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# Field and Laboratory Detection of Clove Sumatra Disease Caused by *Ralstonia syzygii* subsp. *syzygii* in Java, Indonesia

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## ABSTRACT

The significant decrease in clove production in Indonesia is mainly due to the Sumatra disease caused by bacterial *Ralstonia syzygii* subsp. *syzygii*. It is necessary to manage the disease broadly based on disease detection in the field and the laboratory. This study aims to determine the technique for detecting the distribution pattern of Sumatra disease using geographic information systems and validate the presence of *R. syzygii* subsp. *syzygii* in clove plant tissues by molecular analyses based on the endoglucanase gene. This research was conducted by acquiring aerial photos using uncrewed aerial vehicles processed using photogrammetric techniques to produce geographic information system outputs as a thematic map of the clove Sumatra disease distribution pattern. The plant samples were collected for molecular analysis of the pathogens causing clove Sumatra disease in the laboratory. DNA extraction was performed and amplified by polymerase chain reaction (PCR) using UGMRss-specific primers followed by Sanger sequencing. The aerial photo images showed that the distribution of clove Sumatra disease has a random pattern, which tends to spread between healthy and diseased plants. Furthermore, the PCR and phylogenetic analyses revealed that all symptomatic clove plant samples (leaves, twigs, and roots) were positively infected by *R. syzygii* subsp. *syzygii*.

*Keywords*: Endoglucanase, geographic information system, polymerase chain reaction, *Ralstonia syzygii* subsp. *syzygii*, uncrewed aerial vehicle

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## INTRODUCTION

Clove (*Syzygium aromaticum* L.) is a native Indonesian spice plant with many benefits. It can be used as a cooking spice, traditional medicine, pharmaceutical material, and raw material for the food industry. Haro-González et al. (2021) reported that clove plants contain essential oils found in all plant parts (roots, stems, leaves, and flowers), which have many biological activities, including antibacterial, antifungal, insect repellent, and antioxidant properties. Clove oil is traditionally used as a flavoring and antibacterial agent in food. However, many factors are causing the reduction of clove production, one of which is the infection by a bacterial pathogen, *Ralstonia syzygii* subsp. *syzygii* causing Sumatra disease (Roberts et al., 1990).

Sumatra disease was reported for the first time on Sumatra Island, making it known as Sumatra disease (Waller & Sitepu, 1975). At present, clove Sumatra disease has been reported in several clove plantations in Indonesia (Dwimartina et al., 2017). Clove plants infected with Sumatra disease will exhibit symptoms such as leaf drop and wilting die-back of the plant shoot, beginning with the branches at the top followed by the leaves withering. The leaf drop is followed by shoot death due to the blockage of xylem vessels by the pathogens in plant roots and stems (Bennett et al., 1987). Sumatra disease symptoms generally appear after a long incubation period (Danaatmadja et al., 2009). The development of clove plantations to date has not yet experienced a recovery, with production still fluctuating from around 60,000 to 100,000 tonnes each year (Pratama & Darwanto, 2019). According to Bennett et al. (1985), losses due to Sumatra disease have been estimated to have 10–15% annual production losses. However, since 1996 clove production has decreased rapidly, and the average clove yield loss was approximately 40% yearly.

The effective control measures of the clove Sumatra disease are still not yet satisfied, even though insecticide application to its insect vector can suppress disease spread in a short time (Hartati et al., 1991). Likewise, applying biocontrol agents on diseased clove plants reduced the population of *Hindola striata* and the percentage of plant damage and stimulated the production of young leaf shoots (Mardiningsih et al., 2020).

Detecting Sumatra disease is an important key in making disease management decisions. Detection can be conducted directly in the cultivation area by observing the incidence of the disease and its distribution pattern, or it can be done in the laboratory to determine the cause of the disease. Observing disease incidence in a wide area requires more effort, time, and cost. Therefore, implementing an uncrewed aerial vehicle (UAV), complemented with geographic information systems (GIS), is potentially suitable. Detection in the field will provide current information regarding disease conditions existing in an area. On the other hand, detection in the laboratory is also needed to validate the causes of plant diseases. The polymerase chain reaction (PCR) technique is widely used to detect

plant diseases. This technique is quite sensitive for detecting and identifying plant pathogens (Joko et al., 2019).

This study aims to determine the distribution pattern of Sumatra disease in a wide area by applying UAV technology and GIS. Furthermore, this study also aims to validate the presence of *R. syzygii* subsp. *syzygii* in some clove plant tissues using the PCR technique.

