The bacteriophage isolates used are a collection from the Plant Pathology Laboratory, Gadjah Mada University. Colonies of *R. pseudosolanacearum* suspended in sterile water (OD_{600} value 0.1) were added to 0.6% CPG medium as much as 0.5 mL. A total of 1 mL of the bacteriophage suspension was added to the same 0.6% CPG medium. Then, 0.6% CPG media which has been added with *R. solanacearum* and bacteriophage was then poured into a Petri dish containing CPG media as the base. The media was incubated for 24 to 48 hrs at 30ºC. The plaque formed was observed and indicated the ability of bacteriophages to infect *R. solanacearum.*

RESULTS AND DISCUSSION

Colony Morphology of *Ralstonia pseudosolanacearum*

The colony morphology of the four strains of *Ralstonia pseudosolanacearum* tended to be similar. Colonies on CPG medium were cloudy white, fluid, irregular, and convex (Figure 1A). The morphology of *R. pseudosolanacearum* on CPG medium was matched with the description of He et al. (1983) and Pawaskar et al. (2014). Slimy white colonies, non-transparent, and irregular shapes are characteristics of *R. solanacearum* which are still virulent (Arwiyanto 2014).

Colonies that were virulent on tetrazolium chloride (TZC) medium had a bright red or pink center with wider white margins (Figure 1.B). The form of this colony was based on the ability of the virulent strain to reduce formazan compounds in the media with the addition of TZC. This was to distinguish virulent strains from avirulent strains which were round, small, dry, and the entire colony is red (Arwiyanto 2014).



A **B**

Figure 1. *Ralstonia pseudosolanacearum* colonies aged 48 hours on CPG medium (A) and TZC medium (B)

Table 3. Bacteriophage isolates used in this study

Bacteriophage isolate	Isolated from plants	Sample origin
NGW	Tomato	Ngawen, Yogyakarta
MLG	Tomato	Malang, East Java
JTNM	Tomato	Jatinom, Central Java
CAB1	Chili	Boyolali, Central Java
CAB2	Chili	Boyolali, Central Java
GRLY	Tomato	Girimulyo, Central Java
BLKT	Tomato	Bulukerto, Central Java
MGMK	Tomato	Margo Mekar, West Java
ASV1	Grafted Tomato	Yogyakarta
ASV2	Grafted Tomato	Yogyakarta
HSV2	Grafted Tomato	Yogyakarta
HSV3	Grafted Tomato	Yogyakarta

Table 2. List of primers for identification of phylotypes and sequevar of *Ralstonia solanacearum*

Primer	Sequence	Target	Description
Nmult:21:1 F	5′-CGTTGATGAGGCGCGCAATTT-3′	144 bp	Phylotipe I (Asiaticum)
Nmult:21:2 F	5′-AAGTTA TGGACGGTGGAAGTC-3′	372 bp	Phylotipe II (Americanum)
Nmult:22:InF	5′-ATTGCCAAGACGAGAGAAGTA-3′	213 bp	Phylotipe IV (Tropical)
Nmult:23:AF	5′-ATTACGAGAGCAATC GAAAGATT-3′	91 bp	Phylotipe III (African)
Nmult:22:RR	5′-TCGCTTGACCCTATAACGAGTA-3′		Amorce reverse unique
759 R	5′-GTCGCCGTCAACTCACTTTCC-3′	280 bp	Specific primer for *Ralstonia*
760 F	5′-GTCGCCGTCAGCAATGCGGAATCG-3′		*solanacearum*
ENDO-F	5′-ATGCATGCCGCTGGTCGCCGC-3′	750 bp	*egl* gene
ENDO-R	5′-GCGTTGCCCGGCACGAACACC-3′		

Physiological - biochemical characteristics and Biovar of *Ralstonia pseudosolanacearum*

The four strains tested in this study had similar physiological-biochemical characteristics. All strains were Gram-negative, showing a positive reaction on the hypersensitivity test, catalase, nitrate reduction, and oxidative growth, but negative on the fermentative growth and arginine dihydrolase activity test (Table 4). The results of physiology-biochemical testing in this study were matched with the characteristic of *R. solanacearum* (Hayward 1964; He et al. 1983; Pawaskar et al. 2014; Razia et al. 2021).

