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Rapid detection of *Ralstonia syzygii* subsp. *syzygii* on cloves vascular disease and identification potential alternate host

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Abstract

Vascular diseases caused by bacterial is one of the main problems of clove production in Central Sulawesi, Indonesia. In this study, rapid detection using polymerase chain reaction (PCR) was used to detect and characterize the causal agent of the diseases. The pathogenicity of pathogens was tested on clove, guava, banana, chili, tomato, heliconia, and three weeds that grow on clove plantations. Furthermore, the plants were checked using PCR detection to determine potential alternate hosts if they can be a pathogen-host without any symptoms. A specific primer *for Ralstonia syzigii* subsp. *syzygii* was used to amplify the bacterial pathogens in plant Deoxyribonucleic acid (DNA). PCR detection showed that all plant parts collected from clove plantations were infected by *R. syzigii* on DNA fragments (378 bp in size). Pathogenicity test indicates vascular disease symptoms on cloves, and there were no symptoms on other plants tested. Interestingly, PCR detection showed that the DNA of all plants tested was associated with *R. syzigii*. This study figured out that vascular diseases on clove plantations in Central Sulawesi Indonesia are associated with *R. syzigii*. In addition, this study also found a potential alternate asymptomatic host for *R. syzigii* on several plants.

Keywords: Clove, Host, Pathogenicity, Polymerase chain reaction, Sumatra disease

1. Introduction

Indonesia is the largest clove-producing country, accounting for almost 75% of its production globally [1]. Aside from fulfilling domestic needs, cloves are also a commodity that dominates Indonesia's exports. From 2017 to 2021, the clove production in Indonesia grew by 24.58% or increased from 113,178 to 140,997 tons [2]. One of the main cultivation areas in Indonesia is Central Sulawesi Province, which contributes 74,740 ha of clove plantations and produces 17,897 tons annually [3].

However, there is a fluctuation in clove production in some provinces [4,5], leading to an urgent need to study the problem behind it. Herein, we focused on the decline in clove production that is affected by a plant pathogen. One of the main problems is a vascular disease called Sumatra disease, which is caused by *Ralstonia syzygii* subsp. *syzigii* [6-8]. Recent studies have focused on managing vascular diseases; however, little is known about the pathogen's molecular characteristics and host range. This study used a rapid detection and identification of pathogens through polymerase chain reaction (PCR) detection with specific primers. Moreover, the host range was determined, and the pathogenicity was assessed.

The vascular disease on cloves was first reported in clove plantations in Sumatra island in 1975; therefore, it is called Sumatra disease [9,10]. It is one of the most destructive causes of clove yield losses, which can reach up to 40% per year [11].

This vascular disease is caused by xylem-limited bacteria *Pseudomonas syzygii* [12]. Its taxonomic classification was further revised as *R. syzygii* subsp. *syzygii* [13]. The disease is challenging to detect since its symptoms are similar to those due to the lack of water or extreme environmental conditions. Its symptoms are easy to recognize during the rainy season or when plants show wilt symptoms and die [14]. Its spread can be

caused by several factors, including transfer of disease from plant roots, presence of initial bacterial inoculum, and insect vectors, such as *Hindola fulva*, which may allow a broader range of hosts for *R. syzygii* [6,15].

To date, information about the pathogens of clove vascular diseases and their host range in Central Sulawesi is not well-established.

The most important questions include the following: Are cloves the only host of *R. syzygii* subsp. *syzygii*? Are there alternative hosts that can act as a reservoir for the pathogen? PCR techniques through specific gene amplification can possibly determine the presence of the pathogen in certain plants faster and more accurately [7,16-19]. Our previous finding supports PCR's use in discovering *R. syzygii* subsp. *celebesensis* (formerly *R. solanacearum*) on wilt banana [16]. The PCR technique can detect pathogens through DNA duplication with the help of polymerase enzymes and a pair of primers specific to the target DNA. Thus, this method has a high accuracy compared to conventional methods, which are less accurate and require a long laboratory time; therefore, it can be used for the rapid detection of *R. syzygii* subsp. *syzygii* [7,13,16].

