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Research Article
Testing of Biovar and Pathogenicity *Ralstonia solanacearum* in Banana (Kepok: Local Indonesia) in South Kalimantan, Indonesia

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Abstract

Background and Objective: *Ralstonia solanacearum* is a bacterium that causes wilt disease in bananas (Kepok: Lokal Indonesia), this disease is very dangerous and has spread evenly and already at high attack rate causing 100% loss. This bacterium has not been known for its characteristics and its pathogenicity from South Kalimantan, Indonesia. The objective of the study was to determine *R. solanacearum* including biovar (strain) and pathogenicity, bacteria capability and know the pathogenicity of isolate from banana origin in South Kalimantan. **Materials and Methods:** This research was conducted by tracing bacterial isolate in carbohydrate medium and allowed to form acid by showing the change of medium color from green to yellow as a positive reaction of carbon source usage and pathogenicity test by describing plants showing symptoms of pain and counting time of incubation period. **Results:** That isolate of *R. solanacearum* bacteria from Kota Banjarbaru, Banjar, Tapin, Tabalong and Kotabaru Districts were able to use carbon source from lactose, maltose, mannitol, dulcitol and sorbitol. *Ralstonia solanacearum* tested was able to cause sick plants and showed symptoms of wilt averaging 60 days after inoculation, isolates from Banjarbaru, Banjar, Tapin and Kotabaru, while Tabalong isolates caused wilting symptoms after an average of 66 days. **Conclusion:** Isolate *R. solanacearum* from Banjarbaru, Banjar, Tapin, Tabalong and Kotabaru, Indonesia were able to use carbon source from lactose, maltose, mannitol, dulcitol and sorbitol and belong to biovar group 3, very high pathogenicity level and incubation period average 60-66 days after inoculation.

Key words: *Ralstonia solanacearum*, biovar, pathogenicity, carbon source, wilt disease

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Banana cultivation is one of the efforts to meet the needs of banana production. The 2009 and 2017 national banana (Kepok: local Indonesia) production decreased to 618,460 tons, while the banana production in South Kalimantan decreased to 26,890 t between 2009 and 2017.

Based on the data from the Directorate General of Horticulture, Tapin and Banjar are two regions in South Kalimantan considered as plantain suppliers. It was found out that *R. solanacearum* infected plantain plantation in Banjarbaru, Tapin and Tabalong causing major loss for the plaintain[1]. As the result, researchers and farmers are supposed to know the characteristics of *R. solanacearum*[2], a type of bacteria infected plantain plantation in South Kalimantan in order to find effective solutions, ones that suitable with the characteristics of the bacteria, before eradicating them[3,4]. Important characteristics of bacteria to describe are biovar and pathogenicity in the form of their incubation and how infectious they are to plants. Pathogen is closely related to virulence or how much infectious a bacterium is *R. solanacearum* has various different hosts[3-6]. Based on the host, the bacterium can be divided into five groups. Furthermore, based on disaccharide oxidation and hexose alcohol, the bacterium is divided into 5 biovars.

The study is conducted because strand of *R. solanacearum* found in South Kalimantanese plantain that causes wilt disease has yet been identified thoroughly[6-8].

Different strands and biovars of *R. solanacearum* have different characteristics[9,10]. It happens because the bacterium is able to infect different hosts and can utilize carbon. The purpose of this study was to describe ability of *Ralstonia solanacearum* isolate from South Kalimantan plantain in utilizing carbon source as well as to describe its biovar and Pathogen.

MATERIALS AND METHODS

Research location: Taking and surveying plant samples in South Kalimantan, material for biovar and Pathogen tests is *R. solanacearum* isolate bacterium from plantain grown in three different locations namely, (1) Loktabat Selatan, Banjarbaru Selatan, Banjarbaru, (2) Sawang, Tapin Selatan, Tapin and (3) Muang, Jaro, Tabalong. Diseases affected by wilt were identified at the Laboratory of Biological Control, Faculty of Agriculture, Lambung Mangkurat University Banjarbaru. The research was carried out for 6 months, January to June, 2018.

Materials: Samples of plants with bacterial wilt, other media are carbohydrate media (each medium contains lactose, maltose, dulcitol, mannitol, sorbitol), NA (Nutrient Agar) media, TZC (Triphenyltetrazolium Chloride) media, KOH 3%, alcohol 70%, sterilized aquades, spirtus, tissue, cotton, aluminium foil, plantain seed from plant tissue isolation methods, manure and soil.