All isolates of *R. pseudosolanacearum* used were able to trigger hypersensitivity reactions in tobacco leaves. Necrotic spots on leaves appear 24 hrs after pathogen inoculation. The ability of *R. pseudosolanacearum* to trigger hypersensitivity reactions in tobacco is one of the characteristics of pathogenic bacteria. *Ralstonia pseudosolanacearum* was able to trigger a hypersensitivity reaction in tobacco leaves because this bacterium has an Hrp-type III secretion system (T3SS). This secretion mechanism plays a role in the pathogenesis process which can trigger a hypersensitivity reaction (Razia et al. 2021).

Gram testing using 3% KOH produces a mucoid string as a characteristic of Gram-negative bacteria. The isolate of *R. pseudosolanacearum* was also able to break down hydrogen peroxidase which was indicated by the formation of the air bubbles. *Ralstonia pseudosolanacearum* was able to utilize carbon sources only in conditions of oxygen available. This was indicated by the color change in the oxidative medium to yellow and not in the fermentative medium after 48 hrs of incubation. The positive reaction in the nitrate reduction test was seen in the change in color of the medium to red with a cracked surface after 24 hs of incubation and the addition of reagent a (α-naphthylamine) and reagent b (sulfanilic acid). Negative results only occur in the arginine dihydrolase test. The medium did not change color after 48 hours of incubation. This condition indicates the inability of *R. pseudosolanacearum* to degrade arginine.

All *R. pseudosolanacearum* strains in this study were identified as biovar 3. This was indicated by the ability of the four strains to utilize monosaccharides (dextrose), 4 disaccharides (lactose, trehalose, cellobiose, and maltose), and 3 hexose alcohols (sorbitol, dulcitol, and mannitol) as a carbon source (Table 5). The color change of the medium from green to yellow in 3 to 28 days after inoculation indicates a positive reaction in this test. This condition is characteristic of *R. solanacearum* biovar 3 (Hayward, 1964; He et al. 1983). Based on the distribution of *R. solanacearum* biovar 3, 4, and 5 are common in Asian countries (Huang et al. 2012).

Virulence of *Ralstonia pseudosolanacearum* on tomato and eggplant

The virulence of the four *R. pseudosolanacearum* isolates tended to be different on tomato and eggplant. This is indicated by a different incubation period, incidence, and severity of bacterial wilt in the test plants (Table 6). Strains RS19 and RS24 had the shortest incubation period in Servo and Kaliurang tomatoes. *Ralstonia pseudosolanacearum* RS24 was able to cause wilting symptoms starting at 3 days after inoculation (DAI) in Kaliurang tomatoes and 4 DAI in Servo tomatoes. The wilt caused by the *R. pseudosolanacearum* RS19 on Kaliurang tomatoes appeared at 4 DAI, while in Servo at 5 DAI. The four isolates needed a longer time to cause wilting in eggplant EG203. The fastest incubation period in EG203 was shown by isolates *R. pseudosolanacearum* RS19 and RS23 at 12 DAI. The incubation period in both tomato varieties tended to be faster than previously reported results. Laeshita and Arwiyanto (2017) reported that the incubation period of *R. pseudosolanacearum* in Servo and Kaliurang tomatoes was 6 days after inoculation. Both varieties also tend to be moderately resistant to *R. pseudosolanacearum*. It was suspected that the four isolates used in this study had higher virulence in tomatoes.

Table 4. Physiology and biochemistry characters of 4 isolates of *Ralstonia pseudosolanacearum*

Isolate	Hypersensitivity reaction	Gram	Catalase	Arginine dihydrolase	Oxidative	Fermentative	Nitrate reduction
RS18 (Tomato)	+	-	+	-	+	-	+
RS19 (Tomato)	+	-	+	-	+	-	+
RS23 (Eggplant)	+	-	+	-	+	-	+
RS24 (Eggplant)	+	-	+	-	+	-	+

Note: +: Positive reaction, (-: negative reaction.

Table 5. Biovar testing of 4 isolates of *Ralstonia pseudosolanacearum* utilizing different monosaccharides, disaccharides, and hexose alcohols

Isolate	Dextrose	Lactose	Trehalose	Cellobiose	Maltose	Sorbitol	Dulcitol	Mannitol	Biovar
RS18 (Tomato)	+	+	+	+	+	+	+	+	3
RS19 (Tomato)	+	+	+	+	+	+	+	+	3
RS23 (Eggplant)	+	+	+	+	+	+	+	+	3
RS24 (Eggplant)	+	+	+	+	+	+	+	+	3

Note: +: Positive reaction, -: negative reaction.

