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Identification and Characterization of *Ralstonia solanacearum* Species Complex from Ginger (*Zingiber officinale*) in Semarang Regency, Indonesia

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Abstract. The *Ralstonia solanacearum* species complex is a highly destructive plant pathogen with a remarkably broad range of hosts, and ongoing discoveries continue to expand its host list. In May 2023, a new type of bacterial wilt affecting ginger (*Z. officinale*) crops in Semarang Regency, Central Java was reported. Early symptoms included sudden withering of leaves in adult plants followed by complete wilting and darkening of the vasculature, ultimately leading to plant death. This research specifically investigates the spread of *Ralstonia solanacearum* Species Complex within ginger cultivation in Semarang Regency. Twenty bacterial isolates were collected from soil and diseased *Z. officinale* plants at twenty different locations. Physiological and biochemical analyses confirmed that the causative agent for *Z. officinale* bacterial wilt was *Ralstonia solanacearum* belonging to biovar 3 and 4. The study also revealed that the distribution of this pathogen remains focused in the Banyubiru and Sumowono sub-districts. These findings will enhance our understanding of how *Ralstonia solanacearum* Species Complex spreads among ginger crops and its impact on them.

Keywords: bacterial wilt; ginger; biovar; pathogenic bacteria; *Ralstonia solanacearum* species complex

INTRODUCTION

Tropical farming faces significant vulnerability to pests and diseases as a result of the ideal environment created by high temperatures and humidity. However, climate change presents unique obstacles for tropical agriculture, resulting in greater threats from pests and diseases due to rising temperatures and fluctuating patterns of rainfall (FAO, 2021; Newlands, 2018; Savary et al., 2011, 2018; Strange & Scott, 2005; Thornton et al., 2014; Widiyatmoko et al., 2022). Singh et al. (2023) and FAO (2021) highlighted the substantial annual losses of US$220 billion globally in tropical agriculture caused by pests and diseases, resulting in decreased crop yields, heightened pesticide usage, environmental contamination, and threats to agricultural sustainability (Oerke, 2006; Pardey et al., 2014; Parnell et al., 2015). Effective measures are crucial for decreasing the occurrence and spread of tropical agricultural pests to guarantee sustainable farming practices in tropical regions while also combatting climate change impacts on agriculture (Alene et al., 2018; Schleußner et al., 2018; Schneider et al., 2022).

Previous research has established the remarkable adaptability of microorganism pests to their surroundings (Malhi et al., 2021; Richard et al., 2022). This study specifically focuses on the adaptability of plant pathogenic bacteria within the bacterial kingdom. Unlike nonpathogenic bacterial counterparts, these organisms can induce disease in susceptible plants through physiological damage. With an estimated 150 out of 7,100 identified species responsible for various plant diseases globally, plant pathogenic bacteria are prevalent worldwide and can be found in three leading families: Xantomonadaceae, Pseudomonaceae, Enterobacteriaceae, as well as Pectobacteriaceae, which includes genera such as *Dickeya*, *Ralstonia* and others including new species that have been designated over time (Bar-On et al., 2018; Chung et al., 2017; Kannan & Bastas, 2015; Mansfield et al., 2012). The latest developments highlight three significant plant pathogenic bacteria, with *Pseudomonas syringae* pathovars, *Ralstonia solanacearum*,

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and *Agrobacterium tumefaciens* being the top-ranked in terms of importance.

Ralstonia solanacearum stands out for its extensive host plant variation and is widely known as one of the most destructive bacterial plant pathogens due to its aggressiveness. *R. solanacearum* causes vascular wilt disease in almost 200 plant species, including food and high-value cash crops worldwide, such as potatoes, tomatoes, tobacco, ginger, bananas, and vegetables (Denny, 2006; Genin, 2010; Genin & Denny, 2012; Paudel et al., 2020; Setiawan, 2019). The onset of bacterial wilt disease begins when *R. solanacearum* enters plant roots through wounds or natural openings, where it then flourishes in the xylem vessels and generates extracellular polysaccharides (EPS). This subsequently obstructs water supply and leads to wilting of the plants (Xue et al., 2020). As most plants affected by *R. solanacearum* are commonly grown in tropical regions, further research into their current status is crucial, especially in Indonesia, where an extensive range of plants is developed.

