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Diversity of *Ralstonia pseudosolanacearum*, the causal agent of bacterial wilt on *Eucalyptus pellita* in Indonesia

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Abstract. *Siregar BA, Giyanto, Hidayat SH, Siregar IZ, Tjahjono B. 2021. Diversity of* Ralstonia pseudosolanacearum, *the causal agent of bacterial wilt on Eucalyptus pellita in Indonesia. Biodiversitas 22: 2538-2545.* The *Ralstonia* species complex was initially classified into five races and five biovars but the classification could not accommodate the isolates' phylogenetic history or geographic origins. A phylotype and sequevar system is based on the geographic distribution and characteristics of endoglucanase (*egl*) and hypersensitive response and pathogenicity (*hrp*) gene sequences. This study aims to describe pathogen diversity of the causal agent of bacterial wilt on *Eucalyptus pellita* F. Muell. Pathogens were isolated from wilting seedlings and trees in several *Eucalyptus* plantations. The phenotypic diversity analysis included biovar, exopolysaccharide quantification and virulence tests, while genotypic diversity included phylotypes and sequevar determination based on *egl* gene sequences. A total of 35 strains were isolated from the field and nurseries of *Eucalyptus* in various locations. All isolates were confirmed as *Ralstonia* species complex based on morphological characteristics and molecular studies using species-specific primers. These isolates were dominantly classified as biovar 3 and 4 and had a high variation on virulence and EPS production. Based on the *egl* sequence's alignment, 29 strains of Phylotype I are grouped into four sequevar references (sequevars 14, 17, 18, 30) and new a sequevar 58. This study shows that strains of *R. pseudosolanacearum* causing bacterial wilt on *E. pellita* in Indonesia have high phenotypic and genotypic diversities.

Keywords: Diversity, endoglucanase, exopolysaccharide, *Eucalyptus pellita*, species complex

INTRODUCTION

Bacterial wilt on eucalypt was first reported in China (Cao 1982) and Brazil (Sudo et al. 1983), then in Indonesia in 1985 (Machmud 1985). Subsequently, massive attacks on eucalypt plantations occurred in Australia, Venezuela, Paraguay, South Africa and Indonesia (Santiago et al. 2014; Coutinho et al. 2000; Siregar et al. 2020). The studies of the disease were mostly on *Eucalyptus camaldulensis*, *E. dunii*, *E. grandis*, *E. urophylla*, *E. saligna*, and their hybrids. Little research has been dedicated to bacterial wilt disease on *E. pellita* and its few developed hybrids, despite the importance of the species to Indonesian forestry, where losses due to outbreaks of the disease both in nurseries and plantations in 2010-2011 were estimated at IDR 16 billion (Siregar et al. 2012).

The causal agent of bacterial wilt disease on *E. pellita*, *Ralstonia solanacearum*, was further identified as race 1 biovar 3 (Siregar et al. 2012; Tjahjono et al. 2011). Carstensen et al. (2017) reported bacterial wilt disease on eucalypts in Asia and Africa as *Ralstonia pseudosolanacearum* (Phylotype I), which refers to changes in the nomenclature of the *Ralstonia* species (RS) complex (Safni et al. 2014). The diversity of phenotypic and genotypic traits of this pathogen is high enough that it is referred to as a complex species. The RS complex was initially classified into five races and five biovars (Denny and Hayward, 2001). However, this approach cannot classify isolates according to their phylogenetic history or geographic origin (Fonseca et al. 2014). Therefore, a phylotype and sequevar system was formed, considering the geographic distribution, the diversity of the endoglucanase (*egl*), and the hypersensitivity response and pathogenicity (*hrp*) gene (Fegan and Prior, 2005). This classification can study the population of pathogens in a specific geographic area and their virulence properties.

The RS complex produces several virulence factors, including plant cell wall degrading enzymes (e.g. endoglucanase) and exopolysaccharide I (EPS); thus, the virulence of each strain is phenotypically different. Enzymes that destroy plant cell walls make a strain more virulent by increasing the invasion and colonization of vessel tissue (Saile et al. 1997), but these enzymes are unnecessary for disease development. Meanwhile, EPS is essential for the appearance of wilt symptoms and plant death (McGarvey et al. 1998). EPS is produced in excessive amounts both in vitro and in planta and is believed to function by clogging the xylem vessels causing wilting. The EPS quantification produced by pathogens is one of the indicators of pathogen's virulence in causing disease symptoms.

Molecular methods have been used in further understanding of RS diversity (Chesneau et al. 2018; Sharma et al. 2021). Molecular identification of the causal agent of bacterial wilt on *Eucalyptus* in Indonesia at the phylotype and sequevar levels has not been attempted. Besides, knowledge of phenotypic diversity is also essential to see virulence level of each dominant pathogen isolate in a population. This study aims to describe diversity of the causal agent of bacterial wilt on *E. pellita*.

MATERIALS AND METHODS

Sample collection

Samples were taken from *E. pellita* seedlings and trees from nursery and field plantation areas, in Riau, Jambi, South Sumatra and East Kalimantan, Indonesia. Plants showing bacterial wilt symptoms (i.e., yellow leaves followed by wilting branches) were selected and had their stem cut transversally. A bacterial exudate coming out from the plant vessels confirmed the bacterial wilt disease. The infected tissues were then put into plastic bags, labeled, and taken to the lab.

Pathogen isolation

Pathogen isolation was carried out from wilted symptomatic *Eucalyptus* trees and seedlings from different locations, ages, and *Eucalyptus* clones following the procedure described in Tjahjono (2015). Infected stems were cut transversely and placed in a soaked container and sterilized with 5.25% NaOCl twice, then rinsed with sterile water. The stem pieces were then inserted into a test tube containing 10 mL of sterile distilled water and stirred in an incubator shaker for 30 min. 0.1 mL of the solution was transferred to a Petri dish containing TZC growth medium (2,3,5-triphenyl tetrazolium chloride) and incubated at 28 °C for 72 hours. A single colony of virulent bacteria with irregular shape, fluid, and pink in color was taken and rejuvenated in another Petri dish containing TZC medium. Some RS isolates from chili, tomato, acacia, and ginger were used as an out-group. Confirmation of RS species complex was performed using PCR species-specific primers (explained later in a separate section).

