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Morphological and Molecular Characterization of Sumatra Disease of Clove in Central Java, Indonesia

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Highlights

- The Sumatra disease has spread widely throughout the Java Island.
- The use of *egl* gene as molecular marker is reliable to correct identification.
- The causal pathogen of Sumatra disease belongs to *Ralstonia solanacearum phylotype* 4 as revealed in phylogenetic analysis.

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Morphological and Molecular Characterization of Sumatra Disease of Clove in Central Java, Indonesia

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Abstract: A wilt clove disease has been reported recently that triggered a widespread devastation among clove plantings in Central Java, Indonesia. In the early stage, the younger leaves showed wilt suddenly, falling from the twig tip of branches in the crown, leaving the twig tip bare. However, older leaves may also turned brown and stay attached to the branches of infected plants. Several causative pathogens have been previously reported; including oomycetes, fungi, and bacteria. The aim of this research was to confirm the pathogen and to characterize the disease from clove plantings in the field. The research involved on morphological characterization, histology of infected plants, and molecular characterization of pathogens based on the endoglucanase (*egl*) gene. Bacterial ooze was observed on the tip of infected stems, and bacterial colonisation of xylem tissue was observed in the infected plants by cross-sections histology comparison. The pathogenic strain, designated KD1, was isolated from diseased tissue by dilution method and confirmed by Koch's postulates. It showed hypersensitive reaction positive on tobacco and pathogenic to host plants. Partial *egl* sequence analysis revealed that the pathogen was closely related to *Ralstonia syzygii* subsp. *syzygii* (causative agent of Sumatra disease of clove) with sequence similarity of > 99%.

Keywords: Clove, Endoglucanase, *Ralstonia syzygii* subsp. *syzygii*, Sumatra disease of clove

INTRODUCTION

Sumatra clove disease caused by *Ralstonia syzygii* subsp. *syzygii* has been reported to attack the clove plantings in Indonesia, resulting in severe damage, including those in Solok (West Sumatra), Sukamantri (West Java) (Bennet *et al.* 1985), and Jombang (East Java) (Hidayah *et al.* 2014). According to the recent

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taxonomy, besides *R. syzygii* subsp. *syzygii*, the species *R. syzygii* includes two other subspecies, e.g., *R. syzygii* subsp. *celebesensis*, a pathogen of banana blood disease; and *R. syzygii* subsp. *indonesiensis*, a pathogen of solanaceae wilt disease. In contrast to these two closely related subspecies, *R. syzygii* subsp. *syzygii* is limited to the xylem vessel, i.e., in xylem cells or tracheary elements (Safni *et al*. 2018). Early symptoms of Sumatra disease are twig die-back and leaf-fall from the clove's bud. All the young leaves fall, leaving only the twig intact. Moreover, infected plants appear old; thus, it is easier to distinguish them from healthy crops because infected plants have fewer leaves. Another symptom seen in the infected plants is twigs becoming dry and dried leaves remaining on the twig (Waller & Sitepu 1975).

R. syzygii subsp. *syzygii* is a gram-negative, nonspore-forming, nonencapsulate, nonmotile, aflagellate bacterium appearing as straight rods with rounded ends (0.5–0.6 × 1.0–2.5 μm). It occurs singly, in pairs, or occasionally as short chains and exhibits aerobic growth, which is poor (colonies < 1 mm in 7 days) or absent on many common bacteriological media (Roberts *et al.* 1990). Characterization of *R. syzygii* subsp. *syzygii* on several aspects is necessary to elucidate an effective and efficient means of control against Sumatra disease, since no such method has yet been found (Dwimartina *et al.* 2017). Physiological and biochemical aspects of this bacterial pathogen (Danaatmadja *et al.* 2009) and pathogenicity aspect against xylem cell of 6–12 year-old clove plants (Bennet *et al.* 1987) were previously characterised. A molecular characterization is necessary to properly and accurately identify the cause of Sumatra disease, because although it is caused by the same bacterium, Sumatra disease could be disseminated by different vectors carrying *R. syzygii* subsp. *syzygii,* e.g., *Hindola fulva* in Sumatra and *Hindola striata* in Java (Eden-Green *et al.* 1992).