R. solanacearum can infect a wide variety of hosts due to its various strains, placing it within the *R. solanacearum* species complex (RSSC) (Genin, 2010; Safni et al., 2014). Bacteria in this complex are characterized using methods such as host range (race), geographic origin (phylotype), capability to metabolize carbon sources (biovar), and variation in endoglucanase (egl) gene sequences (sequevar) (Lee & Kang, 2013). *R. solanacearum* is divided into 5 races based on their ability to infect specific hosts, and 5 biovars based on their capacity to utilize or oxidize certain sugars like disaccharides (trehalose, maltose, cellobiose) and hexose alcohols (mannitol, sorbitol, dulcitol) (García et al., 2019; She et al., 2017). Research on the identification of *R.solanacearum* in Indonesia has made significant progress, studies have characterized the genetic diversity of the bacterium in Java, with most isolates belonging to phylotype I and one to phylotype II (Hemelda et al., 2019). Real-time PCR assays have been developed for the detection of *R. solanacearum* in ginger rhizomes, a common host of the bacterium (A. Kumar et al., 2002; Thammakijjawat et al., 2006). However, the specific identification of R. solanacearum in ginger in Indonesia is still an area that requires further research.

In an initial effort to investigate the presence of RSSC in Indonesia, researchers conducted a preliminary study with a narrower focus on ginger cultivation in Semarang Regency. The selection of ginger was based on Indonesia's significant global export position as the fifth-largest exporter of this crop (ARISE+, 2022; Elpawati et al., 2022; Nurhidayati et al., 2022; Rusnaldi et al., 2023). In addition, several ginger farmers in the Semarang district have reported instances of wilt disease affecting their cultivated ginger plants. This information was brought to light during the implementation of a Community Service Program in the area. This forms the foundation of this study as Semarang Regency is significant in Central Java Province due to its substantial ginger production across four sub-districts: Banyubiru, Sumowono, Getasan and Tengaran (BPS, 2022a, 2022b, 2022c).

For effective disease management, precise identification, diagnosis, and assessment of the pathogen's strength are crucial. Due to limited data on the prevalence and biovar distribution of *R. solanacearum* in Indonesia, this study aimed to analyze the bacterium and assess the occurrence of biovars in ginger farming across Semarang Regency under different climatic conditions and edaphic factors. The findings will assist farmers in developing strategies to mitigate crop losses. The proposed research aims to address the knowledge gap regarding the prevalence and biovar distribution of *R. solanacearum* in ginger farming in Semarang Regency.

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METHODS

Collection of sample

The sampling took place in four subdistricts within Semarang Regency, Central Java Province: Banyubiru (478 MASL), Sumowono (955 MASL), Getasan (1086 MASL) and Tengaran (741 MASL). In each subdistrict, samples were purposely collected from five ginger farmers' fields where plants exhibited wilt symptoms. Total of twenty soil and plant samples were gathered at these locations, and their respective collection points were documented for reference.

Observation of visual symptoms

Initially, the specimens were cleansed and placed in a refrigerator with temperatures ranging from 4°C to 10°C (EPPO, 2022). Subsequently, infected plant samples underwent examination for oozing using a sterilized knife and a tube filled with purified distilled water, representing an uncomplicated technique (Danks & Barker, 2000; Elphinstone et al., 1996). The identification of diseases was then validated through isolation, diverse biochemical tests, and biovar characterization of all gathered samples by standard procedures.

Isolation of the bacterial isolates

R. solanacearum was isolated primarily from the infected stems and soil obtained from various fields in twenty locations. The stems, specifically from the collar region, were cut into 10-cm sections and then surface sterilized with 70% ethanol before being chopped into smaller pieces. These smaller fragments were subsequently placed in sterilized distilled water and shaken at room temperature for 5 minutes, soil samples (10 g soil in 100 mL distilled water) from the rhizosphere were turned into a soil solution using distilled water. A sample of 100 µL of bacterial suspension from each isolate was individually inoculated onto HiMedia tetrazolium chloride media (TZC), evenly distributed, and placed in an incubator at 30°C for 48 hours to facilitate bacterial growth (Kyaw et al., 2022; Ni'matuzahroh, 2021; Sharma, 2018). The plate's dominant colonies exhibited slimy, convex features with a red center and whitish edges on TZC (Razia et al., 2021; She et al., 2017). Subsequently, the strains underwent comparison based on hypersensitivity response, biochemistry, and biovars.