## MATERIALS AND METHODS

### Study Sites

The research was conducted in a clove plantation in Kalisidi Village, West Ungaran District (coordinates 110°21'47,287"E and 7°8'20,471"S), Central Java Province, and at the Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. The location insets were made to identify the location of the research area. The observation area's location and the study's administrative borders were determined using satellite images. Using satellite imagery in the location inset provides wider spatial information so conditions around the land can be known. The satellite images were obtained through a data mining process against the freely available imagery available in the SASPlanet software (https://bitbucket.org/sas_team/sas.planet.bin). The satellite images obtained were then overlaid with polygon vectors to show the location of the observation block. The polygon vector was then saved in shapefiles (shp.) format, resulting in an observation block shape (Figure 1).

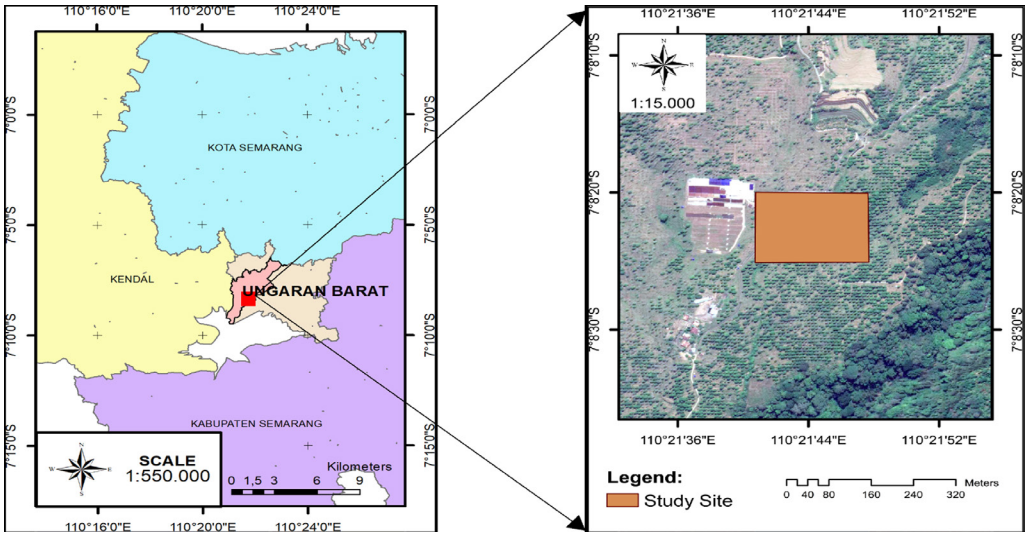


*Figure 1*. The location insets of the study sites located in Kalisidi Village, West Ungaran District, Central Java Province, Indonesia

### Acquisition of Aerial Photography and Photogrammetry

Aerial photography was taken to observe the incidence of Sumatra disease using the UAV. Aerial photo images were obtained using the DJI Phantom 3 Standard quadcopter type UAV (China), with the flight on autopilot following the planned flight plan. The main purpose of flight planning was to obtain navigational data to be transferred to the UAV vehicle. The flight plan was set using the Pix4D Capture software on the smartphone and connected to the remote control (Hung et al., 2019). The Pix4D Capture application created a flight plan to create a 2D map model with an 80% overlap ratio, a camera tilt angle of 60°, and a flight altitude of 80 m (Figure 2). The coverage of aerial photos to be observed was 3 ha. Each flight took 7 min 30 s and produced 69 images. Each image has coordinated data processed using photogrammetry software, namely Agisoft Metashape (Ribeiro-Gomes et al., 2016). Through the Agisoft Metashape application, images previously acquired were processed for alignment. Processing using the Agisoft Metashape application produced a georeferenced (orthomosaic) photo mosaic.