In this study, we describe the current situation of vascular diseases of cloves in Indonesia. Causative pathogens were isolated, cultured, and identified rapidly using PCR. The isolated strains were used in subsequent pathogenicity tests. The plants tested were clove, weeds that grow on clove plantations, guava (*Psidium guajava*), banana (*Musa paradisiaca*), chili (*Capsicum annum*), tomato (*Lycopersicum esculentum*), and heliconia (*Heliconia* sp.). Subsequently, the plant DNA tested for pathogenicity was amplified using a specific primer to determine the possible association of *R. syzygii* subsp. *syzygii* for their alternate hosts.

2. Materials and methods

2.1 Study site and sample collection

Plant samples were collected from Tolitoli Regency, Central Sulawesi Indonesia (1°5′9″ N; 120°48′13″ E). The following samples were taken: roots, stems, twigs, and leaves that show vascular disease symptoms. The samples were photographed and delivered to the Laboratory of Plant Pathology, Tadulako University. In addition, different weeds that grow on clove plantations (*Mentha canadensis, Verbesina alternifolia,* and *Verbesina* sp.) were also collected. These were also tested as alternative hosts. Additionally, guava, banana, chili, tomato, and heliconia were also included for pathogenicity test as described below.

2.2 DNA extraction and PCR amplification

In a ball mill, each sample of clove root, stem, twig, and leaf gathered from the field was ground separately (Retsch MM 2000, Haan, Germany). The total DNA was extracted using a Dneasy plant micro kit, following the manufacturer's instructions (Qiagen, Hilden, Jerman). An IMPLEN nanophotometer P300 was used to quantify the DNA concentrations (Implen, Schatzbogen, Germany). To suspend the DNA, free nuclease water (Sigma-Aldrich Corp., St. Louis, MO, USA) was employed as a solvent. For a more accurate photometer reading, the DNA samples were diluted 10-fold. For the PCR experiment, 2 µL of 50 ng DNA were utilized as a template.

The UGMRss-F (5′-GCTCACCATCGCCAAGGACAGCG-3′) and UGMRss-R (5′TTCGATCGAACGCCT GGTTGAGC-3′) primers were used to amplify the endoglucanase (EG) gene of *R. syzygii* subsp. *syzygii* (Trianom et al., 2018). Each PCR reaction (25 µL) contained: 12.5 µL of Master Mix Top Taq (Qiagen, Germany), 2 µL of forward primer, 2 µL of reverse primer, 2 µL of DNA, and 6.5 µL of nuclease-free water. DNA amplification was carried out using a thermocycler (Labsyclar 011-103 Sensquest, Germany) with the following PCR conditions: initial denaturation at 96°C for 5 min, followed by 30 cycles at 94°C for 15 sec, 59°C for 30 sec, and 72 °C for 30 sec, and a final extension cycle at 72°C for 10 min [7].

The amplified DNA fragments were visualized by electrophoresis (Scientific Company, Del mar, California), which was done using 1% agarose gel in TBE buffer (Tris-HCL, boric acid, EDTA) 1 × (Lonza, Rockland, USA) at 50 Volt DC for 50 min.

Amplification product sizes were estimated using a 100 bp DNA ladder (Bioline) marker. Samples were prepared by mixing 10 µL of DNA, 1 µL of loading dye (Qiagen, Germany), and 1 µL of Sybr Green (Lonza, Rockland, USA); then, it was filled into a gel. The gel was placed on the negative pole if an electric current is applied using a buffer solution. The DNA was moved to the positive pole. The electrophoresis results were photographed using a gel doc (Uvitec, Cambrige, UK).

