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Morphological and Physiological Characterization of *Ralstonia syzygii* from Clove

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Abstract : *Ralstonia syzygii is causes Sumatra disease of clove plants. The existence of this pathogen has been reported there in several areas, but has not been much research report related to this issue. The aim of this study was to characterize isolates of R. syzygii isolated from clove plant in East Java. Bacteria that can be isolated from the clove plant tested morphological, physiological, hypersensitivity, and patogenesitas. Test result showed that bacteria growing on NA medium thin and clear like dew drops, form round colonies, the size of 1mm or less, convex, and not slimy. Also bacteria are gram-negative, aerobe, does not emit fluorescent pigment, not yellow on YDC medium, are not able to utilize arginine, did not grow in the medium D1M agar, and does not grow at 40 ° C. Bacteria causing necrotic on tobacco leaves 4-7 days and cause clove seedlings to wilt within 161 days after inoculation.*

Keywords : *Clove, Bacterial Vessels Wooden Cloves disease, Ralstonia syzygii*

I. Introduction

Sumatra disease or what is now known as Bacterial Vessels Wooden Cloves (BVWC) diseases is still a major problem in the cultivation of cloves in Indonesia. After attacking the clove plant in Sumatra and West Java in the 1980s, recently the disease has spread to Central Java and East Java regions and lead to a decrease in yields of up to 7.3% annually (Tjahyono, 2013 [1]). Diseases caused by bacteria that are identified as Pseudomonas syzygii (Roberts et al., 1990[2]), now known as Ralstonia syzygii (Vaneechoutte et al., 2004[3]).

Bacteria multiply in wooden vessels (xylem) which would then shut down the flow the flow of the vessels. Closing the flow of vessels is thought to be the main cause of the symptoms of the disease. Symptoms diversity and spread of the disease may be caused by differences in shade, temperature, sunlight, and season. In addition, there are the virulence different BPKC disease bacterial strains (Semangun, 2000[4]).

Research on the bacteria that causes this disease is very rare and last conducted in Central Java by Danaatmaja *et al.*, (2009)[5], while in East Java has never been done so that the control techniques became difficult and result in the destruction of cloves in some areas. Morphological and physiological characterization of pathogenic bacteria needed to find a proper way to control the disease, according to the location of the disease.

II. Materials And Methods

2.1 Sources of diseased plant

Diseased plant material derived from clove cultivation centers in Jombang, East Java. Withered Symptomatic plants branches cut on the section between the healthy and the diseased, discarded outer skin, then cut into small pieces the size of 5x5 mm. The piece is disinfected with 70% alcohol for 1 minute and then rinsed with sterile water 2 times. Furthermore, the snippet drained using filter paper and then soaked in sterile test tubes containing sterile water for 3 hours. The suspension was streaked on NA medium and incubated for 7 days.

2.2 Morphology test

Bacterial colonies morphology test conducted on colonies color, colonies size, colonies form, colonies circumstance, and forms the periphery of the colonies.

2.3 Gram test

One loop bacteria were mixed with 1 drop of KOH 3% on objects glass. Both are blended until smooth and then loop removed and observed what happened. Gram test was also carried out by staining. One loop bacteria streaked on objects glass were clean, and then added sterile distilled water to a thin flattened. The bottom of the object glass is then passed over the spiritus lamp until the entire surface of the object glass dry. Crystal violet solution was dripped and then flattened on the surface of the glass object for 60 seconds. Glass objects washed with running water for a few seconds and then dried. Iodine solution was dripped and flattened on the objects glass for 1 minute. Objects glass washed with running water for a few seconds and then dried.

Furthermore, the decolorization with ethyl alcohol for about 30 seconds. Objects glass washed with running water for approximately 2 seconds, and then dried. Safranin solution dripped and flattened on the objects glass for 10 seconds. Objects Glass quickly washed with running water, and then dried. Gram staining results were observed with a microscope.

2.4 Anaerobe growth: Anaerobe bacterial growth is tested by inoculating bacteria on OF media in a test tube. Bacterial inoculation was conducted in two tubes, one tube closed with sterile liquid paraffin, while the other tube without liquid paraffin. Observations were made to change the color of the media.

2.5 Fluorescent pigments at King's B medium : Pathogenic bacteria cultures were taken with loop needle aseptically, and then inoculated streaks on King's B medium. Cultures were then incubated at a temperature of 30^0C. Bacterial colonies growing observed under ultraviolet light whether issued a fluorescent pigment or not.