Table 6. Virulence testing of 4 isolates of *Ralstonia pseudosolanacearum* on Servo, Kaliurang tomatoes, and EG203 eggplant

Isolate	Incubation Period (DAI)			Incidence (%)			Disease Severity (%)		
	Servo	Kaliurang	EG203	Servo	Kaliurang	EG203	Servo	Kaliurang	EG203
RS18 (Tomato)	7	4	15	50.00	80.00	10.00	36.00	56.00	6.00
RS19 (Tomato)	5	4	12	60.00	80.00	20.00	36.00	56.00	20.00
RS23 (Eggplant)	8	4	12	40.00	70.00	30.00	28.00	46.00	24.00
RS24 (Eggplant)	4	3	13	60.00	70.00	30.00	50.00	56.00	24.00

In general, the incidence of wilt disease caused by the four isolates was not too different. The *R. pseudosolanacearum* RS19 and RS24 strains produced the highest wilt incidence in Servo tomatoes (60%), while in the Kaliurang tomatoes, *R. pseudosolanacearum* RS18 and RS219 tended to have a higher incidence (80%). The incidence of bacterial wilt in eggplant EG203 tends to be low, with the highest being 30% in *R. pseudosolanacearum* RS23 and RS24. Similar results regarding eggplant EG203 were previously reported. Eggplant EG202 infected with two different *R. solanacearum* strains showed a low disease incidence of bacterial wilt. *Ralstonia solanacearum* strain Pss97 strain was recorded to cause the bacterial incidence of only 8.9%, while the *R. solanacearum* strain Pss2016 caused a 20% incidence Rakha et al. (2020).

Based on the resulting disease severity, *R. pseudosolanacearum* RS24 tended to be the most virulent because it produced the highest severity in all of the test plants (Servo 50%, Kalurang 56%, and EG203 24%). *Ralstonia pseudosolanacearum* RS18 and RS19 caused disease severity the same disease severity in Servo and Kaliurang tomatoes, but *R. pseudosolanacearum* RS19 was more virulent to eggplant EG203. The value of the area under the disease progress curve (AUDPC) also shows that *R. pseudosolanacearum* RS19 and RS24 tend to be more virulent (Figure 2). The highest AUDPC values in Servo and Kaliurang tomatoes were seen in RS19 infection. While the highest AUDPC value on EG203 is shown by *R. pseudosolanacearum* RS24.

Based on the severity of the disease, Servo tomatoes tend to be moderately susceptible and Kaliurang tomatoes are susceptible to *R. pseudosolanacearum* RS18, RS19, and RS24. Meanwhile, eggplant EG203 was only moderately susceptible to *R. pseudosolanacearum* RS23 and RS24, but moderately resistant to *R. pseudosolanacearum* RS18 and RS19 (Table 7). The plant species that became the original host of the four isolates were thought to affect the ability to infect the test plants. *Ralstonia pseudosolanacearum* RS19 and RS24 which were the 2 most virulent strains in eggplant and tomato, were then identified for their sequevar and susceptibility to bacteriophages.

The ability of the four isolates to infect tomatoes and eggplant is one of the characteristics of *R. solanacearum* race 1. The wide range of hosts, especially those from the *Solanaceae* family such as tomatoes and eggplants, is characteristic of race 1 (Paudel et al. 2020). *R. pseudosolanacearum* RS19 and RS24 had higher virulence in tomato and eggplant than *R. pseudosolanacearum* RS18 and RS23. Various references show differences in the resistance of Servo tomato, Kaliurang tomato, and EG203 eggplant to *R. solanacearum*. Servo is classified as susceptible to *R. solanacearum* phylotype 1, race 1, and biovar 3 (Navitasari et al. 2020). However, there are also reports that the Servo and Kaliurang tomato are moderately resistant to *R. solanacearum* (Laeshita and Arwiyanto 2017). Rakha et al. (2020) and Namisy et al. (2019) mention that EG203 eggplant was resistant to *R. solanacearum* strain Pss97 and moderately resistant to strain Pss2016. This resistance level of EG203 matches the description from AVRDC. Eggplant EG203 is generally classified as resistant to *R. pseudosolanacearum* and is often used as a resistant rootstock in grafting techniques. Arwiyanto et al. (2015) reported that the use of eggplant EG203 as a rootstock in grafting with susceptible tomatoes showed a lower bacterial wilt incidence than local tomatoes without grafting.