2.3 Bacterial preparation, pathogenicity, and Koch's postulate tests

R. syzygii subsp. *syzygii* was isolated from root, stem, twig, and leaves of cloves that were collected from infested plantations. All plant parts were sterilized with 70% ethanol and water. Then, the samples were sliced into small parts ($\pm$0.5 cm^2) in a 10 mL reaction tube with sterilized water for 24 h. Then, the suspension was streaked onto three different media, including casamino acid (CA), sucrose peptone agar, casamino acid peptone

glucose (CPG), and yeast peptone glucose agar (YPGA) to observe which media are more suitable for *Ralstonia* species complexes, as these were reported to grow in different media [13,20-23]. The CPG medium was selected to grow *R. syzygii* subsp. *syzygii* since it shows a better performance. Then, the isolated bacteria were incubated at 28°C. A single bacterial colony closely related to *R. syzygii* subsp. *syzygii* based on its characteristics was restreaked onto new CPG solid agar media for seven days. Then, this single colony was used as a DNA template for PCR amplification to ensure that the pathogen was *R. syzygii*. Bacteria with positive confirmation in PCR were propagated in CPG liquid media.

The pathogenicity tests were done on cloves, three weeds that grow in clove plantations (*M. canadensis, V. alternifolia,* and *Verbesina* sp.), guava, banana, chili, tomato, and heliconia. Five plants were used for each. These plants are common hosts of *R. species complex* [24,25]. Bacterial inoculation was carried out by making a slight injury to plant roots using a needle and by pouring 10 mL of bacterial suspension (1.5×10^6 CFU/mL^{-1}) to the root zone in a pot [26]. To test Koch's postulate, the bacteria isolated from the plants were streaked on CPG media using the above-mentioned procedure. The disease symptoms appeared and were recorded every day based on symptoms of yellowing, drying, and falling of leaves, which are the characteristics of vascular disease.

2.4 PCR amplification after pathogenicity and Koch's postulate test

After three months, the PCR samples were taken from all inoculated plants, including the negative control plants. The samples were washed with water and 70% ethanol and were rinsed again with water to remove any *R. syzygii* subsp. *syzygii* that might be on plant surfaces or within the soil. The DNA extraction, amplification, and electrophoresis were done as the protocol described above.

3. Results and discussion

3.1 Description of clove vascular disease

Most clove plantations in Tolitoli regency exhibited wilt symptoms due to *R. syzygii* subsp. *syzygii* infections. When observed from a long distance, clove plantation areas looked like sporadic white spots (Figure 1A), while plants not infected with the pathogen looked green in normal plantation areas.

In this study, the symptoms of the vascular disease are illustrated from the current situation of clove plantations in Central Sulawesi, Indonesia. A common symptom seen in the field was the dryness of leaves that looked white. It is an advanced symptom due to vascular tissue damage in the roots and stems. Dead leaves may fall from the tree or become white but still stick to the branch, causing the tree to appear burned or withered (Figure 1B). The twigs were split longitudinally, and necrosis symptoms were found in wooden vessels (Figure 1C). Affected stems can also suffer from severe damage to falling. The outer part of the stems showed white patches that may also show cancer, as shown in Figure 1D. Another symptom that was often encountered was dieback on the sides of certain trees. Eventually, the whole tree will die.