Hypersensitivity test

The hypersensitive response was performed on *Nicotiana tabacum* leaves following (Nakaho et al., 2017) methods. Each isolate was streaked on a nutrient agar medium and cultured for 24 hours. The growth from each isolate was later diluted in sterile water to achieve a cell density of 10^8 $cfu.ml^{-1}$ using a spectrophotometer (Shimadzu UV-Vis Single Beam UV-1280), resulting in 28% light transmission at a wavelength of 580 nm optical density. This bacterial suspension was introduced into fully expanded tobacco leaves through infiltration using a hypodermic syringe (OneMed Disposable Syringe 3 mL) with an external diameter of 0.6 mm. The reactions were observed after both 24 and 48 hours post-inoculation, exhibiting positive outcomes characterized by rapid collapse along with water-soaked symptoms that subsequently transformed into dry, light-brown necrotic tissue (J.-S. Huang & Knopp, 1998; Triwidodo & Listihani, 2021).

Physiological and Biochemical test

The physiological and biochemical tests assessed the morphology, gram stain, fermentative-oxidative reaction, and arginine dihydrolase activity (Thind, 2019). Colony morphology (color, size, border, shape, and consistency) during growth on HiMedia TZC medium was documented for the bacteria (Ray et al., 2021; Sharma, 2018). The gram test was conducted using potassium hydroxide by placing a droplet of bacterial isolate (aseptically taken from 24 - 36 hrs old culture) in 3% KOH solution on a glass slide and gently stirring it. If the suspension became sticky within 50-60 seconds, the bacterium was classified as gram-negative; otherwise, it was categorized as gram-

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positive. For the oxidase test, a 24-hour-old bacterial colony from nutrient agar was used to inoculate filter paper (Whatman no. 1) soaked in freshly prepared 1% Tetramethyl-p-phenylene diamine dihydrochloride solution. To assess Arginine dihydrolase activity, a new culture was inserted into soft agar in Thornley's medium-filled tubes and sealed with paraffin before being placed in an incubator at 28°C. A positive reaction is indicated by a color change from pale pink to red within four days (Hanudin et al., 2014; Scala et al., 2018).

Biovar test

For biovar identification, a sterile basal medium was used for the oxidation of carbohydrates, including 1% $NH_4H_2PO_4$, 0.2% KCl, 0.2% $MgSO_4.7H_2O$, 1% peptone, and 0.3% aqueous bromothymol blue solution. Six different carbohydrate sources—lactose, maltose, cellobiose, mannitol, sorbitol, and dulcitol were filter-sterilized and added to the basal media individually. Bacterial suspensions were inoculated into these prepared media, incubating at room temperature (28^{o}C) while recording daily observations on acid production from oxidation of the carbon source as the color gradually shifted from green to yellow (Hayward & Hartman, 1994; Siregar et al., 2021). This typically happens within a period of 3 to 5 days, while biovars that can oxidize disaccharides take slightly longer to produce a distinct positive outcome. A comparison is made between the inoculated tubes and a non-inoculated control tube.

RESULTS AND DISCUSSION

Symptoms of bacterial wilt diseases in ginger plants

The researched area was an elevated region with consistently high moisture levels (46 - 97%) throughout the year. The research occurred from June to September because bacterial wilt cases were more prevalent in the dry season. Soil attributes at the twenty study sites included: pH levels ranging from acidic to slightly acidic (4.61 - 5.56); generally poor drainage; dominant soil types found were Latosol in Tengaran, Alluvial in Banyubiru and Getasan, and Regosol in Sumowono. Soil acidity level significantly influences the survival and management of Ralstonia solanacearum, the bacteria causing bacterial wilt in ginger. Vudhivanich (2008) discovered that increasing soil pH to a range of 7.0-7.2, combined with urea and calcium oxide amendments, can reduce the population of *R. solanacearum*. Also noted by Jiang et al. (2018), elevated soil moisture levels, which impact soil pH as well, can heighten ginger's susceptibility to *R. solanacearum*. Research has indicated that *R. solanacearum*, a pathogenic bacterium responsible for brown rot disease, can endure in various soil compositions, including alluvial, regosol, and latosol (Horita & Tsuchiya, 1998; Teli et al., 2018). Furthermore, investigations into the genetic diversity and virulence of R. solanacearum have yielded valuable insights into these soil types' potential impact on disease spread and severity (Hayes et al., 2017; Steidl et al., 2021). The sampling sites also revealed a variety of plant species abundance in the ginger farmers' land, in addition to soil characteristics. Farmers intercropped ginger with other crops such as bananas, chilies, cassava, coffee, and eggplant (see **Figure 1**). This mixed planting approach could worsen the spread of bacterial wilt by creating proximity and shared soil conditions among different susceptible crops (Adebayo & Fadamiro, 2009; Messiha et al., 2019; Zhang et al., 2023).