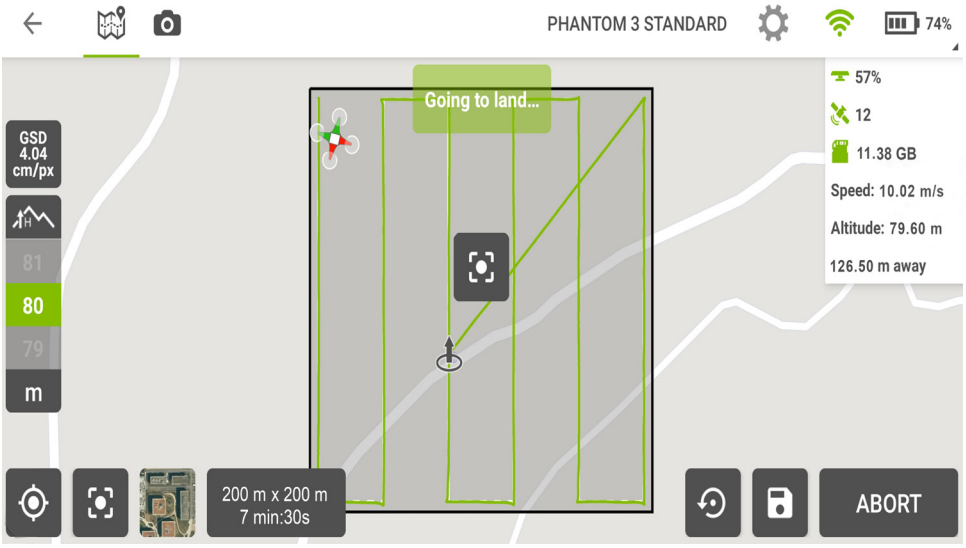


*Figure 2*. Uncrewed aerial vehicle (*autopilot*) flight plan using the Pix4D Capture application

### Digitation on Screen

The photos merged using Agisoft Metashape software were exported to Tag Image File Format (TIFF) referenced with the World Geodetic System (WGS 1984) and Universal Transverse Mercator (UTM Zone 49S) Coordinate System. A coordinate system is a number used to indicate the location of a surface/space, including datums and map projections. The orthophoto results were

processed with on-screen digitization, which converts raster data from remote sensing into vector data accompanied by attributes or additional information about the related object (Figure 3). The digitization process was performed using the ArcGIS 10.8 application.

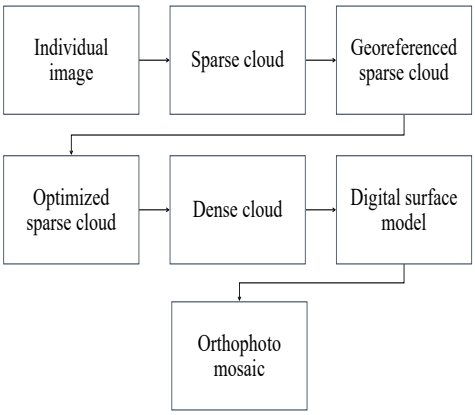


*Figure 3*. The steps in the process of merging individual photos into an orthophoto mosaic using Agisoft Metashape software referring to Ludwig et al. (2020) with some adjustments

The initial step in the on-screen digitizing process was to identify the area that would be the subject of observation. Because photo mosaic images are frequently less distinct or hazy at the margins, polygon features covering an area of three hectares must be used to establish the area of interest/ region of interest (ROI). Once the ROI is known, the incidence of the disease and the distribution pattern of Sumatra disease are identified. The identification process was conducted using a visual assessment technique, referring to the characteristics of the symptoms of Sumatra disease. Identification was performed by marking the individual clove plants, both sick and healthy, appearing on the photo mosaic (orthomosaic). Each clove tree was marked using a point vector in the image. Zooming to a scale of 1:150 in the defined area allowed the conditions of the plants to be visible. Healthy clove plants appeared completely green (Figure 4a), while diseased clove plants showed signs of shedding marked in gray (Figure 4b). The gray color in the aerial photo image of the clove plant represents the part of the twig seen as a result of the leaves drop. Healthy and diseased clove plants were marked with green and red points, respectively. The marking results were made into a geographic information system as a thematic map of the distribution pattern of Sumatra disease with a scale of 1:3500 (Figure 4c).

