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Morphological and biochemical identification of *Ralstonia solanacearum* strains in ginger (*Zingiber officinale* Roscoe) wilt disease

Identifikasi morfologi dan biokimia strain *Ralstonia solanacearum* pada penyakit layu jahe (*Zingiber officinale* Roscoe)

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ABSTRACT
The *Ralstonia solanacearum* Species Complex (RSSC) is a significant plant pathogen affecting various agricultural commodities, including ginger. Understanding the distribution and characteristics of RSSC in ginger plants is crucial for effective disease management. This study aims to determine the distribution of RSSC in ginger plants and identify the pathogen through morphological and biochemical characterization. The research was conducted in Banyubiru and Getasan districts, Semarang Regency, Indonesia. The study involved purposive sampling, isolation, and purification of isolates, followed by morphological characterization through observation and biochemical characterization using Gram staining with KOH, oxidase test, arginine activity test, hypersensitivity test, bacterial density calculation, Koch's Postulates, and biovar characterization. Three isolates from Banyubiru displayed typical morphological characteristics of RSSC, including round, mucoid colonies with red centers and white edges on TZC medium, and rod-shaped bacterial cells. Biochemical characterization identified these isolates as RSSC strains biovar 3 and 4, capable of causing bacterial wilt in ginger plants. The study confirmed the presence of bacterial wilt in ginger in Banyubiru district. The findings reveal the spread of bacterial wilt caused by RSSC in ginger plants in Banyubiru, Semarang Regency. There is a need for measures to control the potential spread of RSSC in the surrounding host plant areas in Tlumpak Village, Banyubiru district.
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ABSTRAK
Ralstonia solanacearum Species Complex (RSSC) merupakan salah satu bakteri patogen tanaman penting yang dapat menyerang komoditas pertanian seperti jahe. Penelitian ini bertujuan untuk mengetahui sebaran RSSC pada tanaman jahe serta mengidentifikasinya melalui morfologi dan karakterisasi biokimia. Penelitian dilakukan di Kecamatan Banyubiru dan Kecamatan Getasan, Kabupaten Semarang, Indoneisa. Tahapan penelitian melalui *purposive sampling,* isolasi dan purifikasi, karakterisasi morfologi dengan pengamatan, serta karakterisasi biokimia melalui uji gram dengan KOH, uji oksidase, uji aktivitas arginin, uji hipersensitif, perhitungan kerapatan bakteri, Postulat Koch, dan karakterisasi biovar. Hasil pengujian menunjukkan 3 isolat dari Kecamatan Banyubiru, Indonesia memiliki ciri morfologi RSSC. Isolat tersebut tergolong bakteri virulen, terbukti pada media TZC koloni bakteri berbentuk bulat, berlendir, dan pada bagian tengah berwarna merah bentuk spiral dengan tepi berwarna putih. Sel bakterinya berbentuk rod/batang. Berdasarkan karakterisasi biokimia, isolat-isolat yang positif merupakan RSSC strain biovar 3 dan 4 dan terbukti dapat menyebabkan penyakit layu bakteri pada tanaman jahe. Hasil penelitian ini, diketahui bahwa penyakit layu bakteri pada tanaman jahe telah tersebar di Kecamatan Banyubiru, Kabupaten Semarang. Perlu adanya suatu tindakan untuk mengatasi adanya potensi penyebaran RSSC pada sekitar lahan inang RSSC di Dusun Tlumpak, Kecamatan Banyubiru, Kabupaten Semarang.

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INTRODUCTION

The *Ralstonia solanacearum* Species Complex (RSSC) is a significant group of plant pathogenic bacteria (PPB) or phytopathogens. RSSC is known for its high tolerance and environmental adaptability (An & Zhang, 2024; Clough et al., 2024). Globally, RSSC is recognized as the causative agent of wilt disease in various cultivated plants. Uncontrolled RSSC can lead to significant losses in agricultural production, causing physiological damage to infected plants. RSSC is a soil-borne pathogen that can spread rapidly through soil, water, roots, agricultural tools, infected plant residues, and even field workers. RSSC causes bacterial wilt, with infections often leading to plant death following initial wilting symptoms (Choudhary et al., 2018; Arwiyanto, 2014; Zhang et al., 2018).