Phenotypic diversity analysis

Biovar determination

Biovar of each strain was determined using a Basal medium containing 1.0 g NH4H2PO4, 0.2 g KCl, 0.2 g MgSO4.7H2O, 1.0 g Peptone, 3.0 g agarose, 80 mg bromothymol blue in 1000 mL distilled water (Denny and Hayward 2001). The pH was adjusted to 7.0-7.1 (green color). The medium was heated to melt the agar, poured into Erlenmeyer, and sterilized in autoclave at 121 °C for 20 min. The 10% aqueous solutions of each different carbohydrate source (dextrose, mannitol, sorbitol, dulcitol, trehalose, lactose, maltose D (+) cellobiose) was sterilized using filter sterilization. Ten mL of the carbohydrate solution was added to 90 mL of warm basal medium to get a final concentration of 1%. Once homogeneous, 3 mL of the molten medium was added to a sterile test tube and allowed to solidify. One hundred µl of a 48 h old culture of RS strains was added into the test tube and incubated at 28°C. The test tubes were examined at 3, 7, 14, and 28 days after inoculation for pH changes (indicated by the color change). The biovars of the RS isolates were determined as described in Denny and Hayward (2001).

Exopolysaccharide (EPS) quantification

Quantification of EPS as a virulence factor for RS bacteria was carried out following the protocol described by Peyraud et al. (2017). A total of 5 mL of 24-48 h old liquid culture of bacterial strains with a minimum concentration of $5x10^8$ cfu/mL was filtered using a 0.22 µm syringe filter and the supernatant was collected on a 1.5 mL microcentrifuge tube. A fraction of 0.2 mL of the supernatant was mixed with 0.004 mL of 5 M NaCl and 0.8 mL of acetone for extraction. The solution was stirred with a vortex for 10 sec and stored at 4°C for 12 hours (overnight). The remaining supernatant was stored at -20°C.

After the 12-hour-period, the suspension was centrifuged for 10 min at 13,000 rpm and 4°C; the supernatant was then removed, and the pellet was air dried. The dried pellets were then dissolved with 0.2 mL of ddH_2O. The suspension can be stored at 4 °C for several days until quantification is carried out. For the EPS quantification, the suspension was heated using a dry bath heater at 65°C for 10 min, then mixed with a Vortex and centrifuged for 5 min at 13,000 rpm, 4°C. The supernatant was transferred to a 2 mL microcentrifuge tube for subsequent EPS quantification.

Two hundred µl of the EPS sample was mixed with 0.15 mL of HCl (37%) and the volume was completed to 0.6 mL with ddH_2O in a 2 mL tube. The tube was shaken with a Vortex, closed tightly and placed on a dry bath heater at 115°C for 30 min. The tube was then allowed to cool down to room temperature and centrifuged for 5 s at 6,000 rpm. 0.4 mL of 2M Na_2CO_3 was added slowly and mix gently. In the final step, 0.5 mL of 2% acetyl acetone solution in 1.5 M Na_2CO_3 was added into solution and the tube was vortexed. CO_2 was released from the tube and the tube was kept open to avoid heating of the solution. The tube was then mixed gently once the CO_2 bubble slows down and vortexed well. The tube was incubated 30 min in the dark condition; the level of turbidity (optical density/OD) of the solution was then read at 530 nm. Pure water was used as a blank control. EPS quantity was calculated based on the relationship between the standard OD curve and the concentration of N-acetylgalactosamine.

Virulence test

The virulence test was carried out through artificial inoculation of 3-month-old eucalypt seedlings in growth chamber. Inoculation was performed using the method described by Fonseca et al. (2015) with some modifications. A 3-day-old bacterial cell suspension (0.5 mL) was injected into an artificial wound at the seedling's stem base (1 mm deep and 10 mm long using a scalpel) 5 cm above the ground.

Table 1. Host resistance score category of virulence test

Score+	Sign* and Symptom		
	Ooze at 6 cm	Ooze at 3 cm	Plant condition
5	Negative	Negative	Healthy
4	Negative	Positive	Healthy
3	Positive	Positive	Healthy
2	Positive	Positive	Wilting / died
1	Negative	Positive	Wilting / died

Note: + A higher score indicates a higher host resistance level, * The bacterial ooze observed (under microscope) on cut stem samples at 3 and 6 cm above the inoculation point

The wound was then covered using wet cotton wool and wrapped with parafilm to keep it moist and to avoid contamination by other microorganisms. Each bacterial strain was inoculated into10 plants and three other wounded plants but with no inoculum were used as control. Inoculated and control plants were incubated in growth chamber at a temperature of 28±2°C, humidity > 80%, and 12 hours of photoperiod, with light intensity of 40 µmol/s/m^2. Plants' symptoms were daily evaluated to observe latent period, disease incidence, and host resistance score (Table 1) until 30 days after inoculation.

Genotypic diversity analysis

Phylotype-Specific Multiplex PCR (m-PCR) amplification

Genomic DNA was extracted using the Bacteria Genomic DNA Kit (Geneaid), according to the manufacturer's instructions. A PCR test using species-specific primers OLI1 and Y2 (Table 2) to amplify a 288 base pair (bp) product was performed to confirm that all isolates belong to the RS species complex. Each 25 µL reaction mix contained 1 x reaction buffer, 1 U Taq DNA-polymerase (Green GoTaq), 25 mM $MgCl_2$, 2.5 mM dNTP mix, 10 µM of each primer, and 20 ng of DNA template. Amplification was carried out in a 2720 Thermal Cycler (Applied Biosystems). The reaction conditions used were heating at 94 °C for 2 min followed with 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Water control was included in every PCR assay to confirm that there was no contamination in the assay.

The mPCR developed by Fegan & Prior (2005) was used to classify the bacterial isolates into phylotypes using a set of four primers for each specific phylotype (Table 2). Each 25 µL reaction mixture contained 1 x reaction buffer, 1 U Taq DNA-polymerase (Green GoTaq), 25 mM $MgCl_2$, 2.5 mM dNTP mix, 10 µM of each primer and 20 ng of DNA template. Amplification was carried out in a 2720 Thermal Cycler (Applied Biosystems). The reaction conditions used were heating at 96 °C for 3 min followed with 30 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1.5 min and a final extension at 72 °C for 10 min. The 144-; 372-; 91-; and 213-bp products generated by m-PCR amplification belong to phylotypes I, II, III and IV, respectively (Fegan & Prior 2005).