Based on the results of visual marking, the number of diseased and healthy clove plants was calculated using the features in ArcGIS 10.8 software, namely the data attribute. The percentage of the incidence of Sumatran disease was calculated using the following formula:

$$\text{Disease incidence (\%)} = \frac{\textit{Number of infected plants units}}{\textit{Total number of units assessed}} \times 100\%$$

(Widyaningsih et al., 2019)

## Plant Materials Sampling

For molecular detection of Sumatra disease, plant samples were taken for laboratory testing. Plant samples were collected using the purposive sampling technique on plants with symptoms of Sumatra disease and

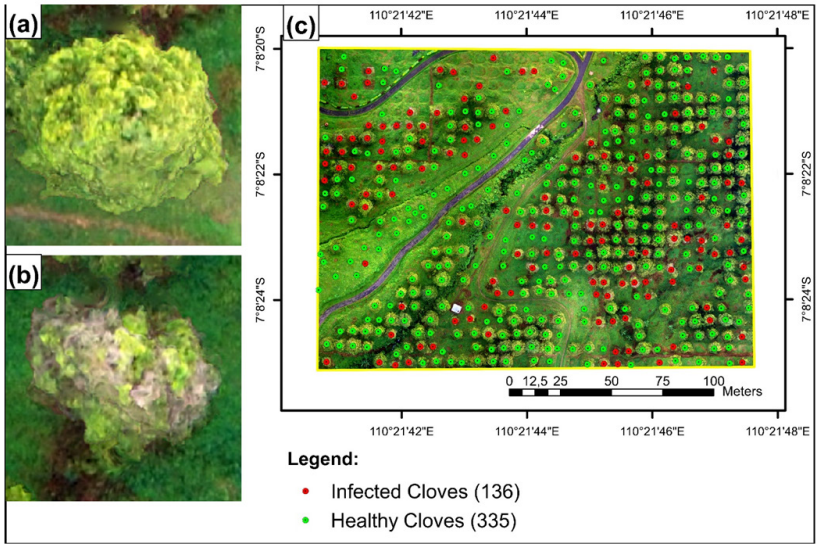


*Figure 4*. The process of identification of clove plants using aerial photography with a magnification (zooming) to a scale of 1:150 (a) healthy clove plants; (b) diseased clove plants; and (c) map for identification of symptoms of Sumatra disease (scale 1:3500)

healthy plants as control. The plant parts included the clove plant's twigs, leaves, and roots with three replicates. The samples were then brought to the laboratory and stored in a refrigerator.

## Polymerase Chain Reaction (PCR) Amplification

DNA extraction was carried out using the ZymoBIOMICS™ DNA Mini Kit (USA) following the protocol contained in the Mini Kit. A total of 0.15 g of samples of twigs, leaves, and roots were added with liquid nitrogen and then crushed with a sterile mortal gun. Furthermore, the detection of *R. syzygii* subsp. *syzygii* in plant tissue samples was conducted by DNA amplification using the PCR technique. The extracted DNA was amplified using a specific primer designed by Trianom et al. (2018), namely UGM RssF: 5'-GCTCACCATCGCCAAGGACAGCG-3' and UGM RssR: 5'-TTCGATCGAACGCCTGGTTGAGC-3'. The PCR reaction process was run using a Bio-Rad T100 machine (Germany) with the following reaction composition: GoTaq Green master mix (Promega) (5 µl); UGMRss-F primer (1 µl); UGMRss-R primer (1 µl); and DNA (3 µl). The mixtures were mixed in a microtube and homogenized gently. Amplification by PCR machine was performed at 96°C for 5 min for initial denaturation, followed by 30 cycles of a denaturation process at 94°C for 15 s, an annealing process at 59°C for 30 s, and an extension process at 72°C for 30 s, which was then proceeded with the final extension at 72°C for 10 min.

The amplification results were analyzed to observe DNA fragments through

electrophoresis using agarose gel in 1× TBE (Tris-HCl, boric acid, EDTA) buffer at 70 V for 45 min. DNA fragments were measured using a 1 kb DNA ladder marker (Promega, USA). The gel was then placed in an electrophoresis machine with an electrophoresis buffer, and the machine was run at 70 V for 45 min. After completion, the gel was immersed in ethidium bromide (EtBr) solution for 20 min. The gel was observed under ultraviolet (UV) light using a UV transilluminator (Optima, Japan) to observe the DNA bands formed (Navitasari et al., 2020).

### Sequencing and Phylogenetic Analysis

The PCR results were then sent to a DNA sequencing service provider (1st BASE, Malaysia) to determine the nucleotide sequence. Sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to be compared with the type strains references through the data mining process at GenBank. Then the similarity analysis was conducted by making a phylogenetic chart using the Mega 11 program. The phylogenetic tree was arranged using the maximum likelihood (ML) tree method (Rahma et al., 2020).