Numerous cultivated plants worldwide have been identified as hosts of RSSC. However, research on RSSC, particularly in Indonesia, over the past five years has primarily focused on tomato (*Solanum lycopersicum* L.) (Nurdika et al., 2022), banana (*Musaceae*) (Ray et al., 2021), pepper (*Piper* spp.) (Kwon et al., 2021), eggplant (*Solanum melongena* L.) Nurdika et al., 2022), and cosmos (*Cosmos caudatus* Kunth) (Hanudin et al., 2014). Research on RSSC in Indonesia remains limited, both in terms of isolation techniques and efficient control methods. Like other plant pathogenic bacteria, such as *Xanthomonas oryzae* causing rice leaf blight, *Ralstonia solanacearum* is known to have specific bacteriophages (Choliq et al., 2020; Navitasari et al., 2022). However, further research is needed on controlling RSSC in the future.

To support further research, it is essential to conduct foundational studies that provide specific insights, such as the identification and characterization of RSSC in various commodities like ginger (*Zingiber officinale* Roscoe). Ginger is a high-potential commodity in Indonesia with significant productivity and export value, yet comprehensive studies on its association with RSSC as a host are lacking (Elpawati et al., 2022; Kannan et al., 2015; Nurhidayati et al., 2022; Rusnaldi et al., 2023). Existing research indicates that *Ralstonia* infecting ginger in Indonesia includes *R. solanacearum* strains biovar 3 and 4 (Hemelda et al., 2019), with biovar 3 phylotype 1 strains being the most prevalent, also affecting Solanaceae crops such as tobacco, tomato, and eggplant. According to Wicker et al. (2012), bacterial wilt caused by *R. solanacearum* biovar 3 phylotype 1 is found in Japan, Thailand, India, and Indonesia. Meanwhile, *R. solanacearum* biovar 4 phylotype 1, more commonly infecting ginger, has spread widely in Asia, particularly in China, Japan, Thailand, India, Indonesia, and even Australia (Paudel et al., 2020; Prameela, 2020; Ramesh et al., 2014).

Banyubiru and Getasan districts are among the top five ginger-producing areas in Semarang Regency, Indonesia, with annual production reaching 1,030,000 kg and 4,250,000 kg, respectively (BPS, 2022). This justifies selecting Banyubiru and Getasan districts for studying the characteristics of RSSC in ginger plants. Understanding the adaptation potential of RSSC is crucial for assessing its impact on agricultural commodities. Therefore, this study aims to determine the distribution of RSSC in ginger plants in Banyubiru and Getasan districts, Semarang Regency, Indonesia, and to identify and characterize RSSC morphologically and biochemically in ginger plants in these districts.

MATERIALS & METHODS

Research location and time

The research was conducted in Banyubiru and Getasan districts, Semarang Regency, from July to January 2024. Analysis was carried out in the Physiology Laboratory and Kartini Experimental Garden Laboratory, Faculty of Agriculture and Business, Satya Wacana Christian University, Salatiga, Indonesia.

Materials

The primary materials used were ginger plant samples and soil from around ginger plants showing RSSC symptoms in Banyubiru and Getasan districts, Semarang Regency. Media for testing included tetrazolium chloride (TZC) (Merck, Germany), yeast peptone glucose agar (YPGA; Himedia, India), arginine broth (Merck, Germany), 1% tetramethyl paraphenyliene diamine dihydrochloride (Merck, Germany), and basal medium (Merck, Germany). Other chemicals were obtained from a local chemical supply store in Salatiga and were of analytical grade.