Recently, a nearby farmer reported the first signs of bacterial wilt on ginger. However, farmers in the area still need to prioritize this issue or find practical solutions. Limited awareness and understanding among local farmers about the bacterial wilt of ginger is believed to have contributed to the increased spread and severity of the disease in the surveyed region (Baral et al., 2021; Guji et al., 2019). Field surveys recorded a 20% incidence of the disease across all visited sites, indicating a moderately high spread in this area, likely due to intercropping with susceptible crops.

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Figure 1. Intercropped ginger with wilting symptoms was suspected of being infected by bacterial wilt at the farmer's field.

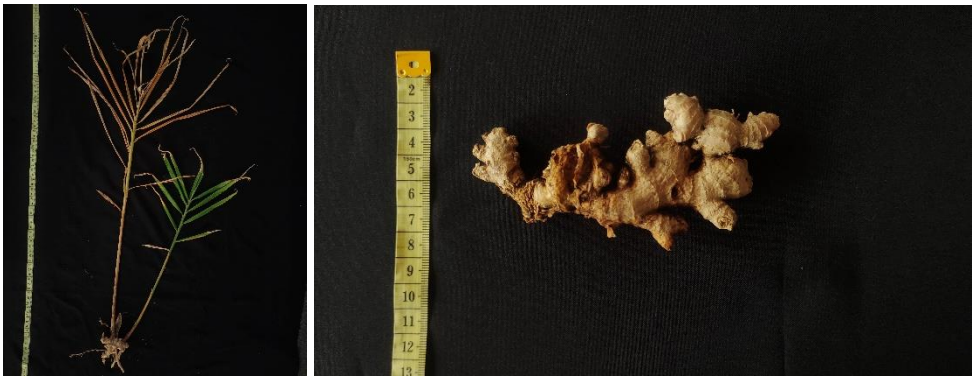

Figure 2. Wilt symptoms and discoloration (left) of a ginger leaf suspected infected with *R. solanacearum*, infected ginger rhizome (right) with some parts starting to wrinkle

Safni (2018) also found similar results, reporting that *Ralstonia solanacearum* isolated in Indonesia was most prevalent during hot seasons. Samples of potentially infected plants were collected and documented. Characteristic wilting symptoms were observed on the affected plants (see **Figure 1**), with leaf discoloration shown in **Figure 2**. Similar symptoms have been observed in Solanaceae plants when infected by *R. solanacearum*, as reported by other researchers (Brown & Allen, 2004; Okiro et al., 2017; Shen et al., 2020). Bacterial wilt infection mainly occurs through wounded tissues; however, some studies suggested that infection could occur without injury, although injuries may accelerate root tissue infections further (Caitilyn et al., 2005). In the early stages of the disease, ginger plants exhibit collapse of the growing apex and discoloration. When infected stems and roots are soaked in sterile water for a few minutes, they excrete cloudy white bacterial fluid resembling condensed milk from their tissue (Meenu et al., 2019).

Isolated bacteria from soil – plant and their morphological characteristic

Isolating *R. solanacearum* from ginger plants and rhizosphere identified twenty isolates based on colony morphology. This Gram-negative bacterium is characterized by short rod-shaped cells as single colonies, and its appearance includes irregularly rounded shapes with a milky white color, slimy or shiny texture, uneven edges, and convex elevation. According to Sharma (2018), *Ralstonia* sp. typically grows on TZC media (see **Figure 3**), producing irregularly shaped colonies with slightly convex elevation that are fluidal and white (virulent colonies exhibit a pink center). Based on these distinct characteristics consistent with those listed in Bergey's Manual of Determinative Bacteriology and reported by (Sharma, 2018), the bacteria isolated in this study were suspected belonging to the Ralstonia genus.

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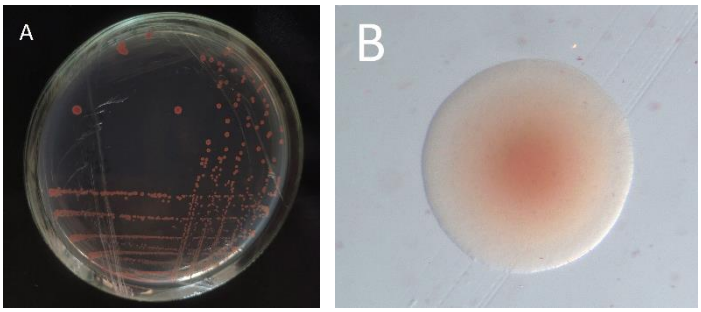