Five µl of the amplified product was separated on a 1.5 % (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer, stained with 0.5 µg/ml of ethidium bromide for 20 min at 60 V constant voltage and the gel was photographed under ultraviolet light using Alfa imager Gel Doc system. For each gel, a 100 bp DNA ladder (Promega) was used as marker to determine the amplicons' size.

Partial sequencing of the endoglucanase (egl) genes

Partial sequencing of the *egl* gene region was performed to identify sequevar groups (Fegan and Prior, 2005). The *egl* gene region was amplified using Endo-F and ENDO-R primers (Table 2). The reaction composition and PCR process conditions followed the method described by Fonseca et al. (2014). Each 25 µL reaction mix contained 1 x reaction buffer, 1 U Taq DNA polymerase (Green GoTaq), 25 mM MgCl2, 2.5 mM dNTP mix, 10 µM of each primer and 20 ng of DNA template. Amplification was carried out in a 2720 Thermal Cycler (Applied Biosystems). The reaction conditions used were heating at 96°C for 9 min followed with 30 cycles 94°C for 60s, 70°C for 60s, 72°C for 120s, and a final extension at 72°C for 10 min. The PCR products were purified and sequenced by Macrogen, Inc.

Sequence analysis

Phylogenetic analysis was carried out on 33 *egl* gene sequences. The sequences were edited using BIOEDIT v.7.2.5 sequence alignment editor and aligned using MEGA version 10. The phylogenetic tree was generated using the neighbor-joining (NJ) method analysis with bootstrapping 1000 replications using MEGA version 8. All the *egl* sequences from the RS strains used in this study were deposited into GenBank (Table 3).

Table 2. Primers used in this study

Level	Primers	Sequence	Target	Reference
R. solanacearum complex	OLI1	5'-GGGGGTAGCTTGCTACCTGCC-3'	16s rDNA	Seal et al. 1993
	Y2	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'		
Phylotype	Nmult: 21:1 F	5'-CGTTGATGAGGCGCGCAATTT-3′	ITS region	Fegan dan Prior 2005
	Nmult: 21:2 F	5'-AAGTTATGGACGGTGGAAGTC-3′		
	Nmult: 22: Inf	5'-ATTGCCAAGACGAGAGAAGTA-3′		
	Nmult: 23: AF	5'-ATTACGAGAGCAATCGAAAGATT-3′		
	Nmult: 22:RR	5'-TCGCTTGACCCTATAACGAGTA-3′		
Sequevar	ENDO-F	5'-ATGCATGCCGCTGGTCGCCGC-3′	*egl* gene	Ji et al. 2007
	ENDO-R	5'-GCGTTGCCCGGCACGAACACC-3′		

RESULTS AND DISCUSSION

Confirmation of *R. solanacearum* species complex isolates

A total of 39 strains were isolated from wilting eucalypt plants in plantations and nurseries in various locations (Table 3). Morphological observations of the colonies on semi-selective media and molecular observations using specific primers of RS species complex were carried out to confirm the strains. On TZC medium the virulent RS bacteria strains were round, irregular, fluidal, and pink in color, surrounded by a white fluid. As a comparison, RS strains were isolated from tomato, chilli, acacia (*Acacia crassicarpa*), and ginger, which showed similarity to the strains isolated from eucalypts. PCR assays with OLI1 and Y2 specific primers confirmed a positive result with the expected 288-bp-long fragments (Figure 1). All isolates tested in this study were molecularly confirmed to belong to the *Ralstonia* species complex and were therefore used for further tests.

Phenotypic diversity of *R. solanacearum*

Biovar variability

Twenty-seven out of the 39 tested strains utilized D-(+)-cellobiose, dulcitol, lactose, maltose, mannitol, and sorbitol and thus are classified as biovar 3, while 12 strains utilized dulcitol, mannitol, and sorbitol, but not D-(+)-cellobiose, lactose, and maltose and thus are classified as biovar 4 (Table 3). There were no other biovars identified.

EPS quantification

The EPS production varied from 0.13 to 8.38 µg/mL across the 39 strains used in this study (Table 4). This variation might be related to the virulence or latency period of wilt symptoms in the host plant.

Virulence diversity of R. solanacearum

Virulence differentiation of RS strains from *E. pellita* in a 3-month-old susceptible clone, named EP0335WK, was observed in this study. The first symptoms appeared at four days post-inoculation (dpi), on the 2 youngest leaf pairs of inoculated seedlings. The wilting started from the top leaves, then spreading down to the entire plant, followed by defoliation and plant's death. Control plants showed no visible symptoms. The latent period for each strain varied from 4 to 29 dpi.

Besides the wilting symptoms, RS-infected plants had the inner stem discolored, getting a dark brown appearance, followed by abundant secretion of bacterial ooze. A combination of wilt symptoms and the bacterial ooze secretion was used to assess the isolate's virulence and host's phenotypic response (Table 4). Host's response and disease incidence (DI) also varied in the virulence test. Twelve strains had high virulence levels (DI 80-100%), while eight strains had low virulence levels (DI 0-25%) at 30 dpi (Table 4).