Procedures

The research was conducted using purposive sampling techniques. Five sampling locations were determined in each district. Ten sampling points were marked using a global positioning system (GPS) Manggihan Village Getasan District (07.35909ºS 110.442214ºE), Karang Ombo Village Getasan District (07.35471ºS 110.45959ºE), Blongoran Village Getasan District (07.36282ºS 110.45908ºE), Pendem Village Getasan District (07.35997ºS 110.44901ºE), Nogosaren Village Getasan District (07.35988ºS 110.41447ºE), Njengkol Village Banyubiru District (7.342712ºS 110.424964ºE), Gesing Village Banyubiru District (7.3261000ºS 110.4053000ºE), Puwono Village Banyubiru District (7.3255870ºS 110.4020335ºE), Plalar Village Banyubiru District (07.32551ºS 110.40198ºE), and Tlumpak Village Banyubiru District (7.34955ºS 110.38711ºE). Samples collected included soil and parts of ginger plants showing bacterial wilt symptoms, such as roots, stems, and leaves. Samples were taken to the laboratory for isolation and morphological and biochemical characterization tests.

Isolation and purification

Isolation was performed from symptomatic plant tissues and the rhizosphere of symptomatic plants using TZC medium with the spread plate method and incubated at 23-35℃ for 3-5 days. Suspected RSSC bacterial colonies were then purified using YPGA medium with the streak method (Razia et.al., 2021).

Morphological characterization

Bacterial isolates were identified by observing colony morphology, including color, shape, edge, and consistency. Bacterial isolates were observed using a stereo microscope (Olympus SZX7, Japan) and a light microscope (Olympus CX43, Japan) at 100× magnification (Razia et.al., 2021; Saridewi et al., 2020).

Biochemical characterization

Gram test with KOH

The Gram test was performed to differentiate whether bacteria were Gram-negative or Gram-positive. A 3% KOH solution was placed on a glass slide. A positive reaction indicated Gram-negative bacteria if the mixture of bacteria and KOH became viscous like a thread, while a negative reaction indicated Gram-positive bacteria if no viscous formation occurred (Kumar et al., 2017; Pooja & Rw, 2023).

Oxidase test with Kovac's reagent

The oxidase test was conducted using Kovac's method to determine the presence of the oxidase enzyme in bacteria. A filter paper was placed in a petri dish, and 3-4 drops of 1% tetramethyl paraphenyliene diamine dihydrochloride solution were added to the center of the paper. Bacterial isolates were then placed and streaked on the filter paper. A color change to purple within 60 seconds indicated a positive result. No color change or a color change taking 60 seconds or longer indicated a negative result (Khan et al., 2024).

Arginine activity test

The arginine dehydrolase activity test determines bacterial growth under anaerobic conditions in a medium containing arginine. The test was performed by inoculating bacteria in the center of arginine broth medium (peptone 1.0 g, NaCl 5.0 g, K_2HPO_4 0.3 g, agar 3.0 g, phenol red 1.0 mg, arginine HCl 10 g) in a test tube. The test tube was then sealed with vaseline/paraffin. A positive result was indicated by a color change to red (Nurdika et al., 2022).

Hypersensitivity test

The hypersensitivity reaction test determines whether a bacterium is a plant pathogen. The test was conducted by infiltrating bacteria into healthy tobacco leaves until they spread within the leaf tissue. If the infiltrated leaves showed necrosis or tissue death within 24-48 hours, the bacterium was confirmed as a plant pathogen (Schachterle & Huang, 2021).

Bacterial density calculation

Bacterial density was measured to determine the number of colonies, indicating the living organism index in the sample. Colony numbers were calculated using two methods: total plate count (TPC) and turbidimetry. The TPC method involved serial dilution up to 6 times to achieve a concentration of 10^{-6}. Then, 0.5 ml of suspension was inoculated on sterile nutrient agar (NA) medium at a 10^{-5} dilution concentration. The number of colonies growing on the medium after 48 hours of incubation was counted to determine the colony forming unit per milliliter of suspension (CFU/ml) using the formula:

$$\text{CFU/mL} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of inoculum in mL}} \quad (1)$$

The turbidimetry method used a spectrophotometer (Shimadzu UV Vis 1280, Japan) at a wavelength of 620 nm. The absorbance value was proportional to the optical density (OD) in the cuvette (Schug et al., 2020; Seniati et al., 2019).