Figure 3. Morphology of *Ralstonia solanacearum* colonies on TZC media aged 48 hours (A) avirulent strain, (B) virulent strain 400 times magnification

Table 1. Physiological - biochemical characteristic of twenty isolates

Isolate	Location	Origin	Gram test	HR	KO	AA
Manggian 1	Getasan	Rhizosphere	Neg	+	-	-
Pendem 1	Getasan	Plant	Neg	-	-	+
Pendem 2	Getasan	Rhizosphere	Neg	-	+	+
Karang Ombo 1	Getasan	Rhizosphere	Neg	-	-	-
Karang Ombo 2	Getasan	Plant	Neg	-	-	+
Karang Ombo 3	Getasan	Rhizosphere	Neg	-	+	+
Njengkol 1	Banyubiru	Rhizosphere	Neg	+	+	+
Njengkol 2	Banyubiru	Rhizosphere	Neg	-	+	+
Tlumpak 1	Banyubiru	Rhizosphere	Neg	+	+	-
Tlumpak 2	Banyubiru	Rhizosphere	Neg	+	+	-
Tlumpak 3	Banyubiru	Rhizosphere	Neg	+	+	+
Tlumpak 4	Banyubiru	Rhizosphere	Neg	+	-	+
Tlumpak 5	Banyubiru	Rhizosphere	Neg	+	+	-
Bodehan 1	Banyubiru	Rhizosphere	Neg	+	-	+
Kebon Agung 1	Sumowono	Rhizosphere	Neg	-	-	+
Kebon Agung 2	Sumowono	Rhizosphere	Neg	+	+	-
Krajan 1	Tengaran	Rhizosphere	Neg	+	+	+
Rejosari 1	Tengaran	Rhizosphere	Neg	+	-	+
Wates Kulon 2	Tengaran	Rhizosphere	Neg	+	-	+
Wates Kulon 3	Tengaran	Rhizosphere	Neg	+	-	+

Remarks: Abbreviation HR means hypersensitive reaction test, KO means Kovac's Oxidase test, and AA means arginine dihydrolase activity test.

Physiological - biochemical characterization of isolated bacteria

Twenty isolates were collected from four separate sub-districts and were subjected to a series of tests to evaluate their physiological and biochemical traits can be seen on table 1. The Gram test using 3% KOH revealed that all specimens displayed gram-negative bacterial characteristics by producing viscous strands. Moreover, only thirteen of these samples demonstrated positive hypersensitive reactions on tobacco leaves, with differing results in Kovac's oxidase and arginine dihydrolase activity tests observed during the investigation. These findings highlight the diversity and variability among the pathogenic bacterial isolates, suggesting the presence of distinct species within the sub-districts. Preliminary findings from physiology-biochemical testing of multiple

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isolates in this research corresponded to the characteristic features of *R. solanacearum* (Caitilyn et al., 2005; Hayward & Hartman, 1994; Pawaskar et al., 2014; Razia et al., 2021).

Hypersensitive reactions on tobacco leaves are characterized by the appearance of necrotic spots on the leaves 24 hours after injection. Isolates are considered highly pathogenic if the isolate gives a hypersensitive reaction characterized by necrotic symptoms that appear after 24 hours of inoculation. Isolates that show symptoms after 72 hours of inoculation are weakly pathogenic, while isolates that show symptoms after seven days are considered slightly pathogenic. The ability of isolates to trigger hypersensitive reactions on tobacco leaves is one of the characteristics of pathogenic bacteria. *R. solanacearum* can trigger hypersensitive reactions on tobacco leaves because the bacteria have an Hrp-type III secretion system (T3SS). This secretion plays a role in the pathogenicity process that can trigger hypersensitive reactions (Chaudhry & Rashid, 2011; Razia et al., 2021).

Kovac's oxidase test is used to distinguish aerobic and anaerobic bacteria. Positive results are characterized by a change in color to purple when the bacterial mass is applied to filter paper that has been given an oxidase reagent (Kovacs, 1956). Ten out of twenty-one isolates produced a dark purple in 10 - 60 seconds (Table 2). This indicates that the isolates are aerobic bacteria. Kovac's oxidase test detects cytochrome C oxidase (oxidase positive), which plays a role in respiration (Akbar et al., 2015; Scala et al., 2018). *R. solanacearum* has a cbb3-type cytochrome c oxidase gene (cc3-cco), which plays a role in bacterial growth in a microaerobic environment, allowing bacteria to respire even in low oxygen conditions (Colburn-Clifford & Allen, 2010).