R. syzygii subsp. *syzygii* is a member of the *Ralstonia solanacearum* species complex, which forms a cluster of closely related bacteria whose individual members may represent more than one species. The use of phylogenetic markers other than 16S rRNA is necessary to achieve robust identification at the species level. In the present study, we describe characterization of pathogen based on histology, morphology and pathogenicity testings, as well as a molecular method for identifying the causal agent of Sumatra disease using a partial sequence of *egl* gene as a phylogenetic marker.

MATERIALS AND METHODS

Sample Collection and Preparation

Twigs and stems of infected and healthy plants were collected from clove-growing areas in Kendal, Central Java, Indonesia. Samples were only collected from fields that indicated symptoms of wilt diseases, by the purposive random sampling

method (Windari *et al*. 2015; Ismiyatuningsih *et al.* 2016). Samples were used for pathogen isolation and histological analysis of the cross-sections of the xylem tissue.

Pathogen Isolation, Hypersensitivity, and Pathogenicity Test

Prior to bacterial isolation, infected twigs were surface-sterilised with 70% alcohol. The presence of bacterial ooze was verified by submerging the stem tip of infected tissue in sterile water in a test tube. The bacterial ooze that appeared was streaked onto the *Casamino Acid* (CA) medium [supplemented with iron salts (CA+; g/l); acid casein hydrolysate 7.5, sucrose 2.0, magnesium sulphate 0.25, di-potassium hydrogen phosphate 0.5, Bacto agar 15.0, and ferric ammonium citrate 0.25] to isolate the pathogen (Bennet *et al.* 1987). The bacterial suspension was serially diluted from 10^{-1} to 10^{-8}. From each dilution, approximately 50–100 µL was removed and streaked onto the CA medium. Bacterial cultures were incubated for 5–7 days at room temperature (± 27°C). Bacterial observations on CA medium included colony color, shape, size, and gram reaction (Joko *et al.* 2000). Characterization of cell morphology of *R. syzygii* subsp. *syzygii* was carried out using a transmission electron microscope (TEM; JEOL 400s Electron Microscope, Jeol Ltd Tokyo, Japan) (Suharti *et al*. 2017).

In order to differentiate the plant pathogen from saprophytes, the hypersensitivity (HR) test was performed (Klement & Goodman 1967). The HR assay was conducted by infiltrating tobacco leaves tissue with bacterial suspensions. Necrotic symptom development was observed within 7 days (Nurjanah *et al*. 2017). Bacterial isolates which showed a hypersensitivity reaction were then tested for pathogenicity on 4-month-old clove seedlings. Inoculation was carried out using the infectivity titration method, which involves making a lesion hole in the seedling stem then injecting 10–20 µL of bacterial suspensions (1×10^8 CFU/mL) into the lesion hole using a microtip. The inoculated plants were then incubated for 2 months for observation of disease symptom development (Widyaningsih *et al.* 2017).

Cross-Section Preparation of Xylem Tissue by Non-Embedding Method

Cross section was prepared using fresh xylem samples from infected and healthy plant. The stem was sliced at a ± 5 cm distance from the leaf tip. Cross-section preparation was carried out with the non-embedding method (Sutikno 2016), which included fixation with 70% alcohol. Slice of 20–30 µm thickness was prepared by using a sliding microtome and stained with 1% fast green and 1% Safranin O. The slice was placed on a glass slide and covered with cover glass, which had Canada balsam previously added to it. The sample was dried at 45°C on a hot plate until Canada balsam was dried.

DNA Extraction and Amplification

Bacterial DNA was isolated using a modified mini preparation DNA isolation technique (Ausubel *et al.* 2003). A 1.5-mL aliquot of cell culture was centrifuged (Thermo Scientific Sorvall Legend Micro 17, Germany) at 5,000 rpm for 2 min. The pellet was suspended in 540 µL of Tris-EDTA (TE) buffer (0.1 M Tris-HCl, 0.1 M EDTA, pH 8), then 30 µL of 10% SDS was added and incubated at 37°C for 60 min. NaCl (5 M, 100 µL) and CTAB/NaCl (80 µL) were then added and the sample incubated at 65°C for 10 min. Chloroform : isoamyl alcohol (24 : 1, 750 µL) was added and the sample was centrifuged at 12,000 rpm for 5 min. The upper layer was transferred to a 1.5 mL Eppendorf tube, and then 600 µL of phenol : chloroform : isoamylalcohol (25 : 24 : 1) was added to it. After gently shaking, the sample was then centrifuged at 12,000 rpm for 5 min. The upper layer was transferred to a new microcentrifuge tube, and 0.6x volumes of isopropanol were added and the sample centrifuged at 12,000 rpm for 5 min. The pellet was washed with 70% ethanol, dried and resuspended in 30 µL of TE buffer.