Koch's Postulates

Koch's Postulates is a method to prove that a microbe is the causative agent of a suspected disease. If the microbe is indeed the suspected pathogen, it will cause the plant to become ill as hypothesized. Ginger plants were inoculated by pouring the isolate suspension onto wounded roots. Disease assessment on plants was observed after 17 days of inoculation. If the plants showed bacterial wilt symptoms, the isolated bacterium was the same as the one causing bacterial wilt in the field (Ray et al., 2021).

Biovar characterization

The biovar test was conducted to characterize the bacterial biovar. The test used basal medium consisting of 1g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4 \cdot 7H2O$, 12.0 g agar, 0.08 g bromothymol blue, and 1 l distilled water containing 1% carbon sources such as mannitol, sorbitol, lactose, and maltose. RSSC isolates were taken using an ose needle and streaked on the carbohydrate medium, incubated at 32℃. Observations were made on the 5th and 10th days to check for color changes (Schachterle & Huang, 2021).

RESULTS & DISCUSSIONS

Isolation and morphological characterization

RSSC was isolated by cultivating bacteria from plant tissues and the rhizosphere of ginger plants exhibiting bacterial wilt symptoms. Samples were taken from five locations in each of Banyubiru and Getasan districts, Semarang Regency. Symptoms of RSSC-infected ginger plants included yellowing and curling leaves, wilting, wet basal pseudostems and rhizomes, and easily breakable roots from the rhizome. Additionally, when uprooted, the pseudostems of ginger plants would easily detach from the rhizomes (Arwiyanto, 2014; Behera et al., 2020). Examples of ginger plants showing bacterial wilt symptoms are shown in Figure 1. Bacterial isolates were grown on TZC medium using the pour plate method. TZC medium is a differential medium capable of distinguishing two bacterial colony variants of RSSC based on their ability to reduce formazan compounds in the medium (Arwiyanto, 2014; García et al., 2019). Non-virulent RSSC bacteria appeared as small, round colonies with red color covering the entire colony, while virulent colonies appeared as round, mucoid colonies with a central red spiral and white edges.

A total of 20 samples were isolated, 10 from soil and 10 from symptomatic ginger plants. From these tests and observations, 13 bacterial colonies grew from all isolated samples, while 8 samples showed no growth on TZC medium. These colonies were identified as virulent bacteria, with growth characteristics shown in Figure 2b. Of the virulent isolates, 1 isolate originated from ginger plant tissues and 12 isolates from the ginger rhizosphere. Based on morphological observations at 100× magnification, all 13 virulent isolates were rod-shaped (Figure 2c). These isolates matched the morphological characteristics of R. solanacearum cells, which are rod-shaped (Arwiyanto, 2014). Detailed observations of each isolate are described in Table 1.

Figure 1. (a) Yellowing and curling leaves exhibiting RSSC symptoms, (b) Rot symptoms on the roots, (c) Rot at the basal pseudostem, easily detached from the rhizome, (d) Healthy ginger plant, (e) Healthy ginger rhizome, and (f) Basal pseudostem of a healthy ginger plant.