2.6 Growth in the YDC medium: Pathogenic bacteria cultures were inoculated by streaks on YDC medium. Cultures were incubated at a temperature of 30^0C. Bacterial colonies growing observed, whether yellow or not.

2.7 Arginine utilization: Bacterial cultures were inoculated in test tubes that had contained the arginine medium by way poked it to a depth of 0.5 cm, and then covered with sterile paraffin. A positive reaction is indicated by a color change media from orange to pink.

2.8 Growth in D1M agar: Pathogenic bacteria were grown on a D1M medium so then incubated at a temperature of 30^0C. Observations were conducted on D1M agar medium, to determine the ability of the bacteria to grow.

2.9 Growth at 40°C: The bacteria were grown in Nutrient Broth (NB) medium in a test tube, and incubated at 40°C. A positive reaction is indicated by a color change that occurs in the medium, of clear color becomes murky.

2.10 Hypersensitivity test: Hypersensitivity reactions conducted by injecting the suspension of pathogens into tobacco leaf tissue (Fahy and Persley, 1982[6]). Pathogen suspension gained by growing the bacteria in the NA medium then dissolving into sterile water to form a suspension. The development of water soak symptoms (wet) in tobacco leaves and chlorosis were observed up to 7 days.

2.11 Pathogenicity test: Bacterial isolates showed hypersensitive reaction tested pathogenicity on clove seedlings 1 year old. Methods of inoculation of bacteria carried by infectivity titration method, is by making wound holes into the stem of the plant is then inserted tip that contains 10-20µl bacterial suspension (1 x 106 CFU / ml) to the hole section (Schaad *et al*., 2001[7]). Infectivity titration method is carried out at the base of the stem. After the treatment, the plants are covered for 48 hours to maintain the viability of the bacteria and provide optimal conditions for the process of pathogenicity.

III. Results And Discussion

3.1 Disease Symptoms in the Field

Early symptoms include death of twigs and leaf drop of clove bud parts (Fig. 1a). Symptoms continue until all the leaves fall and the plant become bald (Fig. 1b). In addition, those that are symptomatic as plants are old, so the plants attacked more easily distinguished because leaved plants rarely (Fig. 1c). Symptoms can also be the partially drying of plant branches and dried leaves is still attached to the tree (Fig. 1d).

3.2 Morphology bacteria

In the NA medium, bacteria have a characteristic rounded shape of the colonies, the color of translucent colonies like dew drops, the size of 1mm or smaller colonies, convex elevation, circumstance not slimy colonies. These characteristics the same with *Ralstonia syzygii* studied by Danaatmaja *et al*., (2009)[5] and has little difference with the results Vaneechoute *et al*., (2004)[3], ie the colony diameter of 5 mm at a good medium (Table 1)

3.3 Bacteria physiology

In the test with KOH 3% of all bacteria seemed sticky when lifted with a loop needle, so that it can be said that the bacteria are Gram negative (Table 1). On Gram staining, the bacteria are red and short oval.

Gram-negative bacteria have a thin peptidoglycan layer, only 1-2 layers and composition of the cell wall is not compact. The permeability of the cell wall of a larger complex that still allows the release of iodine

crystals. Decolorization with ethyl alcohol has been releasing complex crystal violet paint with iodine solution, so that only the bacterial cell wall binds to the second paint is safranin red (Todar, 2012[8]).Bacteria react negatively to the medium OF closed sterile paraffin oil. In the anaerobic conditions, the bacteria are not able to use a carbon source (glucose) in the medium and produce acid, so that the pH of the media has not changed and resulted in no change in color. The bacteria also reacted negatively on King's B medium, which means that the bacteria do not emit fluorescent pigments. These pigments are usually issued bacteria selected for pyoverdins (siderophores) are compounds of iron binding agents which will be formed on the low iron content medium, such as King's B. Test the growth of bacteria on the media YDC / NA The bacteria are not yellow. D1M is a semi-selective medium for the growth of bacteria Agrobacterium sp. Bacteria can not be grown in the D1M agar medium and at 40 ° C, and this is characteristic of the genus Ralstonia bacteria.