Table 7. Resistance levels of tomato and eggplant against bacterial wilt

Isolate	Resistance Level		
	Servo	Kaliurang	EG203
RS18 (Tomato)	MS	S	MR
RS19 (Tomato)	MS	S	MR
RS23 (Eggplant)	MS	MS	MS
RS24 (Eggplant)	MS	S	MS

Note: S: Susceptible, MS: Moderate Susceptible, MR: Moderate Resistant.

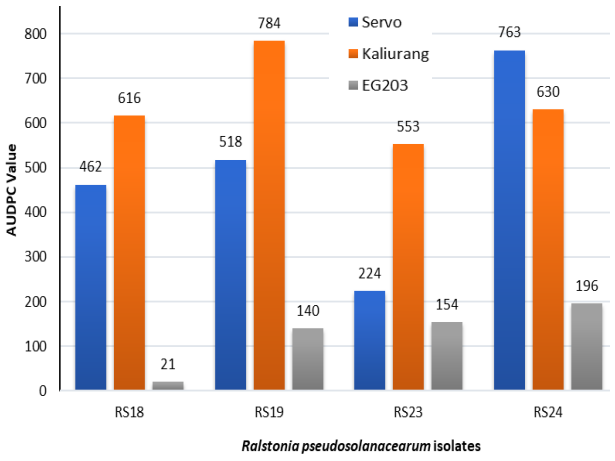


Figure 2. Value of area under disease progress curve (AUDPC). Data were collected at 7 - 21 days after inoculation (DAI)

Phylotype and sequevar of *Ralstonia pseudosolanacearum*

The results of PCR product visualization showed that the four isolates were detected with a specific primer of *R. solanacearum* and belonged to phylotype I (Asiaticum). This was indicated by amplicons with DNA bands with a size of 144 bp and 280bp (Figure 3.A). The DNA band with a size of 144 bp that appears in the visualization of the four samples corresponds to the target of Nmult:21:1:F primer for *R. solanacearum* phylotype I or belongs to the species *R. pseudosolanacearum.* All other comparison isolates were also identified as phylotype I. The phylotype differences in *R. solanacearum* were related to geographic origin. Phylotype I originating from Asia includes *R. solanacearum* biovar 3, 4, and 5 (Sagar et al. 2014). The geographic origin of the isolates influences the host range and the ability to thrive in different environmental conditions (Santiago et al. 2017).

The *R. solanacearum* phylotypes I and III are currently included in a group under the species name *R. pseudosolanacearum* (Safni et al. 2014; Garćia et al. 2019). The other group consisted of phylotypes IIa and IIb which included *R. solanacearum* and phylotype IV including *Ralstonia syzygii*. All isolates of *R. pseudosolanacearum* used in this study had the same phylotype, apart from differences in plant host (tomato, eggplant, and tobacco) and region of origin (Central Java and West Java). The results are consistent with the study of *R. solanacearum* genetic diversity in horticultural crops on Java island (Hemelda et al. 2019). A total of 20 out of 21 *R. solanacearum* isolates from Java Island were phylotype I.

The dominance of *R. solanacearum* phylotype I from samples in Java island illustrates the difference with dominant phylotypes in other regions. Ji et al. (2007) reported that 27 of 30 isolates of *R. solanacearum* that infect horticultural plants in Florida were classified as phylotype II. Only three isolates were phylotype I. Santiago et al. (2017) also reported similar results with 253 isolates of *R. solanacearum* from various regions in Brazil identified as phylotype II, only 48 isolates were phylotype I. Phylotype I as the dominant strain in a region was reported by Li et al. (2016). A total of 89 isolates that infect tobacco in China were detected as *R. solanacearum* phylotype I.

DNA from *R. pseudosolanacearum* RS19 and RS24 were then amplified with ENDO-F and ENDO-R primers targeting the endoglucanase (*egl*) gene region. The result is a DNA band with a size of 800 bp (Figure 3.B). Amplicons were sequenced and aligned with 22 sequences of *Ralstonia solanacearum* species complex phylotype I, IIa, IIb, III, and IV from GenBank (ncbi.nlm.nih.gov). *Ralstonia pseudosolanacearum* RS19 and RS24 based on the results of the phylogenetic analysis were included in *Ralstonia pseudosolanacearum* phylotype I, sequevar 14 clade (Figure 4). RS19 was identical to the *Ralstonia solanacearum* Pss81 sequevar 14 with a bootstrap value of 99. Meanwhile, RS24 tended to be identical with *Ralstonia pseudosolanacearum* RiE-Lk-047 sequevar 14 with a bootstrap value of 99.