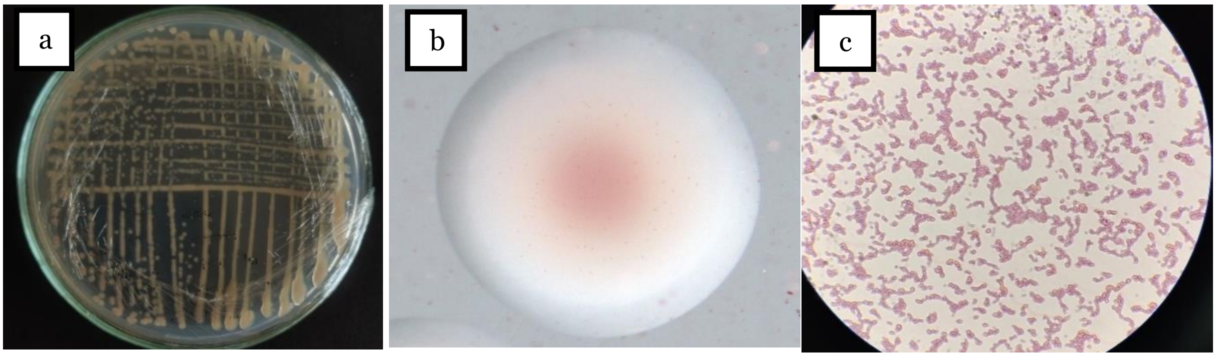

Figure 2. (a) Macroscopic view of virulent RSSC colonies on TZC medium, (b) Microscopic view of virulent RSSC colonies on TZC medium at 100× magnification, and (c) Morphology of RSSC bacterial cells.

Physiological and biochemical properties of isolates

RSSC is a Gram-negative bacterium with oxidative metabolism that causes bacterial wilt disease, giving a positive reaction in oxidase and hypersensitive tests, and a negative reaction in arginine activity tests. The Gram test was performed using KOH dropped onto a glass slide. A positive reaction indicating Gram-negative bacteria occurs if the mixture of bacteria and KOH becomes thread-like with a length of approximately 0.5-2 cm (Arwiyanto, 2014; Pooja & Rw, 2023). The test results showed that 12 isolates gave a positive reaction, indicating that these isolates are Gram-negative bacteria because the mixture of bacteria and KOH became thread-like when lifted with an inoculating needle (Figure 3a).

Table 1. Morphological characterization of bacterial colonies on TZC medium

Isolate	District	Color	Shape	Edge	Consistency	Source
NJK1	Banyubiru	Red with white edges	Round	Entire	Mucoid	Soil
TLP1	Banyubiru	Red with white edges	Round	Entire	Mucoid	Soil
TLP2	Banyubiru	Red with white edges	Round	Entire	Mucoid	Soil
TLP3	Banyubiru	Red with white edges	Round	Entire	Mucoid	Soil
TLP4	Banyubiru	Red with white edges	Round	Entire	Mucoid	Soil
TLP5	Banyubiru	Red with white edges	Round	Entire	Mucoid	Soil
MG1	Getasan	Red with white edges	Round	Entire	Mucoid	Soil
MG3	Getasan	Red with white edges	Round	Entire	Mucoid	Soil
PDM1	Getasan	Red with white edges	Round	Undulate	Mucoid	Plant
PDM2	Getasan	Red with white edges	Round	Undulate	Mucoid	Soil
KAR1	Getasan	Red with white edges	Round	Entire	Mucoid	Soil
KAR2	Getasan	Red with white edges	Round	Entire	Mucoid	Soil
KAR3	Getasan	Red with white edges	Round	Entire	Mucoid	Soil

Note: Isolate MG was obtained from sampling in Manggihan Hamlet, Getasan District; isolate PDM from Pendem Hamlet, Getasan District; isolate KAR from Karang Ombo Hamlet, Getasan District; isolate NJK from Njengkol Hamlet, Banyubiru District; and isolate TLP from Tlumpak Hamlet, Banyubiru District.