DNA amplification was carried out by PCR using a pair of specific primers UGMRss-F (5'-GCTCACCATCGCCAAGGACAGCG-3') and UGMRss-R (5'-TTCGATCGAACGCCTGGTTGAGC-3'), which were designed based on the endoglucanase gene of *R. syzygii* subsp. *syzygii* (Trianom *et al*. 2018). PCR was performed in a PCR machine (Bio-Rad T100, Germany) using GoTaq Green (Promega) in 12.5 µL Go Taq Green Master mix, 1 µL forward primer, 1 µL reverse primer, 1 µL DNA, and 9.5 µL nuclease-free Water. The PCR conditions were as follows: 96°C for 5 min for initial denaturation followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s. PCR was completed with a final extension at 72°C for 10 min (Joko *et al.* 2007a; 2007b).

The PCR products were analysed by electrophoresis on a 1% agarose gel in 1× TBE. DNA fragment measurement was carried out using a 100-bp DNA ladder (Promega). A 5-µL aliquot of the PCR product mixed with 2 µL of 1% gel red was loaded into each well of the agarose gel. Electrophoresis was performed at 70 Volts DC for 50 min and visualised using a gel doc system (Bio-Rad) (Joko *et al*. 2012).

Sequencing and Phylogenetic Analysis

The PCR products were subjected to nucleotide sequencing at the First BASE Laboratories, Malaysia. The nucleotide sequencing result was then analysed and edited using Genetyx Win program, version 4 (Genetyx, Japan). The sequence obtained was then deposited to GenBank (accession number MK240576). Phylogenetic data were obtained by the alignment of different reference sequences (accession number JF702320, KC757083, GU295045, KC820936, KC757081, KP419736, EF192970, KT184806) of *egl* gene retrieved from the Basic Local Alignment Search Tool on http://www.ncbi.nlm.nih.gov. Multiple sequence alignment and phylogenetic tree construction was carried out in the Molecular Evolutionary

Genetic Analysis (MEGA) 7 program. An unrooted phylogram was obtained by the Maximum Likelihood (ML) method based on the Tamura-3 parameter model. An interior branch test was performed (1,000 replications) to check the tree topology for robustness. Some reference strains with similarity close to 100% were determined and used in phylogenetic analysis. Additionally, the Poisson correction was applied to ML analysis for distance estimation, and the complete deletion option was used in handling gaps or missing data obtained from alignments (Joko *et al*. 2014; Mahfut *et al.* 2016).

RESULTS

Sampling and Pathogen Isolation

Non-infected plants (Fig. 1a) were used as control plants and were distinguished from infected plants. Symptoms such as leaf-fall from the twig tip, leaving the twig tip bare, and older leaves looking fresh were observed on infected plants (Fig. 1b). Bacterial ooze was observed on the tip of infected stem placed in sterile water in a test tube (Fig. 1c). The bacterial pathogen grew after incubation for 7 days at ± 27°C in the CA medium. Bacterial colonies that grew were tenacious, muddy white in color, and transparent with a size of ± 1 mm diameter.

Hypersensitivity and Pathogenicity Test

The hypersensitivity response (HR) test on tobacco leaves showed a necrotic lesion 7 days after inoculation (Figure 1d). Initial necrosis began with yellowish leaves, which then became brownish in color and dried. In the pathogenicity assay conducted on a 4-month-clove plant, symptoms of wilting appeared 45 days after inoculation (Fig. 1e). Observation by TEM revealed that the bacterial pathogen had no flagella and non-fluid colonies sized 1 × 1.5–3 µm (Fig. 1f).

Cross-Sectional Morphology of Xylem Tissue

Bacterial colonisation of xylem tissue was observed by comparing the histology of cross-sections of infected and healthy clove stems or twigs. Red colored secondary xylem cells were foun in the infected plants (Fig. 2a) and not in the heathy plants (Fig. 2b). This result suggested that the red colored cells detected were occupied by bacterial colonies of *R. syzygii* subsp. *syzygii.* Fast Green 1% was used for the preparation of cross-sections to clarify the observation of cell structure.