## RESULTS AND DISCUSSION

### Distribution Pattern of Sumatra Disease Using Geographic Information System

Regular monitoring of the incidence of Sumatra disease on cultivated land is one of the epidemiological approaches to prevent the expansion of the infected area. The UAV used to monitor the health condition of clove plants can acquire aerial photos of clove land and produce high-resolution aerial photo data. In this observation, the UAV's acquisition of merged aerial photos (photomosaic) can identify healthy and diseased clove trees (Figure 5). Although it looks blurry due to the overlapping process between photos, the individual clove plants can still be seen clearly. Healthy clove plants showed denser shoots and green leaves, while the diseased clove plants showed a leaf drop. The grey color in the aerial photo image indicates leaf drops in the diseased clove plants. The grey color represents the color of the twigs uncovered by leaves. Plant marking was conducted with the point vector feature in the ArcGIS 10.8 program after the aerial shot image of healthy and diseased clove plants could be recognized.

*Figure 5*. Raster display of aerial images on clove plantation land

The success of clove plant identification is highly dependent on the resolution of the photo used. The spatial characteristics of the aerial photo image of clove plantation conditions can be seen in the raster display (Figure 5). A raster is a data structure that represents the arrangement of color pixels. Caution is needed to identify individual clove plants, especially those still young because their small size makes them look biased when viewed from aerial photo images. A larger zoom is required for clearer observation of the young tree to avoid biases.

Identifying and mapping healthy and diseased plants is needed to monitor disease development and distribution of disease incidence, assess the impact of decreasing productivity, determine possible treatments to extend plant life, and estimate the replanting time (Ouyang et al., 2020). The distribution pattern and incidence of Sumatra disease were determined using spatial analysis based on photomosaic raster data. Based on the results of identification and marking with point vector features, the data were used as a geographic information system containing geographic reference data. Geographic reference data will be useful for direct confirmation in the field. The distribution pattern of Sumatra disease can be seen without direct contact with the object of observation through the identification and marking of the diseased and healthy clove plants using a GIS (Figure 6).

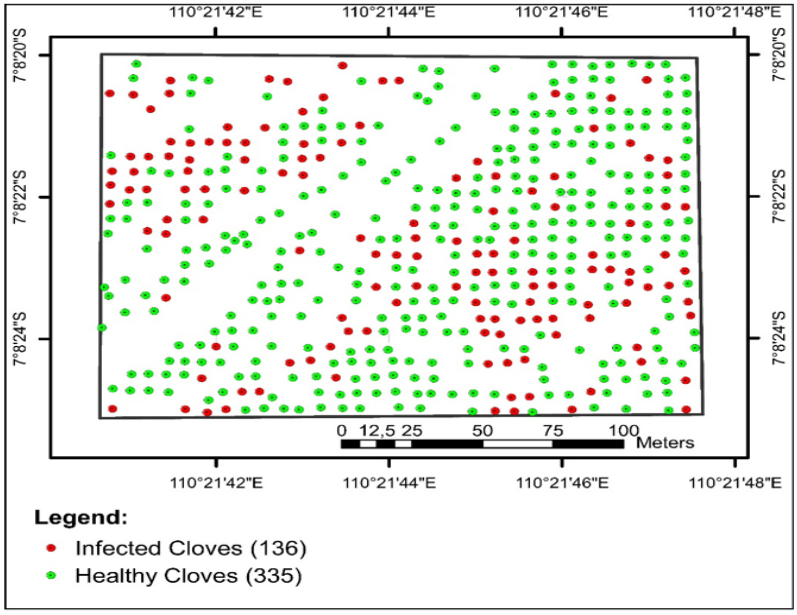


*Figure 6*. Distribution pattern and incidence of Sumatra disease in clove plantations

*Note*. Scale 1:3500; Coordinate System: WGS 1984 UTM Zone 49S; Projection System: WGS 1984; Datum: WGS 1984; Unit: Meters

The Sumatra disease identified with symptoms was randomly distributed and tended to jump between healthy and diseased plants. This result follows Bennett et al. (1985), explaining that the distribution pattern of Sumatra disease on land is the jump-spread pattern. Jump-spread patterns allow the disease to spread quickly in all directions to either highlands or lowlands, even across rivers. This distribution pattern indicates that the distribution of the disease is influenced by the presence of insect vectors, namely *Hindola fulva* and *H. striata*. The Sumatra disease can spread up to several kilometers from its primary source. Besides, there was secondary random distribution to trees adjacent to diseased trees (Eden-Green et al., 1992). This distribution pattern is known as the centrifugal spread. A similar distribution pattern of Sumatra disease is also found in lethal yellowing disease, which infects the phloem of coconut plants. The transmission of the lethal yellowing disease was characterized by a random distribution pattern, commonly known as the jump-spread pattern, carried by a planthopper insect vector (*Haplaxius crudus*). Infections in one or two trees will spread randomly around the infection center. It was explained that the jump-spread pattern indicates the presence of airborne transmission through insect vectors that can spread up to tens of kilometers from the primary infection (Tsai, 1980). Besides, the GIS technique can also be used to determine the percentage of the incidence of Sumatra disease in this study, which was 28.8%.