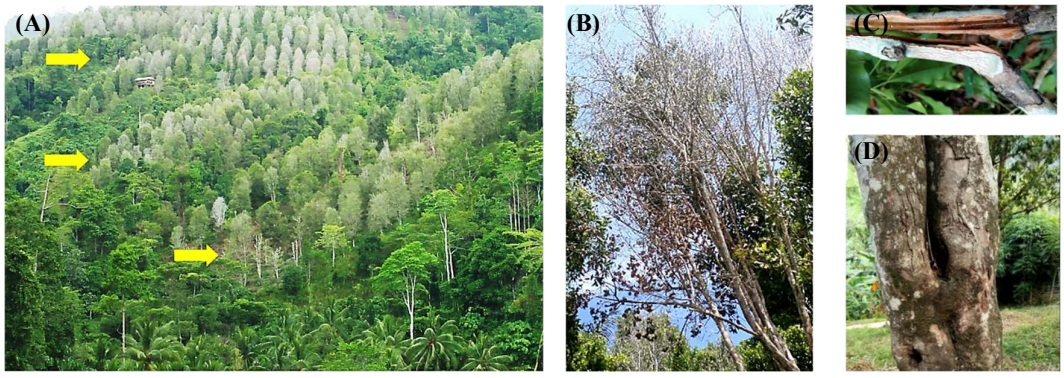

Figure 1 The vascular disease symptoms of cloves in the field: (A) the expanse of clove plantation looks dead with white spots (see arrow), (B) dead parts of the crown and leaves, (C) necrotic twig vessels, and (D) stems damaged by *R. syzygii* subsp. *syzygii* infection.

Assumptions on the virulence of populations in different locations may be related to environmental factors and not to genetic differences between populations. In Indonesia, the vascular disease due to *R. syzygii* subsp. *syzygii* has two main extreme symptoms: rapid or slow death. A rapid death of cloves is commonly found in highlands, as this was reported in West Sumatra, North Sumatra, Lampung, and West Java provinces. In contrast, a slow death is found more frequently in lowlands [22].

The wilting of clove trees due to *R. syzygii* subsp. *syzygii* infection was also observed. Extracellular polysaccharides were reported as a massive virulence factor of *R. syzygii* subsp. *syzygii*. Its accumulation in vascular tissues inhibits the transport of water and nutrients. Moreover, phytohormones, such as ethylene and auxin, were also reported as virulence factors that contribute to plant cell wall degradation [27,28]. In addition, the wilting of cloves may also be due to other causative agents together with *R. syzygii* subsp. *syzygii* infection. For example, in Central Java, stem canker and stem borer by *Nothopeus fasciatipennis* were found to sever the disease [8,29].

3.2 Morphological characteristics of R. syzygii subsp. *syzygii*

Macroscopically, *R. syzygii* subsp. *syzygii* on CPG solid media grew on the fourth day after their isolation as a single colony. The bacterial colonies were round, convex, white, and with flat edges, with a single colony size of 1-1.8 µM after 4-7 days of incubation at 28°C (Figure 2).

Ralstonia syzygii subsp. *syzygii* were reported to grow in CA media [13]. In contrast, the bacteria isolated from cloves with the vascular disease grew well on CPG media. The use of different media for its growth was presented in previous studies, where YPGA [23], sucrose peptone agar [22], and periwinkle wilt [9] were used. Different growth media may reflect the growth performance of the bacteria. In this study, *R. syzygii* subsp. *syzygii* appeared four days after inoculation, and the bacteria on the seventh day are shown in Figure 2.

The causative agent of clove vascular disease is *R. syzigii* subsp. *syzigii* [6]. Its morphological characteristics and growth ability may vary in different synthetic media. Recent morphological and biochemical determination of *R. syzigii* showed that the bacteria can grow at a temperature range of 27-37°C on YPGA, grow into 5 mm-diameter colonies after seven days on YPGA, and use glucose, sucrose, maltose, and mannitol as carbon sources [20]. Compared to the results of this study, the growth of *R. syzigii* on CPG agar media was almost similar.

Figure 2 Colony morphology of *R. syzygii* on CPG solid media.

3.3 PCR detection of the pathogen

PCR amplification from roots, stem, twig, and leaves was positively associated with *R. syzigii* subsp. *syzigii* with ~378-bp DNA fragments. UGMRss-F and UGMRss-R primers were able to amplify the total genomic DNA of *R. syzigii* subsp. *syzigii* mixed with plant DNA. In contrast, the negative control sample (without *R. syzigii* subsp. *syzigii* DNA) remained blank (Figure 3A).