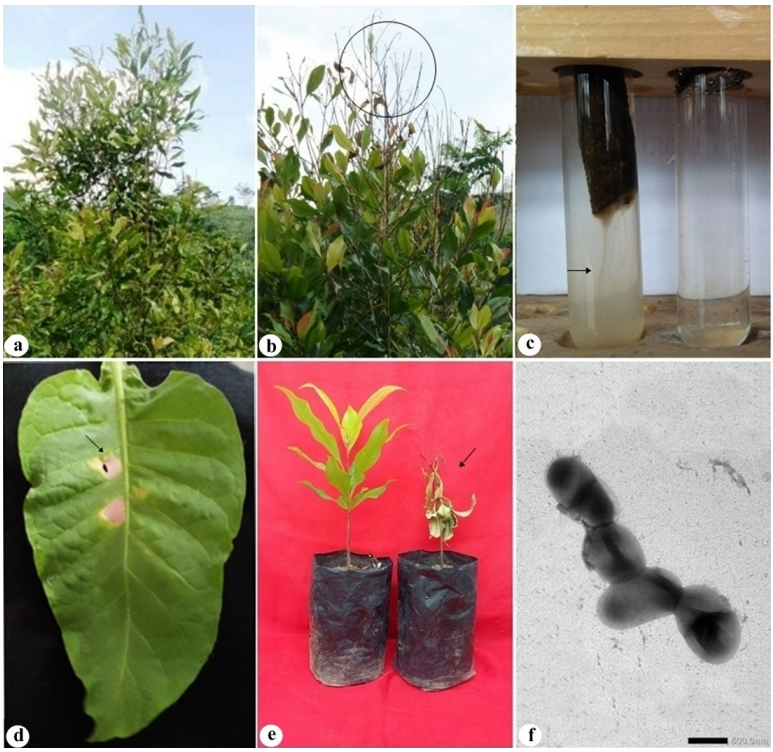

Figure 1: Symptoms of Sumatra disease of clove and cell morphology of *Ralstonia syzygii* subsp. *syzygii*. a: healthy plant, b: symptoms on infected plant, c: bacterial ooze on infected twig of clove plant, d: hypersensitive response on tobacco leaf, e: pathogenicity test of bacterial isolate on non-infected clove seedling (left), clove seedling with pathogen inoculation (right), f: bacterial cell morphology of strain KD1 using transmission electron microscopy.

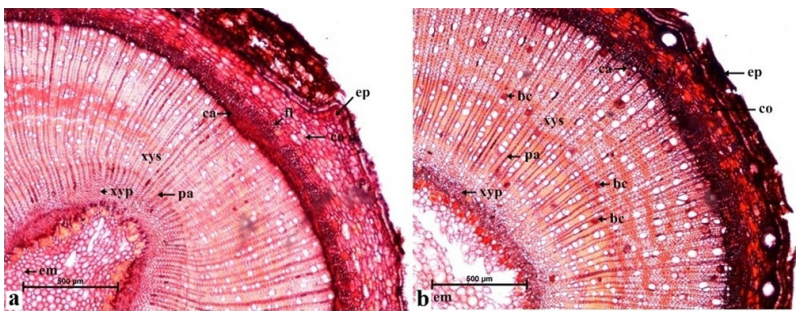

Figure 2: Cross-section of non-infected clove plant at 40× magnification (a); Cross-section of wilt symptoms on clove plant at 40× magnification (b). Letters on the image show the transverse tube structure, i.e., em: empulur, xyp: primary xylem, pa: parencyhme, xys: secondary xylem, ca: cambium, co: cortex, ep: epidermis, fl: phloem, bc: bacterial colonies.

Molecular Analysis of *Ralstonia syzygii* subsp. *syzygii*

Using PCR with specific primers UGMRss-F/UGMRss-R, a 378-bp DNA fragment specific to the endoglucanase gene of *R. syzygii* subsp. *syzygii* was amplified (Fig. 3). Sequence analysis showed that the bacterial pathogen isolate had 100% similarity with *R. syzygii* subsp. *syzygii* R002 and 99.5% similarity with *R. syzygii* subsp. *syzygii* R001. It also had 95.2% similarity with *R. syzygii* subsp. *indonesiensis* UQRS 96 (causative agent of bacterial wilt in tomato) and 95.2% similarity with *R. syzygii* subsp. *celebesensis* R229 (causative agent for banana blood disease). The homology test also revealed that all bacterial groups in the *Ralstonia solanacearum* species complex had ≥ 90% similarity. Meanwhile, the bacterial pathogen isolate only had 46% similarity with the out group strain (*Xanthomonas campestris* pv. *campestris*) (Fig. 4).