Equipment: The equipments used in this study are petri dish, a loopful, measuring cups, beaker glass, test tubes, autoclave, ovens, laminar air flow, vortex, microscope, slides glass, syringe, pipette, tweezers, knives, bunsen lamp, polybag, stationery and cameras.

Biovar test of *R. solanacearum*: The study used the method by scraping isolate bacteria on carbohydrate medium and setting it aside to form acid, which was indicated by change in the color of the medium from green to yellow as positive reaction to the use of the carbon source[11].

Pathogen test of *R. solanacearum*: Method was used for the pathogen test by describing which plants were infected plants and counting incubation time[12]. The pathogen test consisted of 4 treatments and 5 replications, so that there were 20 testing units, where each testing unit has one plantain seed. The treatment was as follows:

- Control (R_0)
- *Ralstonia solanacearum* isolate from the plantain grown in Loktabat Selatan, Banjarbaru Selatan, Banjarbaru (R_1)
- *Ralstonia solanacearum* isolate from the plantain grown in Sawang, Tapin Selatan, Tapin (R_2)
- *Ralstonia solanacearum* isolate from the plantain grown in Muang, Jaro, Tabalong (R_3)

Implementation of the study

Setting and sampling of the study: The setting of the study was selected based on plantain plantation in South Kalimantan infected by *R. solanacearum* while survey was conducted to determine the areas where the samples were taken; the areas were plantain plantation in Banjarbaru, Tapin and Tabalong infected by bacterial wilt disease.

***Ralstonia solanacearum* isolation:** Pathogenic isolate was isolated from the plantation which showed symptoms of wilt disease. Trunk, branch, twig or fruit of the infected plantain were cut and their surfaces were sterilized by soaking them for approximately 15 sec in alcohol 70% and for 3 times in

different sterilized water. Each side of the sterilized pieces of plantains was cut, put inside the petri dish containing the TZC media and finally incubated for 24 h. The bacteria were bred using the NA media incubated in room temperature for 48-72 h[13].

Virulence test for TZC media: The test was conducted to find out the characteristics of *R. solanacearum*, a bacterium causing bacterial wilt disease; its goal was to find out whether the bacterium was virulent or non-virulent. The procedure to breed the bacterium was scrapping it on the TZC media and put them in petri dish. They later were incubated for 48 h and the temperature was between 28 and 30°C[13].

Gram test: The gram test was conducted by mixing ose bacterial culture and one or two drops of KOH 3% solution in the beaker glass.

Biovar test: The biovar test used 5 carbohydrate media where each of them contained 1% of lactose, maltose, mannitol, dulcitol and sorbitol respectively. *Ralstonia solanacearum* isolate was taken using ose needle and then it was scrapped on the carbohydrate media and then incubated in the temperature of 30°C. Observations to check whether there was change of color was conducted on the 2nd, 7th and 14th days[14].

Pathogen test: Soil used as growing media was mixed with manure where the soil-manure ratio was 1:1 prior to that, the soil was sterilized by steaming it inside a barrel 20 seeds of plantain of which height was between 30 and 50 centimeters were grown using plant tissue isolation method by Balai Benih of South Kalimantan. The seeds were grown in polybags of which media were the mixture between soil and manure. While it was growing, the seeds were watered regularly. The grown plantain was inoculated by *R. solanacearum.* The inoculated *R. solanacearum* were two-day-old virulent bacteria. They were suspended in one-milliliter sterilized water of which density was 10^8 cell mL^{-1}. The inoculation for the plantain was conducted using injection (syringe) on its weevil and trunks. One-milliliter sterilized aquades (R_0).

During incubation, observation was conducted every day for three months. The incubation was determined by taking note on which days after the inoculation (his) were the plantain showed the wilt disease symptoms. An average was taken from all of the data obtained from all twenty testing units.

RESULTS

Bacterial virulence and gram test: The results of virulence test and gram test, as shown in Table 1. Bacterial isolate from Banjarbaru, Tapin and Tabalong isolated from its host was *R. solanacearum* and it was virulent (+). It was revealed after observing colony of the bacterium on TZC media. The colony was round, irregular and fluidal (Fig. 1a), some show a deep red in the middle, surrounded by white and jagged edges (Fig. 1b) and It was transparent on the edge and pink in the center (Fig. 1c).