## Visual Assessment Based on Disease Symptom

Clove plants' condition was determined by observing the disease symptoms visually. The symptoms of Sumatra disease observed included the leaves withering from the top of the shoot to the bottom (Figure 7). *R. syzygii* subsp. *syzygii* are classified as xylem-limited bacteria because it exclusively develops in the xylem vessel tissue. Wilting that occurs in symptomatic plants is one of the characteristics of symptoms due to bacterial pathogens that infect the xylem.

*Figure 7*. Symptoms of burning on the leaves that are still attached (on a circle)

The visual assessment by looking at the symptom is the easiest way to detect the early stage of wilting symptoms such as yellowing of leaves. Bacteria colonizing the xylem inhibit the supply of water and nutrients to other parts of the plant so that due to the blockage, the plant wilts and then dies. According to Bennett et al. (1985), leaf drop occurs suddenly from the tip of the lateral branch. The symptomatic part of the branch sheds its dead leaves (yellow), but some leaves are still attached and are generally yellowish (chlorosis). Over time,

the leaves turn brown as if exposed to heat (scorched).

**Molecular Detection of *Ralstonia syzygii* subsp. *syzygii***

Molecular testing in the laboratory is an effort to validate disease detection in the field. Samples were collected from the research location and tested until they were visualized. Before the PCR was started, the first step was to extract the DNA of the clove plant. The DNA extraction results were amplified using PCR with specific primers designed based on the endoglucanase (*egl*) gene, followed by electrophoresis and DNA visualization. It was found that DNA extracted from various plant conditions could be amplified in the range of ~378 bp (Figure 8). This result means that the UGMRss-specific primer can only amplify the *egl* gene from *R. syzygii* subsp. *syzygii*. DNA of *R. syzygii* subsp. *syzygii* was detected in plant parts with mild, moderate, and severe symptoms. All three samples were at the same length of DNA base. It indicates that the bacteria infecting symptomatic plant tissues are caused by *R. syzygii* subsp. *syzygii*. As shown in Figure 8, the bacterial infection of *R. syzygii* subsp. *syzygii* is capable of causing damage on a mild, moderate, to severe scale. Molecular tests were also carried out on healthy clove plant tissues. Based on the results of DNA amplification, there was no bacterial DNA of *R. syzygii* subsp. *syzygii* in the tissue of healthy clove plants. Symptoms of wilt that commonly occur in Sumatra disease are not visible because there is no blockage of *R. syzygii* subsp. *syzygii* in the xylem tissue of the healthy plant.

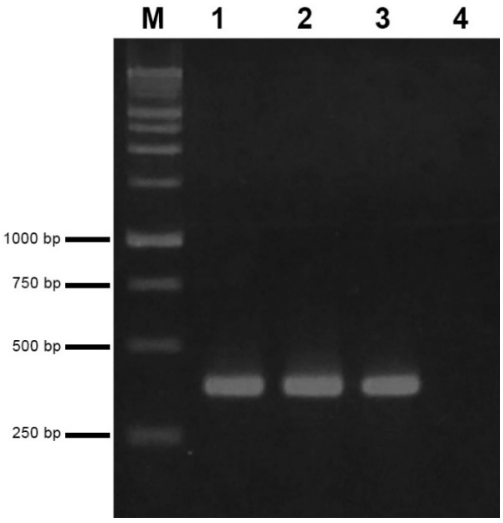


*Figure 8*. Visualization of DNA amplification results of *Ralstonia syzygii* subsp. *syzygii* of twig samples from various plant conditions by extraction using the zymo kit and specific primers UGMRss-F/UGMRss-R on 1% agarose gel

*Note*. M = DNA marker (1 kb ladder); Lane 1 = Samples of diseased twigs with severe symptoms; Lane 2 = Samples of healthy twigs from diseased plants; Lane 3 = Samples of diseased twigs with mild symptoms; Lane 4 = Samples of healthy twigs from healthy plants