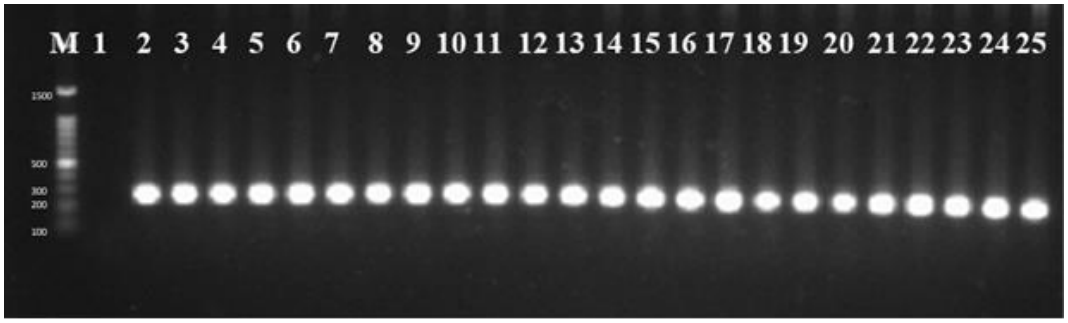

Figure 1. PCR result using OLI1/Y2 specific primers. (M) marker 100bp, (1) negative control, (2) positive control of RS, (3-22) Strains code RIE-Dr-056, RIE-Dr-067, RIE-Ge-078, RIE-Lk-014, RIE-Lk-032, RIE-Pr-051, RIE-Rd-033, RIE-Lk-047, RIE-Pr-052, RIE-Rd-002, RIE-Rd-059, KTE-Sb-061P, KTE-Sb-062P, KTE-Sb-063P, JME-St-069, JME-St-071, JME-St-068, JME-St-076, JME-St-081, PBE-Me-070, respectively, (23) RIA-Mi-091 (*Acacia crassicarpa*), (24) YGT-Sl-099 (tomato), and (25) SBC-Ua-090 (chili).

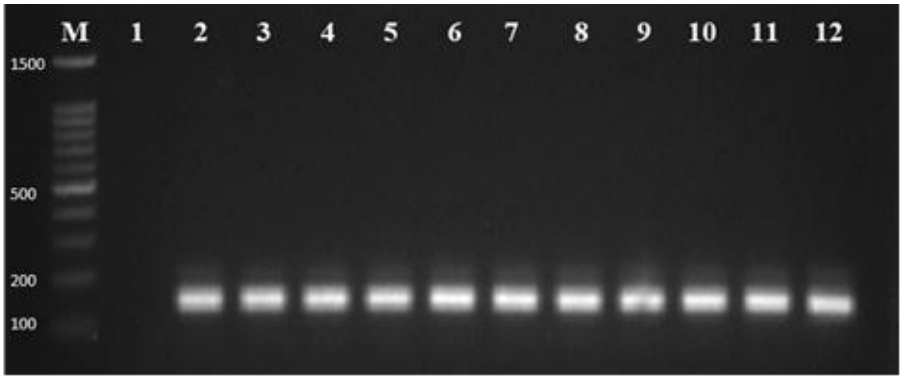

Figure 2. Multiplex PCR result with expected 144 bp specific fragments. (M) Marker 100 bp, (1) Negative control, (2-9) Strains code RIE-Dr-056, RIE-Dr-067, RIE-Lk-047, KTE-Sb-061P, KTE-Sb-063P, JME-St-069, JME-St-076, PBE-Me-070, respectively, (10) RIA-Mi-091 (*Acacia crassicarpa*), (11) YGT-Sl-099 (tomato), and (12) SBC-Ua-090 (chili).

Table 3. List of *Ralstonia* strains associated with bacterial wilt of *Eucalyptus pellita* used for phylotyping and sequevar identification

Strain code	Origin	Host	Bio-var	Phylotype / sequevar	GenBank acc. no.
KTE-Sb-061P	East Kalimanta	*E. pellita*	3	I/18	MW748356
KTE-Sb-062P	East Kalimanta	*E. pellita*	3	I/14	MW748357
KTE-Sb-063P	East Kalimanta	*E. pellita*	4	I/-	-
KTE-Sb-102P	East Kalimanta	*E. pellita*	3	I/14	MW748358
JME-St-068	Jambi	*E. pellita*	4	I/18	MW748351
JME-St-069	Jambi	*E. pellita*	3	I/45	MW748352
JME-St-071	Jambi	*E. pellita*	3	I/nd	-
JME-St-076	Jambi	*E. pellita*	4	I/18	MW748353
JME-St-081	Jambi	*E. pellita*	4	I/18	MW748354
RIE-Dr-056	Riau	*E. pellita*	3	I/nd	-
RIE-Dr-067	Riau	*E. pellita*	3	I/45	MW748360
RIE-Ge-078	Riau	*E. pellita*	3	I/nd	-
RIE-Lk-014	Riau	*E. pellita*	3	I/18	MW748361
RIE-Lk-032	Riau	*E. pellita*	3	I/nd	-
RIE-Lk-043	Riau	*E. pellita*	3	I/18	MW748362
RIE-Lk-047	Riau	*E. pellita*	4	I/14	MW748363
RIE-Pr-013	Riau	*E. pellita*	3	I/nd	-
RIE-Pr-051	Riau	*E. pellita*	3	I/18	MW748364
RIE-Pr-052	Riau	*E. pellita*	4	I/18	MW748365
RIE-Rd-002	Riau	*E. pellita*	4	I/18	MW748366
RIE-Rd-033	Riau	*E. pellita*	3	I/nd	-
RIE-Rd-039	Riau	*E. pellita*	3	I/17	MW748367
RIE-Rd-040	Riau	*E. pellita*	3	I/17	MW748368
RIE-Rd-054	Riau	*E. pellita*	3	I/18	MW748369
RIE-Rd-055	Riau	*E. pellita*	3	I/45	MW748370
RIE-Rd-059	Riau	*E. pellita*	4	I/18	MW748371
RIE-Rd-093	Riau	*E. pellita*	3	I/45	MW748372
RIE-Rd-177B	Riau	*E. pellita*	3	I/30	MW748373
RIE-Rs-010	Riau	*E. pellita*	4	I/18	MW748374
RIE-Rs-015	Riau	*E. pellita*	3	I/18	MW748375
RIE-Rs-016	Riau	*E. pellita*	3	I/14	MW748376
RIE-Rs-018	Riau	*E. pellita*	3	I/14	MW748377
RIE-Rs-019	Riau	*E. pellita*	3	I/18	MW748378
RIE-Rs-046	Riau	*E. pellita*	4	I/nd	-
RIE-Rs-048P	Riau	*E. pellita*	3	I/14	MW748379
RIE-So-017	Riau	*E. pellita*	4	I/14	MW748380
RIE-So-103	Riau	*E. pellita*	3	I/nd	-
RIE-Tp-023P	Riau	*E. pellita*	4	I/18	MW748381
PBE-Me-070	South Sumatra	*E. pellita*	3	I/nd	-
JTT-Ml-101	East Java	Tomato	3	I/14	MW748355
RIA-Mi-091	Riau	*A.crassicarpa*	3	I/18	MW748359
JBJ-Bg-092	West Java	Ginger	4	I/nd	-
SBC-Ua-090	West Sumatra	Chili	4	I/nd	-
YGC-Sl-100	Yogyakarta	Chili	4	I/14	MW748382
YGT-Sl-099	Yogyakarta	Tomato	3	I/45	MW748383

Note: -: The strain did not determine in the sequevar analysis.

