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Preliminary Studies towards Identification of Ginger Wilt Disease in Sabah, Malaysia

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ABSTRACT

Bacterial wilt is a major ginger disease in Sabah after rhizome rot. The disease has affected the production of ginger in Sabah since 2005. In this study, the ginger plants with foliar symptoms (yellowing and wilting), collected from six ginger-growing areas in the Tambunan and Ranau districts, were observed to have signs of bacterial pathogen (i.e., rhizome rot and bacterial ooze). A total of 19 bacterial strains were isolated, and all of the isolates were characterised as rod-shaped and Gram-negative by Gram-staining and potassium hydroxide test and microscopic examination. MALDI-TOF analysis identified six species from the isolates: *Enterobacter cloacae* complex (57.9%), *Ralstonia pickettii* (10.5%), *Agrobacterium tumefaciens* (10.5%), *Bacillus pumilus* (10.5%), *Stenotrophomonas maltophilia* (5.3%) and *Serratia marcescens* (5.3%). In pathogenicity test, *E. cloacae*, which constituted most of the isolates, induced mild rot symptoms (discoloration) on ginger rhizome slices, but no disease symptoms were produced in ginger plants. Further studies on the interaction of *E. cloacae* with other isolated species are required to confirm the causes of ginger wilt disease in Sabah, Malaysia.

Keywords: ginger, ginger wilt disease, *Enterobacter cloacae* complex

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INTRODUCTION

Ginger (*Zingiber officinale* Rosc) is one of the important spices in Malaysia. It is a high-value crop, with a potential yield of 9 – 12 tonne per hectare; at the price of RM 3 – 5 per kilograms, farmers can earn net

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income per hectare of RM 20,595–53,595 (Department of Agriculture, 2011). The type of ginger that is grown in Sabah is Sabah cultivar (Mahdi et al. 2013). The main growing seasons are on March to April. Sabah ginger is known to be the best of its kind, and the main ginger-producing areas in Sabah are Ranau and Tambunan districts. Tambunan district is recognised as the main ginger producer in Malaysia (Daily Express, 2006), and the state government aimed to make Sabah the biggest ginger producer in the country (Daily Express, 2012). However, the bacterial wilt disease restricted the production of ginger in Sabah. The planting areas and production of ginger in Sabah declined from 458.3 hectares (5,848.4 tonnes) in 2006 to only 117.7 hectares (956.7 tonnes) in 2014 (Department of Agriculture, 2006–2014). This incident caused the increase of ginger import value from 1,185 tonnes (RM 1,953,000) in 2006 to 3,508 tonnes (RM5,691,698) in 2012 (Department of Statistic, 2006 - 2012). Bacterial wilt is the main ginger disease in Sabah after rhizome rot. The disease has affected the production of ginger in Sabah since 2005. The symptoms observed in the field included yellowing, wilting and rotting of rhizomes, suspected to be bacterial wilt disease caused by *Ralstonia solanacearum*. The objective of this study was to identify the bacteria that cause ginger wilt disease up to species level. Identification of the bacteria was done by culturing in nutrient agar and using MALDI-TOF technology. Identification of the causal agent of ginger wilt disease is important for proper control of the disease in order to improve ginger production in Sabah.

MATERIAL AND METHODS

Pathogen isolations were based on the standard procedure described by Johnston and Booth (1968). Ginger plants with foliar symptoms (yellowing and wilting) (Figure 1) were collected from six different ginger-growing areas in the Tambunan and Ranau districts which were affected by wilt disease in January to February 2014. The samples in each area were collected by random sampling. The ginger plants were inspected for signs of bacterial pathogen (i.e., rhizome rot and bacterial ooze). Bacterial strains were then isolated from rhizome as the suspected bacteria (*R. solanacearum*) is a seed rhizome borne, and bacterial population is generally larger in roots compared to stems and leaves (Lamb et al., 1996; Nelson, 2013). The ginger rhizomes were washed with tap water to remove soil, and then air-dried. The rhizomes were then aseptically cross-sectioned with approximately 5 mm^3 tissue sections cut out from the central cylinder. The tissue sections were macerated in sterile distilled water (SDW) in a glass cube before one loop-full of the suspension was streaked onto a nutrient agar plate and incubated at 28°C to 30°C for 2 days. The single colonies forming after that were transferred to new plates and incubated. Purified cultures were characterised based on their morphological characteristics, Gram staining, potassium hydroxide testing and microscopic examination (Suslow et al., 1982; Breakwell et al., 2007). Identification

of the bacterial strains was completed using matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) technology (Bizzini et al., 2010). Pathogenicity tests were carried out based on the method of Nishijima et al. (2004). Inoculation tests were performed on ginger rhizome slices and young ginger plants grown in polybags. Pathogenicity tests on the slices were done using mature ginger rhizomes purchased from the local market. The rhizomes were cleaned with tap water and dried at room temperature. After that, they were cut into approximately 3 mm slices with a sterilised knife.