Likewise, DNA extracted from various plant parts (leaves, twigs, and roots) can be amplified (Figure 9). It proves that *R. syzygii* subsp. *syzygii* are found in all parts of the plant. The presence of *R. syzygii* subsp. *syzygii* in some clove plant tissues indicates that *R. syzygii* subsp. *syzygii* can spread through vascular tissue. The extraction process involved xylem tissue from symptomatic plant tissue containing bacterial DNA of *R. syzygii* subsp. *syzygii* so

that it could be amplified through the PCR analysis. The results of DNA visualization showed that the DNA band was in the range of ~378 bp.

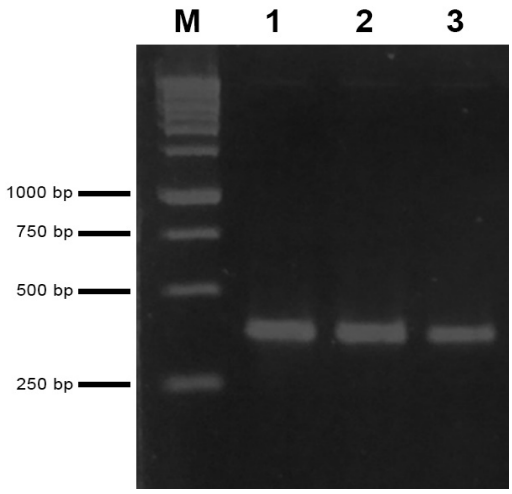


*Figure 9*. Visualization of DNA amplification results of *Ralstonia syzygii* subsp. *syzygii* from samples of several plant parts by extraction using the zymo kit and specific primers UGMRss-F/UGMRss-R on 1% agarose gel

*Note*. M = DNA marker (1 kb ladder); Lane 1 = Leaf samples; Lane 2 = Twig samples; Lane 3 = Root samples

UGMRss-specific primers can amplify about ~378 bp of *R. syzygii* subsp. *syzygii* DNA on clove plants. The use of specific primers based on the unique nucleotide sequence of *R. syzygii* subsp. *syzygii* was only able to detect DNA from the *egl* gene in *R. syzygii* subsp. *syzygii* (Trianom et al., 2019). The thickness of the DNA band varied from each sample. It is due to the differences in DNA concentration. Thick and light bands indicate a high concentration of DNA produced, while thin bands mean a low concentration of DNA produced.

**Sequencing and Phylogenetic Analysis**

Sequence analysis was conducted to confirm the PCR amplification data and to improve the data reliability. The edited nucleotide sequences of the *egl* gene were determined and deposited in GenBank under the accession numbers OK539688 (clove root), OK539686 (clove leaf), and OK539687 (clove twig). The results of DNA sequencing were used for phylogenetic analysis with those in GenBank (Figure 10). The phylogenetic analysis began by analyzing the kinship of *R. syzygii* subsp. *syzygii* on the BLAST results from the National Center of Biotechnology Information (NCBI) page. The results on the BLAST page revealed that the three samples namely leaves, twigs, and roots, had the highest similarity to the type of strain of *R. syzygii* subsp. *syzygii* (strain R001, accession number JF702320). The phylogenetic tree is divided into four clades, with samples taken from root, leaf, and twig tissue in the same node. As shown in Figure 10, the three samples are in the same clade as *R. syzygii* subsp. *syzygii* R001. It shows that the bacteria that infect clove plants in the roots, leaves, and twigs have the same relationship with the *R. syzygii* subsp. *syzygii* R001 type strain. Phylotypes in the same subgroup show similarities in pathogenicity abilities or are derived from the same source (Prakoso et al., 2022; Safni et al., 2014). The following are the results of the phylogenetic analysis using the ML Tree method with 1,000 bootstraps.