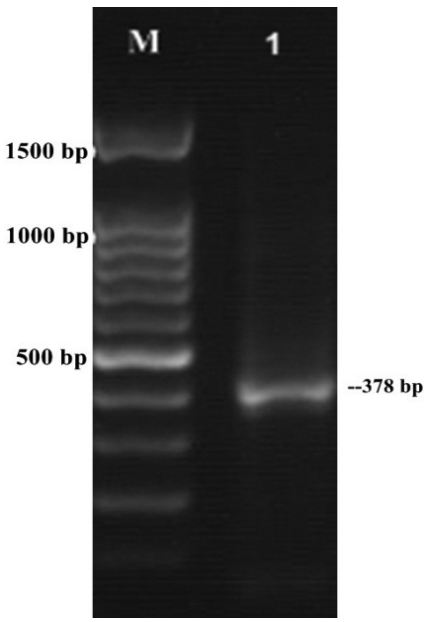

Figure 3: DNA amplification of strain KD1 using specific primers UGMRss-F/UGMRss-R. The PCR product was analysed by electrophoresis on 1% agarose gel in 1× TBE. Lane 1: *egl* gene of *Ralstonia syzygii* subsp. *syzygii* that was amplified at ± 378-bp, M: 100-bp DNA marker (Promega).

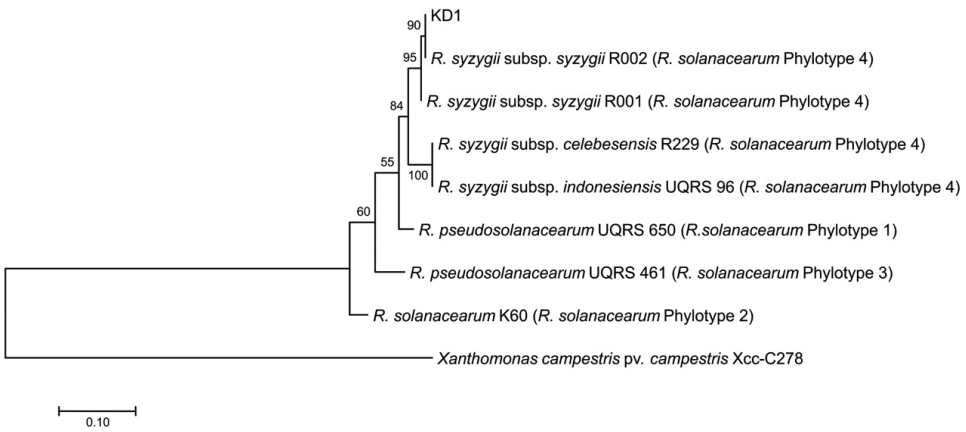

Figure 4: Phylogenetic tree construction of strain KD1 and the closely related species available in GenBank on the basis of the alignment of *egl* gene sequences. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method with the Tamura-3 best fit model. Stability of the tree was assessed by 1,000 bootstrap replications. The sequence of *Xanthomonas campestris* pv. *campestris* was used as an outgroup.

DISCUSSION

The observations, isolation, and several tests performed on the causative agent of wilt disease in clove plants revealed that wilt disease of the clove plants in Central Java was caused by *R. syzygii* subsp. *syzygii*. Symptoms began with the death of twigs, the wilt of young leaves, and fall of almost all of the leaves. It would be difficult to distinguish healthy plants from the infected old plants if the severity is medium; however, dead twigs and the falling of yellow leaves could be an indication of Sumatra disease. Wilt is one of the characteristic symptoms of bacteria that attacks the xylem. These bacteria colonise the xylem, thereby inhibiting the water and nutrition supply to several parts of the crop, causing wilt. Bennet *et al.* (1985) reported that similar bacteria were found in non-infected plants on an infested plantation, but they were not found in plants from a disease-free plantation or in plants killed by drought or stem borer. The existence of bacteria that infect the xylem was also determined by examination of the bacterial ooze when it was placed in water.