Sequevar characterization in *R. solanacearum* species complex was based on sequence differences in the endoglucanase (*egl*) gene (Sagar et al. 2014). *Ralstonia solanacearum* phylotype I, sequevar 14 was previously reported to infect tomato (Jimenez Madrid et al. 2019; Ren et al. 2022), tobacco (Li et al. 2016; Liu et al. 2017), and eucalyptus (Carstensen et al. 2017). RS19 and RS24 were isolated from different plant hosts, tomato and eggplant. This host difference corresponds to the characteristics of *R. solanacearum* phylotype I which was the widest host range compared to other phylotypes (Lin et al. 2014).

RS19 had a high similarity with *R. solanacearum* Pss81 isolate from China, while RS24 was similar to *R. pseudosolanacearum* RIE-Lk-047 from Indonesia. The two isolate belonged to phylotype I sequevar 14. Siregar et al. (2021) in their research mapped the diversity of *R. pseudosolanacearum* that infects *Eucalyptus Pellita* in Indonesia. *Ralstonia pseudosolanacearum* RIE-Lk-047 was isolated from the Riau region. Based on that research, sequevar 14 is one of the *R. pseudosolanacearum* variants commonly found in Indonesia, including Riau, East Kalimantan, East Java, and Yogyakarta.

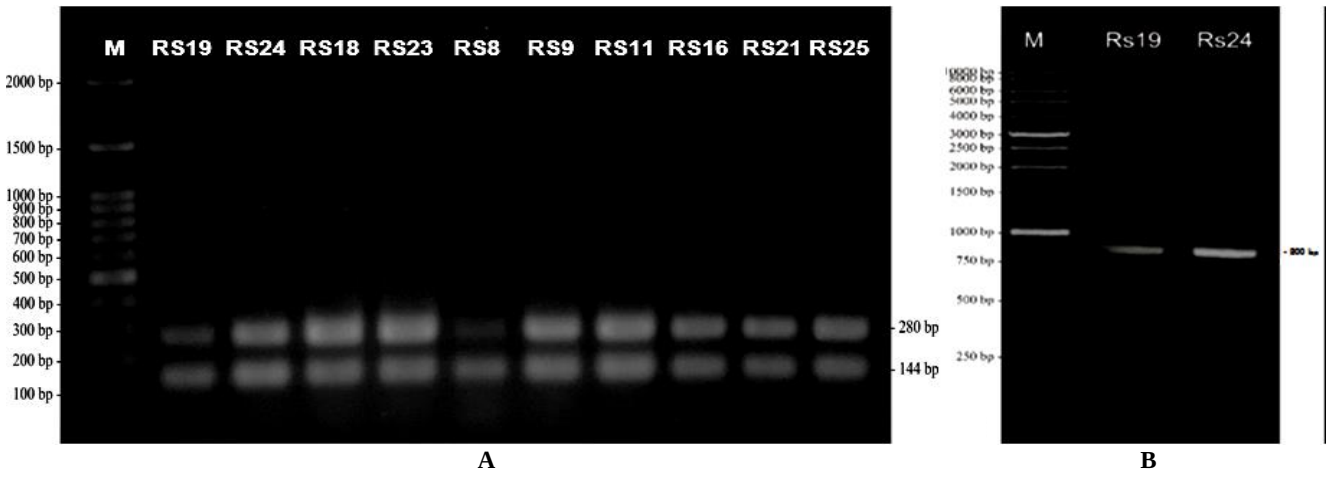


Figure 3. Visualization of DNA amplicons of *Ralstonia pseudosolanacearum:* A. Identification of phylotype by multiplex PCR method, 2 DNA bands with size 144 bp and 280 bp appear, B. Amplification of endoglucanase gene region, DNA band with the size around 800 bp appear