Genotypic diversity

Phylotyping and phylogenetic analysis

The mPCR results showed that all strains of RS from *E. pellita* belong to phylotype I, as seen by the expected 144 bp specific fragments (Figure 2). Based on the new classification system, phylotype I belongs to *R. pseudosolanacearum*. The same result also happened to the RS isolates from tomato, chili, ginger, and *A. crassicarpa*. Thus, the *R. pseudosolanacearum* strains from *E. pellita* used in this study belong to phylotype I and biovar 3 or 4.

Partial sequencing of the *egl* gene region was analyzed to identify sequevar groups. The *egl* gene sequences from the 33 strains shared 99.1%-100% identity. The accession numbers MW748351 to MW748383 were assigned to these sequences in GenBank. Twenty-nine strains from *E. pellita* and four strains from the other different hosts were used as representatives; 47 reference strains of *R. solanacearum* retrieved from GenBank were also included to construct the phylogenetic tree using the *egl* gene. Phylogenetic analysis of partial *egl* gene sequences showed that all of the 33 strains analyzed in this study were classified as phylotype I, consistent with mPCR results (Figure 3).

Table 4. Phenotypic diversity of RS strain from *Eucalyptus pellita*

Strain code	EPS quantification (µg/mL)	Latent period (days)	Host response (score)	Disease incidence (%)
JME-St-068	5.88	-	3.6	0
JME-St-069	1.60	5.6	1.4	100
JME-St-071	4.70	20.0	2.4	60
JME-St-076	1.10	3.5	3.5	50
JME-St-081	0.40	14.0	1.8	100
KTE-Sb-061P	1.70	8.0	1.8	80
KTE-Sb-062P	4.00	17.0	3.3	20
KTE-Sb-063P	3.30	8.7	3.0	66
KTE-Sb-102P	6.30	23.5	3.5	40
PBE-Me-070	4.30	11.5	2.3	80
RIE-Dr-056	5.13	15.0	1.6	100
RIE-Dr-067	5.13	10.6	1.6	100
RIE-Ge-078	4.25	13.0	2.6	60
RIE-Lk-014	5.30	8.4	1.0	100
RIE-Lk-032	6.13	25.0	3.8	20
RIE-Lk-043	1.70	16.6	1.3	100
RIE-Lk-047	5.88	20.0	3.0	50
RIE-Pr-013	2.00	14.0	3.3	25
RIE-Pr-051	1.80	15.0	4.0	20
RIE-Pr-052	4.88	18.3	2.8	50
RIE-Rd-002	3.30	11.3	3.2	60
RIE-Rd-033	0.90	10.0	2.0	80
RIE-Rd-039	3.40	18.0	3.8	40
RIE-Rd-040	0.40	16.3	2.3	66
RIE-Rd-054	2.90	9.0	4.0	20
RIE-Rd-055	0.13	9.8	2.5	80
RIE-Rd-059	*	6.6	1.7	100
RIE-Rd-093	5.70	24.0	3.4	40
RIE-Rd-177B	0.90	16.8	2.0	80
RIE-Rs-010	0.70	-	4.0	0
RIE-Rs-015	1.10	-	4.2	0
RIE-Rs-016	1.70	13.0	1.7	75
RIE-Rs-018	3.88	14.0	2.2	60
RIE-Rs-019	8.38	8.8	3.5	50
RIE-Rs-046	*	15.0	3.6	40
RIE-Rs-048P	2.50	-	4.0	0
RIE-So-017	0.30	17.7	2.3	50
RIE-Tp-023P	5.10	5.7	2.0	75