The internal symptoms for the presence of Sumatra disease of clove could be observed by examining the xylem. *R. syzygii* subsp. *syzygii* is known to only attacks the xylem tissue; therefore, its existence could be detected by observing a cross-section from the twig. Bacteria were present in the secondary xylem cells, and cells colonised by bacteria were stained red with Safranin O. Tortora *et al.* (2000) reported Gram-negative bacteria when stained with Safranin O turned red. Bennet *et al.* (1987) reported detection of *R. syzygii* subsp. *syzygii* on secondary xylem from samples taken along the woody parts of infected plants and in the

vessels of fine roots, flower buds, leaf tissue, and the juveniles from roots and rod stems. Bacterial colonisation was frequently found in the protoxylem vessel. Limited observations of the longitudinal part of the primary and secondary xylem showed that all the infected vessels were usually filled with *R. syzygii* subsp. *syzygii*.

Bacterial growth development on the CA medium was slow as indicated by its appearence after 7 days. This could be one explanation for the slow action of *R. syzygii* subsp. *syzygii* in the field. The slow growth of bacteria could be determined from the size of the colony, i.e., ≤ 1 mm at 7 days after inoculation and the absence of growth in common bacterial medium (Vaneechoutte *et al.* 2004). Colonies became slightly milky, were less domed, and reached a maximum diameter of 0.5–1.0 mm after 7–10 days at 28°C –30°C. Growth did not occur above 32°C and was extremely slow below 25°C (Roberts *et al.* 1990).

The hypersensitivity response, which is encoded by *hrp* gene clusters results in the destruction of all membranes of cells in contact with bacteria, which is followed by desiccation and necrosis of the leaf tissues invaded by the bacteria (Ageichik *et al*. 2002; Lindgren 1997). Tobacco (*Nicotiana tobaccum* L.) is an ideal indicator plant for the hypersensitive test when testing the pathogenicity of most Gram negative phytopathogenic bacterial isolates. Tobacco hypersensitivity is a fast and convenient way to screen bacterial cultures and the reaction is invaluable for the characterization of phytopathogenic bacteria recovered from outside their host or recovered from a host with latent infections (Gitaitis1990).

The pathogenicity test showed symptoms by the tritration method at 45 days after infiltration of axilla. The pathogenicity of *R. syzygii* subsp. *syzygii* causing wilt in cloved is related to the existence of bacteria on the plant tissue. Firstly, the bacteria will penetrate the plant tissue, replicate in the xylem of the stem and spread out to all parts of the plant in the advanced disease stage (Sutrisno *et al.* 2018). In contrast to soft rot bacteria that collapse cell wall tissue (Joko *et al*. 2018), *R. syzygii* subsp. *syzygii* might have another strategy that enables the pathogen to remain for longer duration in the plant vascular tissue.

The degree of similarity and the intraspecies comparison of DNA sequence identity between strain KD1 and *R. syzygii* subsp. *syzygii, R. syzygii* subsp. *celebesensis, R. syzygii* subsp. *indonesiensis, R. solanacearum, R. pseudosolanaecearum,* and *X. campestris* pv. *campestris* were determined by the BLAST algorithm for *egl* gene and can be shown via phylogenetic tree construction. Analysis of homogeneity using MEGA 7 revealed that *R. syzygii* that attacks clove, potato and banana had ≥ 95% similarity; however, from the phylogenetic tree construction, it was shown that *R. syzygii* that attacked clove (strain KD1 and *R. syzygii* subsp. *syzygii*) was in a different cluster from *R. syzygii* subsp. *celebesensis* and *R. syzygii* subsp. *indonesiensis,* which were in the same cluster as the *R. solanacearum* species complex but in a different sub cluster (Fig. 4). This finding suggests that there is considerable genetic diversity in *R. syzygii*. Also, the multiple sequence alignment of these sequences revealed conserved regions at different stretches. This result confirmed that although *R. syzygii* subsp. *celebesensis* and *R. syzygii* subsp. *indonesiensis* were same species (95% genetic similarity), they

were closely related to *R. solanacearum*, according to homogeneity analysis, which showed them to be in the same cluster in the phylogenetic tree. The *R. solanacearum* species complex is composed of at least four genetic groups or phylotypes. Within these phylotypes there are subgroupings, sequevars, which correspond to the clusters of isolates with similar pathogenicity or isolates of a common geographic origin (Fegan & Prior 2005).