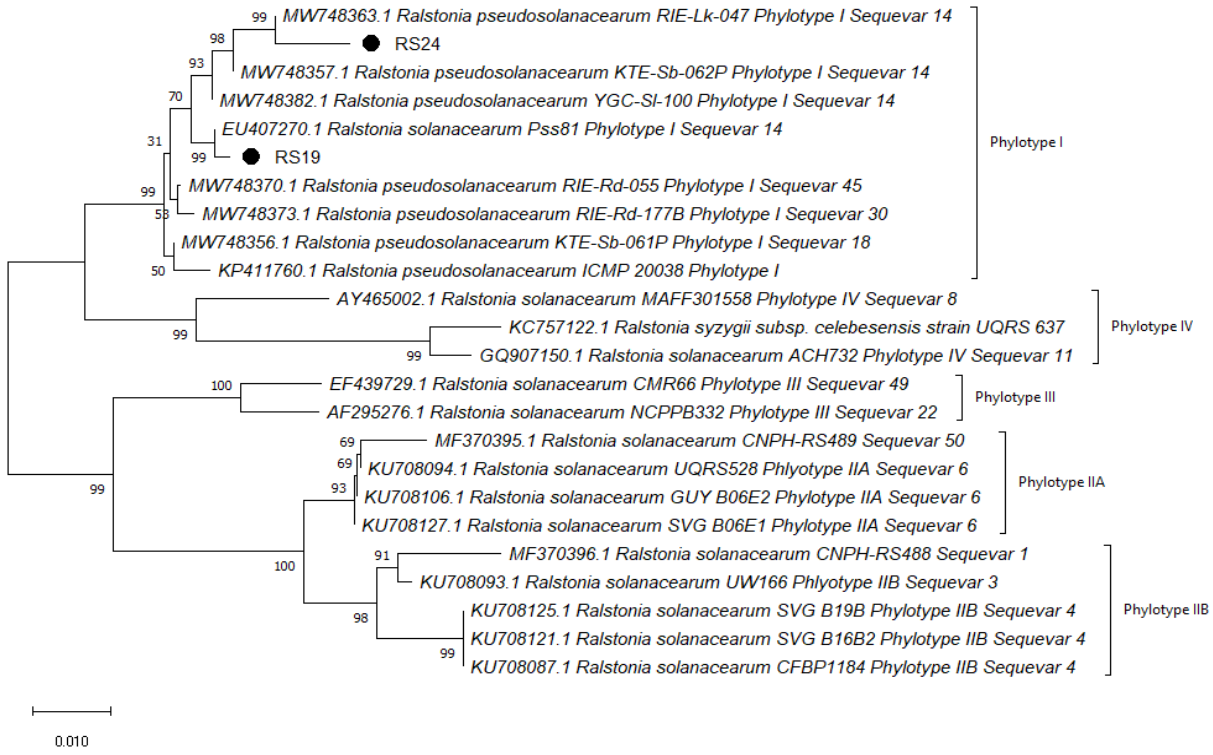


Figure 4. Phylogenetic analysis showed the phylotype and sequevar of *Ralstonia pseudosolanacearum* isolate RS19 and RS24

Susceptibility of *Ralstonia pseudosolanacearum* to Bacteriophages

The double layer agar plaque assay showed that only seven out of a total of twelve bacteriophage isolates can infect *R. pseudosolanacearum* RS19 and RS24 (Table 8). Plaque formed on the surface of the 0.6% CPG medium after 48 hours of incubation. The size of the plaque produced is quite varied, ranging from 1-4 mm. In general, there are 4 distinct plaque morphologies (Figure 5). The plaque that appears has a clear plaque with a round shape, a cloudy round plaque with a point in the middle (bull's eye), a cloudy round with a halo around it, and an irregularly cloudy plaque.

Bacteriophage isolate NGW and JTNM produced plaques that appeared irregular, cloudy, and concentrated on certain sides of the media. ASV1, ASV2, HSV2, HSV3, and MLG phage isolates produced round plaques that spread throughout the media. The morphology of clear round plaque was seen in MLG isolates, while ASV1, ASV2, HSV1, and HSV3 isolates had various round plaque morphology. Bacteriophage CAB1, CAB2, BLKT, and MGMK did not show any plaque formation.