Note: * not detected

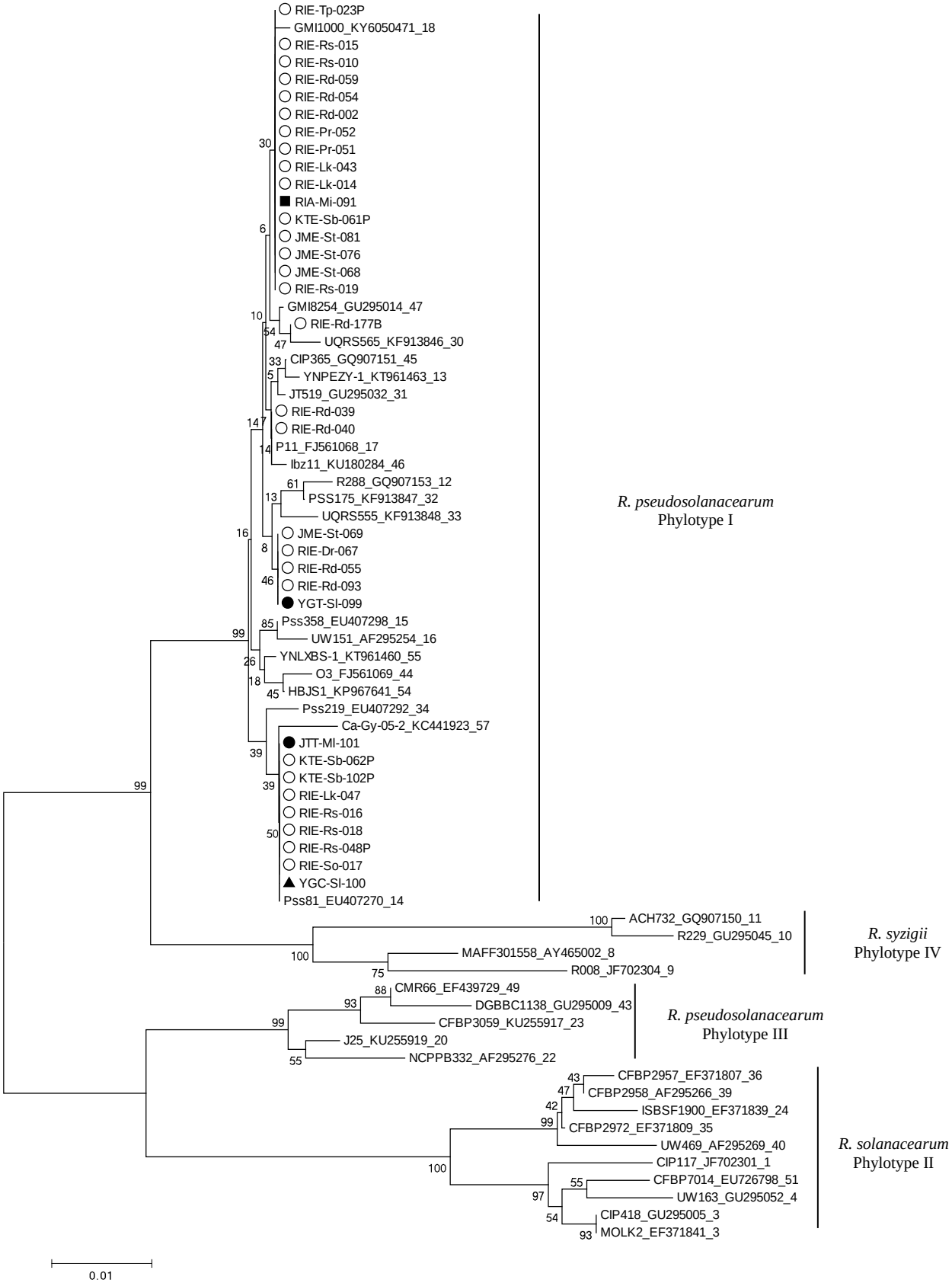

Figure 3. Phylogenetic analysis of 33 strains used in this study (white circle - from *Eucalyptus pellita*; black triangle - chili; black circle - tomato, black square - *Acacia crassicarpa*) and 49 references strains of *R. solanacearum* using the neighbor-joining method. The numbers at the tree branch points indicate the percent bootstrap support for 1,000 iterations. The name of the reference strain followed by the accession number and sequevar number

The *egl* tree showed that *R. pseudosolanacearum* strains from *E. pellita* clustered with four major groups A, B, C, and D. Grup A, which included 15 strains, represents sequevar 18 (reference strains tomato GMI 1000). Grup B, which included seven strains, represents sequevar 14 (reference strains tomato Pss81). Grup C, which included two strains, represents sequevar 17 (reference strains peanut P11). Grup D, which only included one strain, represents sequevar 30 (reference strains ginger UQRS565). Besides, four strains from *E. pellita* were not identical to any previously designated sequevar, named sequevar 58. Strains from chili and tomato were grouped with sequevar 14, while strains from *A. crassicarpa* were grouped with sequevar 18.

Discussion

Bacterial wilt has been reported attacking eucalypt plantations in Indonesia in the last decades (Carstensen et al. 2017; Machmud 1985; Siregar et al. 2012; Siregar et al. 2020; Tjahjono et al. 2011). The disease severity varied depending on locations, plant's age and clones. Initially, attacked plants show yellowish wilting leaves followed by wilting branches, the internal part of the stems turn blackish-brown, and bacterial exudate is seen on the cutted stems (Siregar et al. 2020). Isolation from wilting eucalypt plants from plantation and nursery resulted in 39 bacterial strains showing similar morphological characteristics such as irregular rounded shape, fluidal, and pink in color, surrounded by a white fluid on semi-selective TZC medium. The morphology of isolated colonies was consistent with previous descriptions of *R. solanacearum* (She et al. 2012). Molecularly, all isolates subjected to PCR assays with OLI1 and Y2 specific primers confirmed a positive result of the expected 288 bp specific fragments (Seal et al. 1993). Both approaches confirm that all isolates were species from the *Ralstonia* complex.

The phenotypic diversity analysis of RS isolates includes biovar, exopolysaccharide quantification and virulence test. Biovar determination depends on the isolate's ability to utilize or react with certain disaccharides and hexose alcohol. Biovar 3 isolates were predominant over biovar 4 within the samples isolated from *E. pellita*. Previous research on the RS biovar from another eucalypt species showed that biovar 2T is dominant in 'urograndis' hybrids in Brazil (Marques et al. 2012). In contrast, in Asia and Australia, biovar 3 is the most common (Coutinho et al. 2008), which is in line with results of this study. The isolates from chili and ginger were classified as biovar 4, while tomato and *A. crassicarpa* biovar 3. Biovars 3 and 4 found in Asia are reported to cause wilt disease in various host plants, including the Solanaceae family (Denny and Hayward 2001).

EPS is a major virulence factor of several phytopathogenic bacteria, including *Ralstonia*, producing high amounts of it either in pure culture or during plant multiplication (Alvarez et al. 2010). The EPS quantification is based on the determination of N-acetyl-D-galactosamine concentration produced by isolates. In *Ralstonia*, it has been reported that all virulent wild-type strains produce EPS (Álvarez et al. 2010). The EPS production varied within the strains used in the present study with a value range of 0.13 - 8.38 µg/mL. This number is lower than that in the previous studies, most likely because of the bacterial cell growth in minimum medium used in the studies (Addy et al. 2012, Negishi et al. 1992, Peyraud et al. 2017). Cultures grown on minimum broth medium affect the viscosity of the culture supernatant, which is most likely directly related to the amount of EPS produced by each strain (Denny et al. 1988). In plants, EPS contributes to wilting symptoms by blocking the xylem vessels, directly interfering with the movement of fluid within the plant or destroying the vessel tissue caused by excessive hydrostatic pressure. In addition, EPS plays a role in manipulating the surface structure of bacteria recognized by plant defense mechanisms; this condition causes bacteria to colonize the stems. Therefore, EPS is considered as the main virulence factor in *R. solanacearum* (Schell 2000; Araud-Razou et al. 1998).

The virulence test in this study succeeded in determining the virulence level of each strain. Variations in disease incidence, host response scores, and the latent period might be contributing to the varying disease symptoms incidence by each strain in plantation. The modified method developed in this study could produce disease symptoms faster than the original procedure described by Fonseca et al. (2015). This method is a breakthrough in screening for disease resistance in forest species that used to take years through old methods of observation of infected trees in plantation. This approach can be used in massive disease screening programs in growth chambers to produce superior clones as part of the plant breeding programs.