Table 1: Bacterial virulence and gram test result

Isolate	Virulence test	Gram test
Banjarbaru	+	-
Tapin	+	-
Tabalong	+	-

Virulence test, +: Virulent, Gram test, -: Gram negative

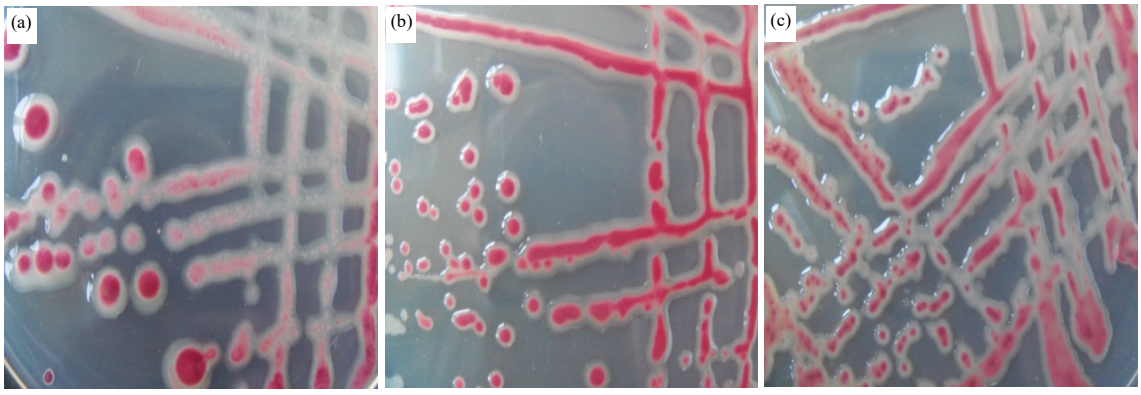

Fig. 1(a-c): Virulent *R. solanacearum* isolate from, (a) Banjarbaru, (b) Tapin and (c) Tabalong

Fig. 2(a-d): Plantain inoculated using (a) Sterilized aquades steril, (b) *Ralstonia solanacearum* from Banjarbaru, (c) *Ralstonia solanacearum* from Tapin and (d) *Ralstonia solanacearum* from Tabalong

Table 2: Biovar test of *R. solanacearum*

	Isolates		
Physiological test	Banjarbaru	Tapin	Tabalong
Using disaccharides			
Lactose	+	+	+
Maltose	+	+	+
Alcohol oxidation			
Dulcitol	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+

+: Able to use carbon source

Table 3: *Ralstonia solanacearum* classification based on biovar and the ability to use disaccharides and hexoses alcohol oxidation

	Biovar				
Physiological test	1	2	3	4	5
Using disaccharides					
Sellobiosa	-	+	+	-	+
Lactose	-	+	+	-	+
Maltose	-	+	+	-	+
Alcohol oxidation					
Dulcitol	-	-	+	+	-
Mannitol	-	-	+	+	+
Sorbitol	-	-	+	+	-

+: Able to use carbon source, -: Unable to use carbon source, Source: Aley and Elphinstone[15]

Table 4: *Ralstonia solanacearum* isolate pathogen test result for plantain

Ralstonia solanacearum	Test result on plantain		
isolates	GP	W	I
R_0	-	>90	-
R_1	+	60	+
R_2	+	60	+
R_3	+	66	+

GP: Infected, +: Wilted plant, -: Not infected, W: Time (days) taken for plantain to be wilted after inoculation or at the end of observations, I: isolation result, +: Successful bacterial isolation, -: Successful bacterial isolation

Biovar test: The biovar test results are as in Table 2, shows all samples can use carbon sources (+), All isolates found from diseased banana plant samples (Banjarbaru, Tapin, Tabalong) showed that they could use carbon sources, the isolates were put into positive groups using carbon sources.

The test result was compared to Table 3, the classification table from *Ralstonia solanacearum* based on biovar and the ability to use disaccharides and hexoses alcohol oxidation to find out the biovar of the bacterium. The result was the *R. solanacearum* isolates from Banjarbaru, Tapin and Tabalong were biovar 3.