The Arginine dihydrolase test is used to determine the ability of bacteria to produce arginine dihydrolase enzyme. A positive reaction is characterized by changing red to purple in the media (Thornley, 1960). In this study, as many as six isolates of negative arginine dihydrolase (Table 1). The change in media color proves that there is a change in alkaline pH in the media covered by liquid paraffin, indicating the ability of bacteria to produce ammonia from arginine under anaerobic conditions. Negative results indicate that bacteria cannot produce ammonia under anaerobic conditions (Thornley, 1960). *R. solanacearum* gave negative results of arginine dihydrolase, as indicated by no color change in the media after 48 hours. This condition indicates the inability of *R. solanacearum* to degrade arginine (Nurdika et al., 2022; Sharma, 2018). This follows the character of *R. solanacearum*, an aerobic bacterium (oxidase positive), so that under anaerobic conditions, no metabolic activity occurs, which gives negative results in the arginine dihydrolase test.

Biovar test

The bacterial isolates underwent a biochemical assessment to classify them, and out of the twenty isolates, only four were identified as *R. solanacearum*. A further test was carried out to distinguish the biovar of these *R. solanacearum* isolates based on their capacity to oxidize various carbohydrate sources. The results of the biovar assessment revealed that all bacterial isolates successfully oxidized sugar alcohols such as mannitol, dulcitol, and sorbitol except for isolate Kebon Agung 2 (Table 2). Based on the study by Ahmed et al., the ability of *R. solanacearum* isolates to oxidize different carbohydrates is a critical factor in determining their biovar classification (Chamedjeu et al., 2018).

Kebon Agung 2 was the only isolate that did not ferment lactose, maltose, and cellobiose to produce acid. If the response was absent, the oxidation reaction could be observed through color changes in the medium from green to yellow or by retaining its original green color. This indicates the generation of acid due to bacterial colony

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carbohydrate oxidation. The ability to isolate Kebon Agung 2 to oxidize lactose, maltose, and cellobiose differed from all other isolates tested, as it did not produce acid from these carbon sources (Q. Huang et al., 2012).

Table 2. Biovar tests on four samples indicated it was *Ralstonia solanacerum*

Isolate	Carbohydrate Sources						Biovar
	LAC	MAL	CEL	MAN	SOR	DUL	
Tlumpak 1	+	+	+	+	+	+	3
Tlumpak 2	+	+	+	+	+	+	3
Tlumpak 5	+	+	+	+	+	+	3
Kebon Agung 2	-	-	-	+	+	+	4

Remarks: LAC means lactose, MAL means maltose, CEL means cellobiose, MAN means mannitol, SOR means sorbitol, and Dul means dulcitol.

Upon initial observation, a faint yellow hue emerged around the colony within 3 to 5 days of inoculation. Subsequently, the medium completely transformed into yellow after being kept at 28 ± 3°C for 21 days. The variation in oxidation reactions on different carbon sources was used to categorize different biovars of bacterial strains (Ahmed et al., 2013; S. Kumar et al., 2017; Razia et al., 2021). The change in color from faint to complete yellow after 21 days of incubation at 28 ± 3°C indicates the oxidation reactions in bacterial biovars.

Three of the four bacterial isolates examined were classified as belonging to biovar three and showed the ability to metabolize both polysaccharides and sugar alcohols. The Kebon Agung 2 isolate was categorized as biovar four and displayed favorable oxidation only on sugar alcohols. Both biovars 3 and 4 preferred growth at higher temperatures (37°C). These characteristics might indicate their natural distribution about the presence of suitable hosts. (Hayward & Hartman, 1994) suggested that geographical isolation along latitudinal lines could contribute to these conditions.

CONCLUSION

Bacterial wilt on ginger (*Zingiber officinale*) in two subdistricts (Banyubiru and Sumowono) within Semarang Regency, Central Java, was attributed to the pathogen *R. solanacearum*. The infected plants displayed stunted growth and wilting, with yellow/brown discoloration and oozing when leaves were submerged in water. Culture analysis revealed mucous, pleomorphic colonies with a red center and whitish periphery on the TZC medium. These rod-shaped colonies tested gram-negative. Based on symptoms observed along with physiological and biochemical evaluations, this particular *R. solanacearum* belonged to biovar 3 and 4, hosted by wild ginger at Semarang Regency - Central Java for the first time reported.

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