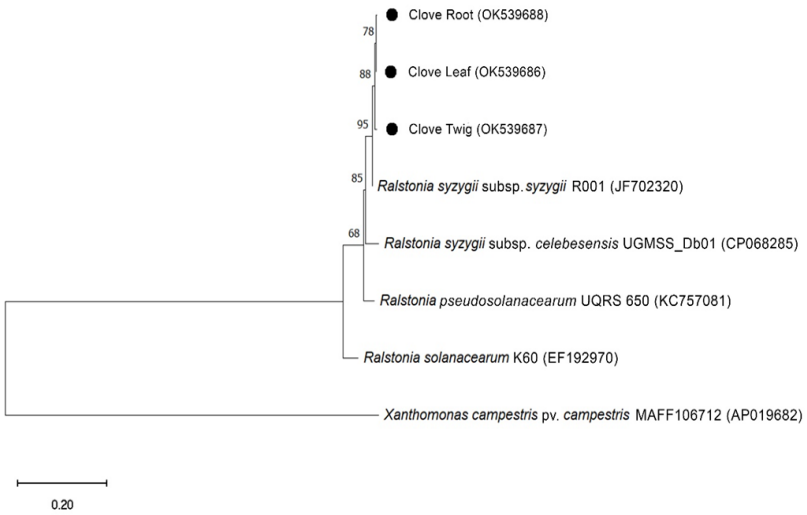


*Figure 10*. Phylogenetic tree of *Ralstonia syzygii* subsp. *syzygii* that infects cloves in root, leaf, and twig tissues with some proximity to strains of the genus *Ralstonia* based on the *egl* gene sequence using the maximum-likelihood method with 1,000 bootstrap times. *Xanthomonas campestris* pv. *campestris* sequences were used as an outgroup

Table 1

*Percentage of nucleotide sequence homology of* Ralstonia syzygii *subsp.* syzygii *with several strains of the genus* Ralstonia *and outgroup* Xanthomonas campestris *pv.* campestris *as comparison*

| No. | Identity | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Clove roots | ID | | | | | | | |
| 2 | Clove leaves | 99.7 | ID | | | | | | |
| 3 | Clove twigs | 99.1 | 99.1 | ID | | | | | |
| 4 | *Ralstonia syzygii* subsp. *syzygii* R001 (JF702320) | 99.1 | 99.4 | 98.5 | ID | | | | |
| 5 | *Ralstonia syzygii* subsp. *celebesensis* UGMSS_ Db01 (CP068285) | 95.1 | 95.4 | 94.4 | 95.5 | ID | | | |
| 6 | *Ralstonia solanacearum* K60 (EF192970) | 89.2 | 89.6 | 88.5 | 90.4 | 88.3 | ID | | |
| 7 | *Ralstonia pseudosolanacearum* NCPPB 253 | 94.8 | 95.1 | 95.1 | 95.5 | 94.2 | 89.7 | ID | |
| 8 | *Xanthomonas campestris* pv. *campestris* MAFF106712 (AP019682) | -23.1 | -24.1 | -21.8 | -22.8 | -27.6 | -22.4 | -22.4 | ID |

Based on the phylogenetic analysis, the DNA extracted from leaves, roots, and twigs are in the same class with a percentage of similarity between leaf and root samples of 99.7%, between roots and twigs of 99.1%, and between leaves and twigs by 99.1%. This result shows that there is a similarity in infecting bacteria. The similarity percentage between roots and twigs is 99.1%, 99.4%, and 99.5%, respectively. The value of the percentage of similarity indicates that the cause of Sumatra disease comes from the same group. According to Frank et al. (2008), the phylogenetic groups are different if there are more varied parts in a gene sequence. The phylogenetic analysis in this study also involved sequences from the genus *Ralstonia* as a comparison with the percentage of similarity between each species (Table 1). All strains used for comparison are the results of sequences obtained from the *egl* gene. According to Trianom et al. (2018), the sequences in the *egl* gene have good gene copy effectiveness, are easy to conserve, and are easy to amplify. In detection and monitoring efforts, *egl* gene sequencing analysis can be a method that can be used to determine the diversity of pathogens. The *Xanthomonas campestris* pv. *campestris* sequence was also used as an outgroup.

## CONCLUSION

The application of UAV technology for aerial photo data acquisition and as a data source for making GIS thematic maps of Sumatra disease distribution patterns facilitated the detection of Sumatra diseases in a wide area. The distribution pattern of Sumatra disease was random and relatively jumped between healthy and diseased plants. Detection of *R. syzygii* subsp. *syzygii* causing Sumatra disease in cloves using the PCR technique with UGMRss-specific primers producing ~378 bp amplicon. In diseased clove plants, bacteria detected in all plant parts, including leaves, twigs, and roots, originate from single species of bacteria. The *R. syzygii* subsp. *syzygii* was also detected in asymptomatic branches in diseased clove individuals.

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