Advances in molecular techniques aid in the rapid reporting of plant diseases for diagnosis. The efficiency of molecular techniques will facilitate the detection of pathogens directly from plant parts suspected to carry pathogens. Molecular analysis can be used to monitor the presence of disturbing microbes in agricultural plants and crops.

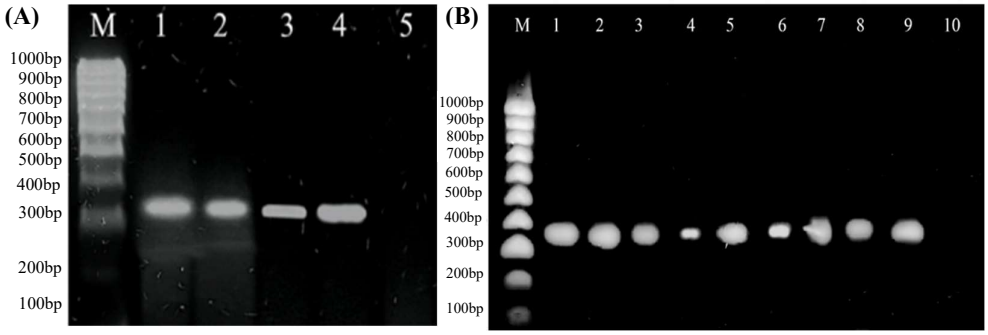

Figure 3 Amplification of *R. syzigii* subsp. *syzigii* from the total genomic DNA using UGMRss-F and UGMRss-R primers: (A) on symptomatic clove section samples, namely: 1) root, 2) stem, 3) twig, 4) leaf, and 5) negative control (no DNA), and (B) from plants used in pathogenicity tests. M is the marker, samples with numbers 1 to 9 refer to sample numbers in Table 1, and 10 is the negative control (no DNA).

3.4 Pathogenicity and Koch's postulate tests of R. syzigii subsp. *syzigii and DNA amplification from their host*

Pathogenicity tests were done to clove seedlings and three different weeds that grow on clove plantations. Guava, banana, chili, tomato, and heliconia were also included. Cloves were the only plants that showed wilt symptoms 25 days after the pathogenicity test using *R. syzigii* subsp. *syzigii*, while other plants remained healthy. Interestingly, although the plants looked healthy except for cloves, the PCR detection results were positive. *R. syzigii* subsp. *syzigii* can associate with plant tissues without causing wilt symptoms. The results of the pathogenicity tests and PCR detection are presented in Table 1 and Figure 3B, respectively. Figure 4 shows that only cloves showed wilt symptoms, while the other plants remained healthy. Interestingly, *R. syzigii* subsp. *syzigii* were successfully isolated from all plants to confirm Koch's postulate.

Table 1 Pathogenicity test on cloves and several plants. Positive means that the infected plants have wilt symptoms. The negative ones refer to healthy plants.

Nr.	Plant tested	Pathogenicity test	PCR test
1.	Clove	Positive	Positive
2.	Guava	Negative	Positive
3.	Banana	Negative	Positive
4.	Chili	Negative	Positive
5.	Tomato	Negative	Positive
6.	*Heliconia* sp.	Negative	Positive
7.	Weed 1 (*M. canadensis*)	Negative	Positive
8.	Weed 2 (*V. alternifolia*)	Negative	Positive
9.	Weed 3 (*Verbesina.* sp.)	Negative	Positive