Plaque is a sign of the lytic activity of bacteriophage against its host cells. Bacteriophages at the end of the infection-replication process in their host cells will encode the production of endolysin. This enzyme plays a role in causing the lysis of bacterial cells (Dy et al. 2018). The plaque morphology produced by 7 bacteriophage isolates tended to be different. The virulence of bacteriophages to their host bacteria is related to the morphology of plaque formed. Jurczak-Kurek et al. (2016) described that clear plaque is a characteristic of virulent lytic phages. Plaque that tends to be cloudy or with a point in the middle is thought to be due to decreased phage lysis ability. This occurs due to the aging of the host bacteria in the culture or inhibited lysis due to host resistance. Variations in the morphology of bacteriophage plaques associated with *R. solanacearum* were also reported by Barua and Nath (2019). Variations that occur due to differences in adsorption rate, the time required for lysis, virion size, and host strain. Determination of the spectrum of bacteria strains that can be infected is very important before combining several bacteriophage isolates. Host spectrum testing is intended to ensure that the bacteriophage mixture used is capable of infecting hosts with high strain diversity (Kalpage and De Costa 2015).

In conclusion, the bacteria that cause bacterial wilt infecting tomato and eggplant in this study were identified as *R. pseudosolanacearum* phylotype 1, biovar 3, and sequevar 14. The susceptibility of *R. pseudosolanacearum* to several local isolates of bacteriophage showed the potential for biocontrol of this pathogen. In the future, further research is needed on the effectiveness of the combination of bacteriophage isolates in suppressing *R. pseudosolanacearum.*

Table 8. Bacteriophage plaque formation on CPG medium with *Ralstonia pseudosolanacearum*

Bacteria isolate	Bacteriophage isolate											
	NGW	MLG	JTNM	CAB1	CAB2	GRLY	BLKT	MGMK	ASV1	ASV2	HSV2	HSV3
RS19	+	+	+	-	-	-	-	-	+	+	+	+
RS24	+	+	+	-	-	-	-	-	+	+	+	+

Note: (+): Plaque is formed, (-): No plaque formed.

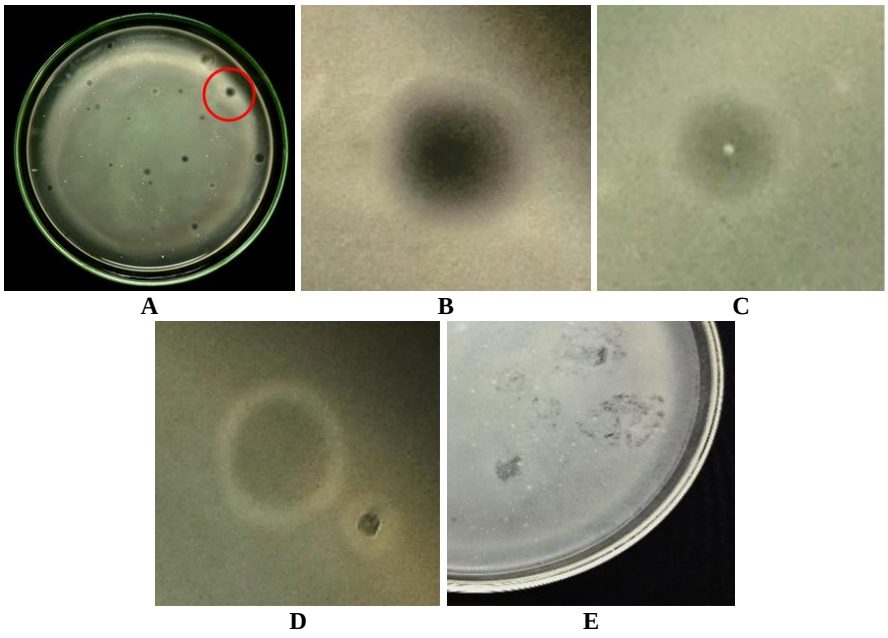


Figure 5. Plaques from Bacteriophage that infect *Ralstonia pseudosolanacearum*: A. Plaques spread on CPG medium, B. Clear and round plaque morphology, C. Cloudy plaque with a point in the center, D. Cloudy with a halo around it, and E. irregularly cloudy plaque

ACKNOWLEDGEMENTS

The data in this publication was a part of the master thesis of the first author, Anggi Anwar Hendra Nurdika. This research was funded by the Indonesia Endowment Fund for Education (LPDP), Ministry of Finance of Republic of Indonesia as part of the first author scholarship fund (LPDP awardee no: 20200811154106). The research was also funded by Universitas Gadjah Mada with the scheme of Rekognisi Tugas Akhir, No: 3143/UN1.P.III/DIT-LIT/PT/2021 dated 10 May 2021. The authors declare that there is no potential conflict of interest.

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