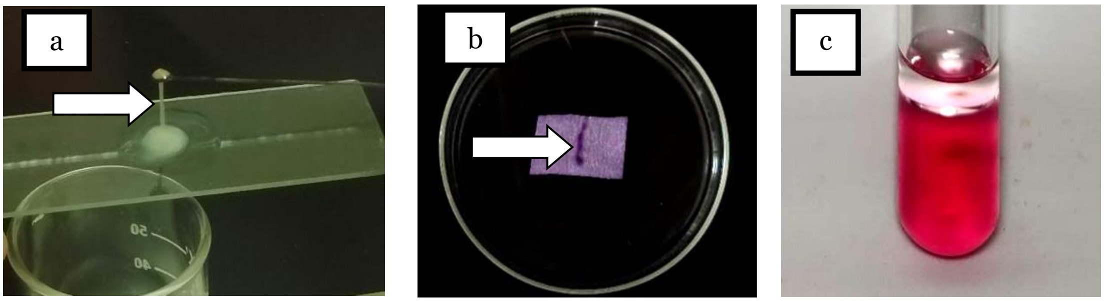

Figure 3. (a) Gram test results with KOH for Gram-negative bacteria, (b) Oxidase test showing a positive result, and (c) Arginine activity test showing a positive result.

The oxidase test shows a positive result if the color of tetra-methyl-p-phenylenediamine dihydrochloride changes to purple within less than 60 seconds; if the color change takes more than 60 seconds, the result is considered delayed positive or negative. The test results showed that 5 isolates gave a positive reaction, while the remaining 8 isolates gave a negative reaction (Table 2; Figure 3b). The oxidase reaction test is crucial for Gram-negative bacteria, including RSSC. Through this test, the presence or absence of cytochrome C associated with the aerobic respiration process in bacteria like RSSC can be detected (Khan et al., 2024).

The arginine dihydrolase activity test shows a positive result if the medium color changes from pink to red. Bacteria will exhibit different metabolic responses in arginine medium engineered to anaerobic conditions by applying a paraffin layer on the medium's surface (Nurdika et al., 2022). Under these conditions, anaerobic bacteria can still metabolize arginine and produce ammonia. The ammonia produced by these bacteria will increase the pH of the arginine medium, causing its color to change to red. Therefore, RSSC, being an aerobic bacterium, will not give a positive result in this test because RSSC cannot metabolize arginine under anaerobic conditions to produce ammonia. The test results showed that 5 isolates did not show a positive reaction, which is consistent with RSSC characteristics (Table 2; Figure 3c).

Hypersensitivity test

The results of the hypersensitivity test showed that 9 isolates gave a positive reaction, indicated by the presence of necrosis symptoms on the second day after inoculation (Table 2). According to Herdiyantoro et al. (2022), if the leaves infiltrated with bacteria experience necrosis or tissue death, it indicates that the bacteria are pathogenic. The pathogenicity of R. solanacearum is due to the secretion of specific proteins through the type II secretion systems (T2SS) and type III secretion systems (T3SS). T3SS plays a crucial role as the main determinant of pathogenicity and is a key factor triggering the reaction in the hypersensitivity test (Schachterle & Huang, 2021).

Table 2. Results of physiological and biochemical tests of isolates and hypersensitivity test

Isolate	Gram test	Oxidase test	Arginine activity test	Hypersensitivity test
NJK1	-	+	+	-
TLP1	-	+	-	+
TLP2	-	+	-	+
TLP3	-	+	+	+
TLP4	-	+	+	+
TLP5	-	+	-	+
MG1	-	-	-	+
MG3	+	-	+	-
PDM1	-	-	+	-
PDM2	-	+	+	+
KAR1	-	-	-	-
KAR2	-	-	+	+
KAR3	-	+	+	+