Strain KD1 was shown to be identical to *R. syzygii* subsp. *syzygii* R002, which had 100% genetic similarity on the phylogenetic tree with bootstrap value of 90%. However, it had 99.55% genetic similarity with *R. syzygii* subsp. *syzygii* R001, was in a different sub cluster in the same cluster. Although both strains were shown to attack clove plants, *R. syzygii* subsp. *syzygii* R001 was the strain previously isolated from clove plantings on Sumatra Island, whereas *R. syzygii* subsp. *syzygii* R002 was from clove plantings on Java island. This result suggested that Sumatra disease observed in clove plantings on central Java might be the same origin with that of found on west Java. Noting that, the *egl* gene sequences have several advantages, including being effectively a single copy gene, highly conserved in *R. solanacearum* species complex and easy to amplify. Therefore, the sequencing and analysis sequence data for the *egl* region could be a reliable tool for detection and monitoring the diversity of pathogens.

CONCLUSION

This study emphasises the importance of using more than one method for the correct and proper identification of *Sumatra disease of clove caused by Ralstonia syzygii* subsp. *syzygii*. Application of PCR-based methods using *egl* gene, coupled with morphological and histological studies and further pathogenicity assays on host plant enable deeper understanding of the Sumatra disease epidemiology.

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REFERENCES

Ageichik A V, Evtushenkov A N and Nikolaichik Y A. (2002). The role of type III secretion system in *Erwinia carotovora* subsp. *atroseptica* virulence. *Plant Protection Science* 38(s2): 523−527.

Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A and Struhl K. (2003). *Current protocols in molecular biology*. USA: John Wiley & Sons, Inc., 1600 pp.

Bennet C P A, Hunt P and Asman A. (1985). Association of a xylem-limited bacterium with Sumatra disease of cloves in Indonesia. *Plant Pathology* 34(4): 487−494. https://doi.org/10.1111/j.1365-3059.1985.tb01398.x

Bennet C P A, Jones P and Hunt P. (1987). Isolation, culture and ultrastructure of a xylem-limited bacterium associated with Sumatra disease of cloves. *Plant Pathology* 36(1): 45−52. https://doi.org/10.1111/j.1365-3059.1987.tb02176.x

Danaatmadja Y, Subandiyah S, Joko T and Sari C U. (2009). Isolation and characterization of *Ralstonia syzygii. Jurnal Perlindungan Tanaman Indonesia* 15: 7−12.

Dwimartina F, Arwiyanto T and Joko T. (2017). Potential of endophytic and rhizobacteria as an effective biocontrol for *Ralstonia syzygii* subsp. *syzygii*. *Asian Journal of Plant Pathology* 11: 191−198.

Eden-Green S J, Balfas R and Sutarjo T. (1992). Characteristics of the transmission of Sumatra disease of cloves by tube-building cercopoids, *Hindola* spp. *Plant Pathology* 41(6): 702−712. https://doi.org/10.1111/j.1365-3059.1992.tb02553.x

Fegan M and Prior P. (2005). How complex is the *Ralstonia solanacearum* species complex?. In: C Allen, P Prior and A C Hayward (eds.), *Bacterial wilt disease and the Ralstonia solanacearum species complex*. St. Paul, MN: APS Press, 449−461.

Gitaitis, R D. (1990). Induction of hypersensitive like reaction in four o'clock by *Clavibacter michiganensis* ssp. *michiganensis*. *Plant Disease* 74: 58−60. https://doi.org/10.1094/PD-74-0058

Hidayah N, Abadi A L and Aini L Q. (2014). Morphological and physiological characterization of *Ralstonia syzygii* from clove. *Journal of Agriculture and Veterinary Science* 8(7): 87−91.

Ismiyatuningsih, Joko T and Hartono S. (2016). Survey and detection of *Pectobacterium atrosepticum* in major potato-growing areas in Central Java Province, Indonesia. *Agricultural Science* 1: 1−6.

Joko T, Subandiyah S and Somowiyarjo S. (2000). The role of extracellular protein on the pathogenicity of *Xanthomonas campestris* pv. *citri*. *Jurnal Perlindungan Tanaman Indonesia* 6: 32−38.

Joko T, Hirata H and Tsuyumu S. (2007a). Sugar transporter (MfsX) of major facilitator superfamily is required for flagella-mediated pathogenesis in *Dickeya dadantii* 3937. *Journal of General Plant Pathology* 73(4): 266−273. https://doi.org/10.1007/s10327-007-0018-8

_____. (2007b). A sugar transporter (MfsX) is also required by *Dickeya dadantii* 3937 for in planta fitness. *Journal of General Plant Pathology* 73(4): 274−280.