Pathogen test: The results of the pathogenicity test of *R. solanacearum* isolates (Table 4), showed all are pathogenic and can cause diseased plants (wilting disease). See R_0 more than 90% as pathogens, R_1, R_2 and R_3 show 60%.

The results of tests on experiments of healthy banana plants inoculated with *R. solanacearum*, showed that testing on healthy banana plants was symptomatic. R_0 healthy plants without treatment (Fig. 2a), R1 Banjarbaru isolates symptoms appear moderate (50%) (Fig. 2b), R_2 symptoms of tapin isolates appear moderate (50%) (Fig. 2c) and R_3 Tabalong isolates symptoms appear larger (60%) (Fig. 2d).

The results of tests on experiments of tobacco leaves inoculated with *R. solanacearum*, showed that testing on showed that the tobacco leaf inoculated in *R. solanacearum* necrosis occurred. R_0 necrosis does not occur (Fig. 3a), R1 Banjarbaru isolates symptoms appear moderate (60%) (Fig. 3b), R_2 Symptoms of tapin isolates appear moderate (40%) (Fig. 3c) and R_3 Tabalong isolates symptoms appear larger (70%) (Fig. 3d).

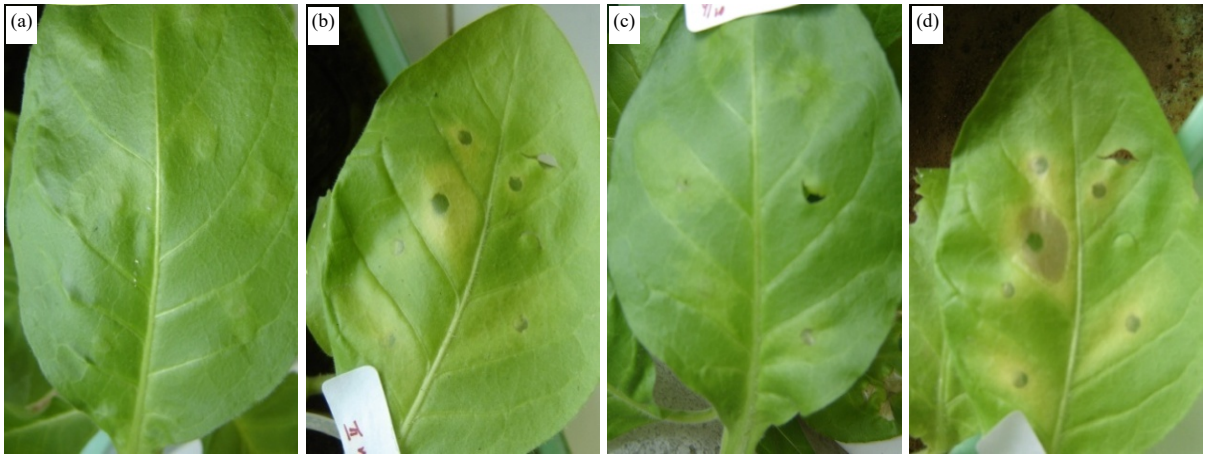

Fig. 3(a-d): Pathogen test on inoculated *R. solanacearum* from (a) Tobacco leaves grown in Banjarbaru, (b) Tapin and (c-d) Tabalong

DISCUSSION

Tests on several uses of carbon and biovar sources, showed that all isolates were able to use them[15]. Biovar testing shows biovar 3 in accordance with the results of the study[8,11]. The virulence test result shows that the tested bacterium is *R. solanacearum* and virulent. *Ralstonia solanacearum* virulence affects how pathogenic it is. Virulent bacteria can infect their hosts and therefore, the tested *R. solanacearum* is considered as pathogen for wilt disease found in the plantain. Based on the observations, the colony started to grow on the second day after the TZC media were isolated. Visually, the size of *R. solanacearum* isolate from Tabalong was smaller compared to that from Banjarbaru and Tapin. It indicates that *R. solanacearum* from the same host, plantain, has different characteristics. It happens because each of the isolates came from different environment.

Different characteristics mean *R. solanacearum* has diverse genetic variation. Based on isolate from similar host may have different genetic variation. Genetic variation will influence *R. solanacearum* phenotype. Phenotype is expression of gene found in bacterial isolate. According to genetic variation of *R. solanacearum* pathogen is shown by its ability to develop new strain different from the original strain, for instance strain with different virulence, physiological and biochemical reaction.