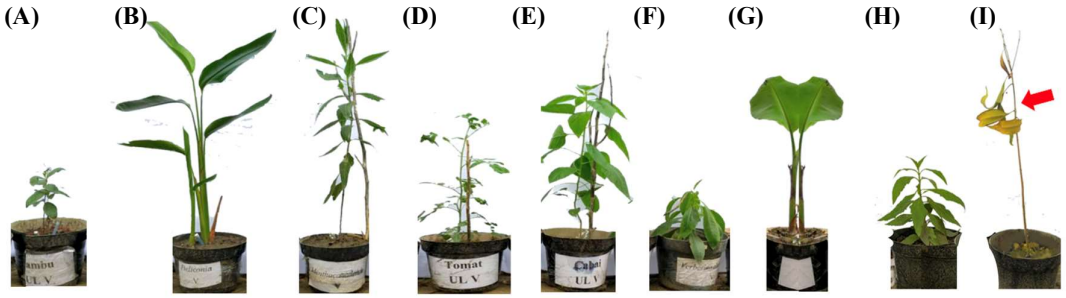

Figure 5 Pathogenicity test shows that only cloves were infected by *R. syzigii* subsp. *syzigii* (red arrow). All plants seedling tested; (A) guava, (B) heliconia sp., (C) *M. canadensis*, (D) tomato, (E) chili, (F) *V. alternifolia*, (G) banana, (H) *V.* sp., and (I) clove.

The development of rapid detection using a molecular approach has been used worldwide. One of the common methods is using specific primers for rapid detection. In this study, UGMRss-F and UGMRss-R primers were designed based on the Enduglacanase (EG) gene [7] and were used to identify the causative agent of clove vascular disease. EG genes have been used to determine *R.* species complexes for decades [30-32]. Since the *egl* gene contains the phylogenetic information about the diversity of [26,28,29], these bacteria were used for the taxonomic classification of bacterial strains from Asia and Africa [13,30].

The isolated and identified pathogen was inoculated to several plants for pathogenicity tests. Cloves, three weeds that originated from clove plantations, guava, banana, chili, tomato, and heliconia were tested for pathogenicity assay. Cloves were the only infected plants that showed wilt symptoms. This is not surprising since *R. syzigii* subsp. *syzigii* was reported as a specific host pathogen.

Interestingly, the rapid detection of PCR-specific primers of UGMRss-F/R on these plants showed positive results. *R. syzigii* can live in other plant hosts without any symptoms. Therefore, this study revealed a possible alternate host of *R. syzigii*.

The pathogenicity test also aimed to find alternate hosts other than cloves for pathogens when the main host is not available. *Egl* gene species complexes are diverse bacterial strains of plant pathogens with a wide range of hosts. *R.* has four phylotypes based on race and host range. In Indonesia, only phylotype IV strains occurs to date. However, this phylotype also attacks plants in Australia, Korea, and Malaysia. *R.* belongs to phylotype IV; then, it was re-taxonomically classified as *R. syzigii* [13]. The species that infected cloves was *R. syzigii* subsp. *syzigii*. *R. syzigii* subsp. *indonesiensis* hosts a wide range of Solanaceae and Zingeberales family plants. In addition, *R. syzigii* subsp. *celebensis* only attacks *Musa* sp. [13,19,31].

Although *R. syzigii* has the most diverse host range, it has less active metabolites as virulence factors to infect plants [32], allowing *R. syzigii* to associate in non-host crops. *R. syzigii* may also be avirulent in non-host crops since it did not produce any disease symptoms in the pathogenicity tests. Crop resistance against *R.* still remains unclear, and the ability of *R.* to infect plants and plant responses to form resistance varies under different environmental conditions [33]. Moreover, *R.* is very diverse, so these plants may have different resistance against it.

4. Conclusion

A rapid detection and assessment of host range are essential to determine the existence of plant pathogens and their management. In this study, PCR can rapidly detect and characterize plant pathogens. The vascular disease was caused by *R. syzigii* subsp. *syzigii*. *R. syzigii* only infected cloves with wilt symptoms, as the other tested plants remained healthy. This indicates that other plants may potentially act as alternative hosts of *R. syzigii.* This finding contributes to the national data on the causes of vascular diseases. The pathogen's identity and host range are important in developing a vascular disease control strategy.

5. Acknowledgments

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