Joko T, Koentjoro M P, Somowiyarjo S, Rohman M S, Liana A and Ogawa N. (2012). Response of rhizobacterial communities in watermelon to infection with cucumber green mottle mosaic virus as revealed by cultivation-dependent RISA. *Archives of Phytopathology and Plant Protection* 45(15): 1810−1818. https://doi.org/10.1080/03235408.2012.707526

Joko T, Subandi A, Kusumandari N, Wibowo A and Priyatmojo A. (2014). Activities of plant cell wall-degrading enzymes by bacterial soft rot of orchid. *Archives of Phytopathology and Plant Protection* 47(10): 1239−1250. https://doi.org/10.1080/03235408.2013.838374

Joko T, Umehara M, Murata T, Etoh H, Izumori K and Tsuyumu S. (2018). Hyperinduction of pectate lyase in *Dickeya chrysanthemi* EC16 by plant-derived sugars. *Journal of Plant Interactions* 13(1): 141−150. https://doi.org/10.1080/17429145.2018.1444206

Klement Z and Goodman R N. (1967). The hypersensitive reaction to infection by bacterial plant pathogens. *Annual Review of Phytopathology* 5: 17−44. https://doi.org/10.1146/annurev.py.05.090167.000313

Lindgren P B. (1997). The role of *hrp* genes during plant bacterial interactions. *Annual Review of Phytopathology* 35: 129−152. https://doi.org/10.1146/annurev.phyto.35.1.129

Mahfut, Joko T and Daryono B S. (2016). Molecular characterization of odontoglossum ring spot virus (ORSV) in Java and Bali, Indonesia. *Asian Journal of Plant Pathology* 10(1–2): 9−14.

Nurjanah N, Joko T and Subandiyah S. (2017). Characterization of *Pantoea ananatis* isolated from garlic and shallot. *Jurnal Perlindungan Tanaman Indonesia* 21: 120−126.

Roberts S J, Eden-Green S J, Jones P and Ambler D J. (1990). *Pseudomonas syzygii*, sp. nov., the cause of Sumatra disease of cloves. *Systematic and Applied Microbiology* 13(1): 34−43. https://doi.org/10.1016/S0723-2020(11)80178-5

Safni I, Subandiyah S and Fegan M. (2018). Ecology, epidemiology and disease management of *Ralstonia syzygii* in Indonesia. *Frontiers in Microbiology* 9: 1−11. https://doi.org/10.3389/fmicb.2018.00419

Suharti T, Joko T and Arwiyanto T. (2017). Detection of seed-borne pathogenic bacteria of northern black wattle (*Acacia auriculiformis* A. Cunn. ex Benth.). *Jurnal Hama dan Penyakit Tumbuhan Tropika* 17: 19−36.

Sutikno. (2016). *Plant microtechnique*. Yogyakarta: Universitas Gajah Mada Press. pp. 51.

Sutrisno, Susanto F A, Wijayanti P, Retnoningrum M D, Nuringtyas T R, Joko T and Purwestri Y A. (2018). Screening of resistant Indonesian black rice cultivars against bacterial leaf blight. *Euphytica* 214: 199. https://doi.org/10.1007/s10681-018-2279-z

Tortora G J, Funke B R and Case C L. (2010). *Microbiology: An introduction* (10th Ed.). San Francisco: Benjamin Cummings, pp. 812.

Trianom B, Arwiyanto T and Joko T. (2018). Development of novel subspecies-specific primers based on the endoglucanase gene for detection of *Ralstonia syzygii* subsp. *syzygii*. *Jurnal Perlindungan Tanaman Indonesia* 22: 124−131.

Vaneechoutte M, Kampfer P, Baere T D, Falsen E and Verschraegen G. (2004). Wautersia gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] *syzygii*. *International Journal of Systematic and Evolutionary Microbiology* 54(2): 317−327.

Waller J M and Sitepu D. (1975). Sumatra disease of cloves in Indonesia. *Pest Articles and News Summaries* 21(2): 141−147.

Widyaningsih S, Utami S N H, Joko T and Subandiyah S. (2017). Development of disease and growth on six scion/root stock combinations of citrus seedlings under huanglongbing pressure. *Journal of Agricultural Science* 9(6): 229−238.

Windari U, Joko T and Subandiyah S. (2015). Detection of bacterial fruit blotch of melon using ELISA. *Jurnal Perlindungan Tanaman Indonesia* 19(1): 1−5.