The abundance of bacterial ooze is also important to the virulence test. This ooze shows bacterial colonization in the stems and, together with EPS production, plays a role in causing wilting in plants. Virulent strains will produce symptoms with a short latent period, high incidence of disease, and low host response scores. The absence of symptoms on the susceptible clone EP0335WK inoculated with some RS strains might indicate a specific interaction between the host and certain strains. This interaction can occur by strains unable to colonization of the vessel tissue and the expression of the strains virulence factor such as EPS production and hrp gene expression in causing wilt symptoms (Zheng et al. 2017). On the other hand, plant recognizing specific signals can affect the induction of plant innate immunity (Hikichi 2016).

In this study, the RS strains obtained from *E. pellita* host plants and different geographical areas of four provinces in Indonesia were characterized for their phylogenetic characteristics. mPCR and phylogenetic analysis findings indicate that RS strains in Indonesia are categorized as phylotype I. This result is consistent with the current taxonomy revision, in which all strains of *R. solanacearum* from Asia are classified into Phylotype I (race 1: bv 3, 4, and 5) (Fegan and Prior 2005, Safni et al. 2014). This finding also proves earlier reports that *R.*

pseudosolanacearum is the primary pathogen causing bacterial wilt on *E. pellita* in Indonesia. From 33 *egl* sequences, four previously described sequevars and a new sequevar 58 were identified. Among those four sequevars, sequevar 30 has not been reported to infect *Eucalyptus* in Asia. Sequevar 17 and 18 have been reported on *Eucalyptus* in Indonesia and sequevar 14 in China (Carstensen et al. 2016). Sequevar 30 was reported in Thailand to infect ginger (Albuquerque et al. 2014). The four strains from *E. pellita* and one strain from tomato are proposed as sequevar 58 because it was not identical to any previous sequevar in Indonesia and Asia. Phylogenetic analysis of the *egl* gene sequence is in line with the phylotype result. It shows that the sequevar grouping is not limited by geographic area and host plants. It should also be noted that there is a potential for cross-infection of isolates from other plants to eucalypt plants or vice-versa.

The second-largest forest species for wood production in Indonesia after acacia species, the eucalypt faces a serious issue of bacterial wilt. This association is widely spread to eucalypt plantations throughout Indonesia. Our study shows that the use of resistant genetic plant materials and suppression of bacterial populations in the nursery environment has so far been the main control measures of the disease on eucalypt (Siregar et al. 2020). Further research should be continued to reduce the risk of losing wood yield due to this disease. In this paper, the phenotypic and genotypic properties of RS strains are shown to vary widely. This will help develop resistant clones as part of the integrated disease management.

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REFERENCES

Addy HS, Askora A, Kawasaki T, Fujie M, Yamada T. 2012. Loss of virulence of the phytopathogen *Ralstonia solanacearum* through infection by φRSM filamentous phages. Phytopathol 102: 469-477 DOI: 10.1094/PHYTO-11-11-0319-R.

Albuquerque GMR, Santos LA, Felix KCS, Rollemberg CL, Silva AMF, Souza EB, Cellier G, Prior P, Mariano RL. 2014. Moko disease-causing strains of *Ralstonia solanacearum* from Brazil extend known diversity in paraphyletic phylotype II. Phytopathol 104: 1175-1182. DOI: 10.1094/PHYTO-12-13-0334-R.

Álvarez B, Biosca EG, López MM. 2010. On the life of Ralstonia solanacearum, a destructive bacterial plant pathogen. In: Méndez-Vilas A (ed.). Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Formatex Research Center, Badajoz, Spain.

Araud-Razou I, Vasse J, Montrozier H, Etchebar C, Trigalet A. 1998. Detection and visualization of the major acidic exopolysaccharide of *Ralstonia solanacearum* and its role in tomato root infection and vascular colonization. Eur J Plant Pathol 104 (8): 795-809. DOI: 10.1023/A:1008690712318.

Cao JD. 1982. Investigation of bacterial wilt in *E. saligna* and *E. grandis* introduced from Brazil. Guangxi For Sci Technol 4: 30-31. DOI: 10.1111/ppa.12577.

Carstensen G, Venter S, Chen S, Wingfield M, Coutinho T. 2017. Two Ralstonia species associated with bacterial wilt of *Eucalyptus*. Plant Pathol 66: 393-403. DOI: 10.1111/ppa.12577.

Chesneau T, Maignien G, Boyer C, Chéron J-J, Roux-Cuvelier M, Vanhuffel L, Poussier S, Prior P. 2018. Sequevar diversity and virulence of *Ralstonia solanacearum* phylotype I on Mayotte Island (Indian Ocean). Front. Plant Sci 8: 2209. DOI: 10.3389/fpls.2017.02209.

Coutinho T, Jolanda R, Karl-Heinz R, Terblanche J. Wingfield M. 2008. First report of bacterial wilt caused by *Ralstonia solanacearum* on eucalypts in South Africa. Forest Pathol 30: 205-210 DOI: 10.1046/j.1439-0329.2000.00205.x.

Coutinho TA, Roux J, Riedel K, Terblanche J, Wingfield MJ. 2000. First report of bacterial wilt caused by *Ralstonia solanacearum* on eucalypts in South Africa. Forest Pathol 30: 205-210. DOI: 10.1046/j.1439-0329.2000.00205.x.

Denny TP, Hayward AC. 2001. Gram-negative bacteria *Ralstonia*. In: Schaad NW, Jones JB. Chun W (eds) Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd ed. APS Press, St. Paul, MN.

Denny TP, Makini FW, Brumbley SM. 1988. Characterization of *Pseudomonas solanacearum* Tn5 mutants deficient in extracellular polysaccharide. Mol Plant-Microbe Interact 1: 215-223. DOI: 10.1094/MPMI-1-215.

Fegan M, Prior P. 2005. How complex is the *Ralstonia solanacearum* species complex? In: Allen C, Prior P, Hayward AC (eds) Bacterial Wilt Disease and The *Ralstonia solanacearum* Species Complex. Amer Phytopathological Society, St. Paul, MN.