3.4 Hypersensitivity test

Bacteria reacted positively to the hypersensitivity test, indicated by the formation of the water soak (wet) area on the leaves which bacteria infiltrated. These symptoms progress to yellow necrotic and eventually become dry brown after 4-7 days (Fig. 2). Hypersensitivity reactions indicate interactions that do not match between pathogens with the plant, because of the strong defense response of the plant. Tobacco plant responds hypersensitive by forming necrotic to inhibit the growth of bacteria to other cells that are not infected. Hypersensitivity reactions resulting in cell death (necrosis), which inhibits the growth and spread of pathogens (Suryani, 2012[9]).

3.5 Pathogenicity test

Pathogenicity test results on seed cloves with the treatment of bacterial infiltration in the base of the stem showing symptoms of wilting within 161 days after inoculation (DAI), with a wilting percentage of 67%. Furthermore symptomatic plants (Fig. 3) are in subculture back to prove Koch's postulates. Bacteria subculture results showed the same colony with inoculated bacteria, which is the size of a small colony, clear, flat edge, convex, and not slimy. Pathogenicity of bacteria that cause wilt on clove is correlated with the presence of bacteria in the tissue blockage of wooden vessels. After a successful entry into the plant tissue, bacteria will multiply in the wooden vessels (xylem) in the stem and then spread to all parts of the plant. Due to blockage of wooden vessels by millions of bacterial cells, the transport of water and minerals from the soil so the plants become stunted wilt and eventually die. Different strains of a pathogen has the different ability to cause disease in host plants were highly susceptible. It depends on the degree of pathogenicity, the ability to grow and develop rapidly in the host plant tissue in the early stages of each strain of the pathogen (Semangun, 2000[4]). Agrios (2004[10]) also states that the amount of pathogen virulence is determined by its ability to attack (invasion) and the toxin produced in the host plant tissue. Likewise, the possibility of the cloves. In addition to blockage by millions of cells, wilting can compounded by a bacterial toxin that is released and the variability depends on the characteristic of genetic and environmental.

IV. Figures And Tables

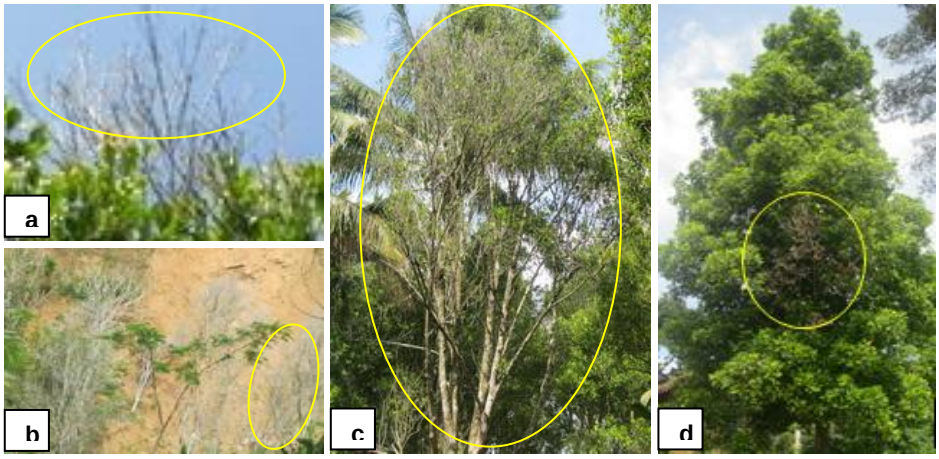

Fig. 1. Symptoms of wilt disease on the cloves. a. Autumn leaves start from the top. b. Plants become bald. c. Rare leafy plants. d. Dried leaves attached in a tree.

Fig. 2. Symptoms of necrosis in tobacco leaf that showed a positive reaction in hypersensitive reaction test.

Figure 3. Symptoms of wilting in the pathogenicity test

Table 1. Character of Morphology and Physiology *Ralstonia syzygii* East Java isolates

Characters	Results
Morphology	
* Shape	Round
* Edge	Flat
* Elevation	Convex
* The color	Transparent
* Consistency	Not slimy
* Colonies (diameter)	1 mm
Physiology	
*Gram	-
* Anaerobic	-
* Yellow on YDC / NA	-
* Fluorescent pigments	-
* Arginine	-
* Growth 40ºC	-
* D1M Agar	-
Note: - was negative reaction	

V. Conclusion

Ralstonia syzygii isolated of cloves in East Java has very different morphological characters with bacteria of the genus Ralstonia in general, but has the same physiological characters.

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