Notes: - indicates a negative reaction and + indicates a positive reaction

Bacterial density and Koch's Postulates test

The results of bacterial density calculations were used to determine absorbance values corresponding to Optical Density (OD) concentrations, which were then used for testing Koch's postulates (Schug et al., 2020; Seniati et al., 2019). In this test, only isolates that, based on physiological and biochemical properties tests, were suspected to be RSSC bacteria were used: isolates TLP 1, TLP 2, and TLP 5. These isolates were obtained from Banyubiru District, Semarang Regency. They showed positive results in oxidase and hypersensitivity reaction tests, and negative results in gram and arginine activity tests, in accordance with previous studies on the properties and characteristics of RSSC (Razia et al., 2021). A comparison with control treatment using sterile aquades was also conducted. Based on the Total Plate Count (TPC) method, the Colony Forming Unit (CFU/ml) values for isolate TLP 1 were 8.4x10^6; for TLP 2, the CFU/ml was 1.29x10^7; and for TLP 5, the CFU/ml was 1.29x10^7. Using the turbidimetric method, the Optical Density (OD) values for isolate TLP 1 were 0.003; for TLP 2, the OD was 0.004; and for TLP 5, the OD was 0.008. R. solanacearum can infect plants with a population of 5x10^5 CFU/ml (Arwiyanto, 2014). These OD values were used as a reference when preparing bacterial suspensions for Koch's postulates test because they had colony counts sufficient to infect plants. Each ginger plant was inoculated by pouring 500 µl of inoculum based on the OD value around the wounded roots.

The identified RSSC isolates were inoculated into healthy ginger plants. If the isolates were indeed the cause of bacterial wilt in ginger, they would induce the disease as hypothesized. Observations showed that ginger plants inoculated with these isolates exhibited initial symptoms of yellowing leaves followed by wilting from the 17th to the 30th day. Other symptoms included easily detachable and rotting basal stems. These symptoms were consistent with those observed in the field during sample collection. In contrast, the control treatment plants remained healthy and did not show any symptoms of bacterial wilt. This proved that the isolated and identified isolates were indeed RSSC bacteria causing bacterial wilt in ginger, supported by the physiological and biochemical tests conducted. These findings are consistent with previous research stating that RSSC infection can lead to ginger plant death, starting with leaf yellowing and wilting, easily detachable stems, and root rot appearing from the 14th to the 21st day (Behera et al., 2020; Zhang et al., 2018). This validation in the study follows the steps of Koch's postulates. Koch's postulates generally refer to a method to prove that a microbe is indeed the suspected cause of a disease (Ray et al., 2021).

Biovar test

The biovar test was conducted last, after obtaining data from previous tests. A positive biovar test is indicated by a change in the medium color from green to yellow between the 5th and 10th days, along with bacterial growth. The results of the biovar test showed that isolate TLP 1 was identified as biovar 4, while isolates TLP 2 and TLP 5 were identified as biovar 3. These results were determined based on the ability of *R. solanacearum* bacteria from each isolate to utilize two disaccharides, lactose and maltose, and two hexose alcohols, mannitol and sorbitol, as carbon sources. Biovar 4 strains can only utilize mannitol and sorbitol as carbon sources, whereas biovar 3 can utilize all four tested carbon sources (Table 3). According to previous research, Ralstonia strains that have been found to infect ginger plants in Indonesia include R. solanacearum biovar 3 and 4 strains (Yusriadi et al., 2020).

Table 3. Biovar test results of identified RSSC isolates

Isolat	Mannitol	Sorbitol	Lactose	Maltose
TLP 1	+	+	-	-
TLP 2	+	+	+	+
TLP 5	+	+	+	+

Note: `+` indicates the ability to utilize the carbon source, `-` indicates the inability to utilize the carbon source

CONCLUSION

This study reveals that the Ralstonia solanacearum species complex (RSSC) has spread to ginger plants in Banyubiru District, Semarang Regency. Identification and characterization results found three isolates, TLP 1, TLP 2, and TLP 5, which exhibit the morphological characteristics of RSSC. These three isolates are classified as virulent bacteria, as evidenced by their round, mucous colonies with a red spiral center and white edges on TZC medium. The rod-shaped bacterial cells further support this identification. Biochemical characterization identified these isolates as belonging to biovar 3 and 4 strains, capable of causing bacterial wilt disease in ginger plants. Given these findings, preventive measures must be implemented to mitigate the potential spread of RSSC to other ginger cultivation areas, Solanaceae plants, and surrounding lands in Tlumpak Village, Banyubiru District, Semarang Regency. Such measures are crucial to protect crops from further infection and ensure the sustainability of ginger farming in the region.

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