Fonseca NR, Guimarães LMS, Hermenegildo PS, Teixeira RU, Lopes CA, Alfenas AC, 2014. Molecular characterization of *Ralstonia solanacearum* infecting *Eucalyptus spp.* in Brazil. Forest Pathol 44: 107-16. DOI: 10.1111/efp.12073.

Fonseca NR, Oliveira LSS, Guimarães LMS, Teixeira RU, Lopes CA, Alfenas AC. 2015. An efficient inoculation method of *Ralstonia solanacearum* to test wilt resistance in *Eucalyptus spp*. Trop Plant Pathol 41: 42-47. DOI : 10.1007/s40858-015-0056-2.

Hikichi, Y. 2016. Interactions between plant pathogenic bacteria and host plants during the establishment of susceptibility. J Gen Plant Pathol 82: 326-331. DOI: 10.1007/s10327-016-0680-9.

Ji P, Allen C, Sanchez-Perez A, Yao J, Elphinstone JG, Jones JB, Momol MT. 2007. New diversity of *Ralstonia solanacearum* strains associated with vegetable and ornamental crops in Florida. Plant Dis 91: 195-203. DOI: 10.1094/PDIS-91-2-0195.

Machmud M. 1985. Bacterial wilt in Indonesia. In: Craswell ET, Pushperajah E (eds) Bacterial Wilt Disease in Asia and The South Pacific. ACIAR, Canberra.

Marques E, Uesugi CH, Ferreira MASV, Rezende D. 2012. Characterization of isolates of *Ralstonia solanacearum* biovar 2, pathogenic to *Eucalyptus* "urograndis" hybrids. Trop Plant Pathol 37: 399-408

McGarvey JA, Bell CJ, Denny TP, Schell MA. 1998. Analysis of extracellular polysaccharide I in culture and in planta using immunological methods: New insights and implications. In: Prior P, Allen C, Elphinstone J (eds.). Bacterial Wilt Disease: Molecular and Ecological Aspects. Springer, New York

Negishi H, Yamada T, Shiraishi T, Oku H, Tanaka H. 1993. *Pseudomonas solanacearum* plasmid pJTPS1 mediates a shift from the pathogenic to nonpathogenic phenotype. Mol Plant-Microbe Interact 6: 203-209. DOI: 10.1094/MPMI-6-203.

Peyraud R, Denny TP, Genin S. 2017. Exopolysaccharide quantification for the plant pathogen *Ralstonia solanacearum*. Bio-protocol 7: 1-8 DOI: 10.21769/BioProtoc.2289.

Safni I, Cleenwerck I, De Vos P, Fegan M, Sly L, Kappler U. 2014. Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *R. solanacearum* and *R. syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. syzygii subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. indonesiensis subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. celebesensis subsp. nov., and *R. solanacearum* phylotypes I and III strains as *Ralstonia pseudosolanacearum* sp. nov. Intl J Syst Evol Microbiol. 64: 3087-3103. DOI: 10.1099/ijs.0.066712-0.

Saile E, McGarvey JA, Schell MA, Denny TP. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology 87: 1264-1271. DOI: 10.1094/PHYTO.1997.87.12.1264.

Santiago TR, Grabowski C, Mizubuti ESG, 2014. First report of bacterial wilt caused by *Ralstonia solanacearum* on *Eucalyptus* sp. in Paraguay. New Dise Rep 29: 2. DOI: 10.5197/j.2044-0588.2014.029.002.

Schell MA. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. Ann Rev Phytopathol 38: 263-292. DOI: 10.1094/PHYTO.1997.87.12.1264.

Seal SE, Jackson LA, Young JPW, Daniels MJ. 1993. Detection of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and Blood Disease Bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J Gen Microbiol 139: 1587-1594. DOI: 10.1099/00221287-139-7-1587.

Sharma K, Kreuze J, Abdurahman A, Parker M, Nduwayezu A, Rukundo P. 2021. Molecular diversity and pathogenicity of *Ralstonia solanacearum* species complex associated with bacterial wilt of potato in Rwanda. Plant Dis 105: 770-779.

She X, Yu L, Lan G, Tang Y and He Z. 2017. Identification and genetic characterization of *Ralstonia solanacearum* species complex isolates from *Cucurbita maxima* in China. Front Plant Sci 8: 1794 DOI: 10.3389/fpls.2017.01794.

She XM, He ZF, Luo FF. 2013. Pathogen identification of *Ageratum conyzoides* bacterial wilt disease and its biological characteristics. Acta Phytophyl Sin 40: 533-539. DOI: 10.3389/fpls.2017.01794.

Siregar BA, Giyanto, Hidayat SH, Siregar IZ, Tjahjono B. 2020. Epidemiology of bacterial wilt disease on *Eucalyptus pellita* F. Muell. in Indonesia. IOP Conf Ser Earth Environ Sci 468: 012033. DOI: 10.1088/1755-1315/468/1/012033.

Siregar BA, Indrayadi H, Mardai. 2012. Ancaman penyakit layu bakteri terhadap produktivitas *Eucalyptus pellita* di dataran rendah tropis. In: Plant Protection (eds) Laporan Tahunan R&D PT. Arara Abadi. PT. Arara Abadi, Perawang, Indonesian. [Indonesian]

Sudo S, Oliveira GHN, Perreira AC. 1983. Eucalipto (*Eucalyptus sp*) e Bracatinga (*Mimosa escabrela*), novos hospedeiros de *Pseudomonas solanacearum* EF Smith. Fitopathol Bras 8: 631.

Tjahjono B. 2015. Isolasi dan Identifikasi Penyakit Layu Bakteri pada Eukaliptus. In: Sinarmas Forestry (eds). Instruksi Kerja R&D PT. Arara Abadi. PT. Arara Abadi, Perawang. Indonesia. [Indonesian]

Tjahjono B, Gafur A, Golani GD. 2011. Occurrence of bacterial wilt on acacias and eucalypts in Indonesia. J ISSAAS 17 (1): 234.

Zheng X, Zhu Y, Liu B, Lin N, Zheng D. 2017. Invasive properties of *Ralstonia solanacearum* virulent and avirulent strains in tomato roots. Microbial Pathogenesis 11: 144-151. DOI: 10.1016/j.micpath.2017.10.046.