Some of the results of previous studies[14], stated that 0.5-2 cm-thread in the gram negative bacteria occurred because their outer membrane could easily be broken once they were mixed to KOH 3% that released thick DNA.

Gram negative bacteria are ones with double membranes and thick walls. Gram of a bacterium also affects pathogeniticity since gram negative bacteria have lipopolysaccharide layer, endotoxin component of bacterium cell walls.

Although *R. solanacearum* is endotoxin or it has ability to infect its host, the main cause of wilt disease in the plantain is the bacterium blocks xylem on the root and stem and as the effect, flow of water and nutrients is also blocked.

The biovar test showed the isolates from Banjarbaru, Tapin and Tabalong were able to use all carbon sources being tested; in other words, they were categorized as biovar 3. Various carbon sources the bacterium can use means *R. solanaearum* can infect various hosts since carbohydrate is the molecule developed as the result of photosynthesis. The evidence is the findings of study conducted in Banjar and Banjarbaru that all types of vegetable mainly peanut, ginger, tomato, eggplant, large chilli and celery are the hosts of *R. solanacearum* because they are grown next to or in the land where plantain used to grow[1].

Argued that the cause of plant diseases is chemoheterotrophs that obtain energy from carbohydrate metabolism, amino acid or other carbon organic substances of which function is as carbon source. Change of color from green to yellow happens because of acid that is the result of bacterial metabolism scrapped on the carbohydrate media.

Pathogeniticity refers to ability of pathogen to infect plants. In the study, pathogeniticity refers to incubation time and how infectious a pathogen is. Based on studies, symptom

of wilt disease appears because bacteria attack vascular system so that water and nutrient transportation system in the xylem is blocked and plants lack of both water and nutrients[13].

Plant age, bacterial concentration, inoculums virulence and environment affect incubation time[15]. Plant age determines phase or time when it is vulnerable to disease. The plants in the study are less than two months, between 30 and 50 cm long and grown using plant tissue isolation method. The plants in such condition show the symptoms of wilt disease when they are inoculated by virulent *R. solanacearum* pathogen. Types of plantain that can be infected by the bacteria and show the symptoms of wilt disease are blooming and young plantains.

Postulated that isolates from the same host have different genetic variation[16,17]. The findings showed that the *R. solanacearum* isolates from Banjarbaru and Tapin have faster incubation time than those from Tabalong even though all three isolates were from plantain. The incubation time of the isolates from Banjarbaru and Tapin was 60 days after inoculation while that of the isolates from Tabalong was 66 days after inoculation.

Besides that, there is different characteristic in the plants infected by the wilt disease. In the *R. solanacearum* isolates from Banjarbaru and Tapin, the leaves were wilted and the leaves covering the branch were blooming. On the other hand, the leaves covering the branch in the *R. solanacearum* isolates from Tabalong were not blooming. The leaves of the isolates were also wilted. Based on the preliminary study, it was found that the isolate of the bacterium being tested is virulent *R. solanacearum* that will infect the plantain. Mentioned that the level of resistance of a cultivar is not fixed[12,17], but is influenced by the rapidly changing virulence and pathogenicity of pathogens that may kill plant tissues.

CONCLUSION

R. solanacearum isolates from Banjarbaru, Tapin and Tabalong can use lactose, maltose, mannitol, dulcitol and sorbitol as the source of carbon. *Ralstonia solanacearum* infected the plantains grown in Banjarbaru, Tapin and Tabalong, South Kalimantan is categorized as biovar 3. Pathogeniticity is indicated by incubation time. The incubation time of *R. solanacearum* isolates from Banjarbaru and Tapin is 60 days after inoculation while that of *R. solanacearum* isolates from Tabalong is 66 days after inoculation.

SIGNIFICANCE STATEMENT

This research has found about *R. solanacearum* isolates in South Kalimantan, as biovar 3 and able to use carbon sources. Determination of biovar isolates is very important to reveal the characteristics of these isolates. This finding is very useful for researchers and the community in general and for local governments, to implement alternative controls. The next action was immediately carried out research to determine bacterial wilt disease in other plants, to prevent the spread to other areas, so that researchers and the government can develop plants that are resistant to the invasion of the biovar bacterial wilt disease 3 (three).

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