The slices were sterilised on both surfaces by flame, then immersed in SDW for rehydration and placed on moistened filter paper in petri plates. The rhizome slices in each petri plate were inoculated with bacterial suspension. Rhizomes inoculated with SDW served as a control. Inoculations were made using two methods. In the first, a sterile toothpick dipped in either bacterial culture or SDW was punctured into the centre of each rhizome slice. In the second method, 100 ul of bacterial suspension at approximately 10^{11}, 10^{10}, 10^{9}, 10^{8} and 10^{7} colony-forming units (CFU)/ml or SDW were pipette-inoculated into the puncture-wounds of the rhizome slices. Each inoculated rhizome slice in the petri plate of both methods was incubated at 30°C until visible symptoms were observed for evaluation. Pathogenicity tests on the ginger plants were carried out using tissue-culture-initiated ginger plants that were grown in polybags containing sterilised Sahara potting media (90% organic matter). The 3-month-old plants were inoculated by pouring 18 ml of bacterial suspension at approximately 10^{11}, 10^{10}, 10^{9}, 10^{8} and 10^{7} CFU/ml or SDW into the media in three replicates. Monitoring of foliar symptoms was done every week.

RESULT AND DISCUSSION

The ginger plants with foliar symptoms (yellowing and wilting) collected from six ginger-growing areas in the Tambunan and Ranau districts was observed to have signs of bacterial pathogen (i.e. rhizome rot and bacterial ooze). A total of 19 bacterial strains were isolated, and all of the isolates

Figure 1. Ginger plants with foliar symptoms (yellowing and wilting)

were characterised as rod-shaped and Gram-negative by Gram-staining and potassium hydroxide test, and microscopic examination. MALDI-TOF analysis identified six species from the isolates: *Enterobacter cloacae* complex (57.9%), *Ralstonia pickettii* (10.5%), *Agrobacterium tumefaciens* (10.5%), *Bacillus pumilus* (10.5%), *Stenotrophomonas maltophilia* (5.3%) and *Serratia marcescens* (5.3%).

The bacteria with the highest abundance were *E. cloacae* with 57.9% isolates, while *R. solanacearum* which was thought to be the cause of ginger wilt disease was not detected among the isolates. *E. cloacae* is a Gram-negative, facultative anaerobic, rod-shaped bacterium that has been reported to cause ginger rhizome rot in Hawai and Brazil (Nishijima et al., 2004; Moreira et al., 2013). *Enterobacter* species have also been reported to cause ginger rhizome rot in Australia (Stirling, 2004). *E. cloacae* had been reported to have antagonistic effects and to effectively suppress the growth of *R. solanacearum* (Xue, 2009; Liu et al., 2013). Previous study showed that two species of *Enterobacter, E. asburiae* and *E. cloacae,* were frequently isolated from ginger rhizomes, and in fact there was overgrown *R. solanacearum* in culture (Alvarez et al., 2003). Moreover, *E. cloacae* had been reported to replace *R. solanacearum* as the causal pathogen for bacterial wilt of mulberry in China (Wang et al*.,* 2010). This result suggested that *R. solanacearum* could have been suppressed or overgrown by *E. cloacae*.

The other four plant pathogenic bacteria, *R. pickettii*, *A. tumefaciens, B. pumilus* and *S. marcescens*, were also isolated from the samples. *B. pumilus* is a secondary pathogen of ginger rhizomes that causes rhizome rot and foliar symptoms at later growth stages (Peng et al*.,* 2013). *R. pickettii, A. tumefaciens* and *S. marcescens* are not known to be the pathogen for ginger. *R. pickettii* is a pathogen for leaf spot and blight disease of Bird of Paradise tree (Polizzi et al*.,* 2008). *R. pickettii* also known as a biocontrol for *R. solanacaerum* and it can be found in soil, water and plants (Stelzmueller et al*.,* 2006; Wei et al*.,* 2013). *A. tumefaciens* is a pathogen for crown gall disease of many plant species (Suma et al*.,* 2008; Gohlka & Deeken, 2014). *S. marcescens* is a pathogen for cucurbit yellow vine disease and corn whorl rot (Besler & Little, 2015; Wang et al*.,* 2015).

S. marcescens also known as a biocontrol agent and a rhizobacteria that can be found in the ginger rhizosphere (Bini et al*.*, 2011; Yang et al*.* 2012). The other bacteria *S. maltophilia* is not a plant pathogenic bacteria. *S. maltophilia* is a biocontrol agent and a rhizobacteria that can be found in the ginger rhizosphere (Bini et al*.,* 2011; Yang et al*.,* 2012).

Pathogenicity test of *E. cloacae* was conducted on rhizome slice and ginger plants to determine it as the causal pathogen. Results showed that obvious disease symptoms in rhizome slice were observed in both inoculation methods that were used. However, rhizome slices inoculated using

Method 1 exhibited disease symptoms earlier (i.e., after 10 days of inoculation) compared to rhizome slices inoculated using Method 2 (i.e., after 13 days of inoculation). Rhizome slices infected with *E. cloacae* showed discoloration and decaying at the punctured wound, which are common symptoms of rotting, while control slices showed no rotting symptoms (Figure 2).

There was no disease symptoms observed in any ginger plants inoculated with *E. cloacae*. A previous study by Nishijima et al. (2004) reported that *E. cloacae* did not produce disease symptoms in ginger plants when the temperature, humidity, aeration and soil moisture are not optimal for infection. Environmental condition such as high temperature (35 to 37^{0}C), high humidity and low oxygen atmosphere (i.e. stagnant and waterlogged soil) are needed for optimal infection (Nishijima et al*.,* 2004). The soil used during the time of the test was aerated (90% organic matter), the average air temperature was 33^{0}C and the plant was placed under shade. This environment condition was not optimal for infection.

The bacteria from the infected rhizome slices in the pathogenicity test was re-isolated and cultured in nutrient agar and

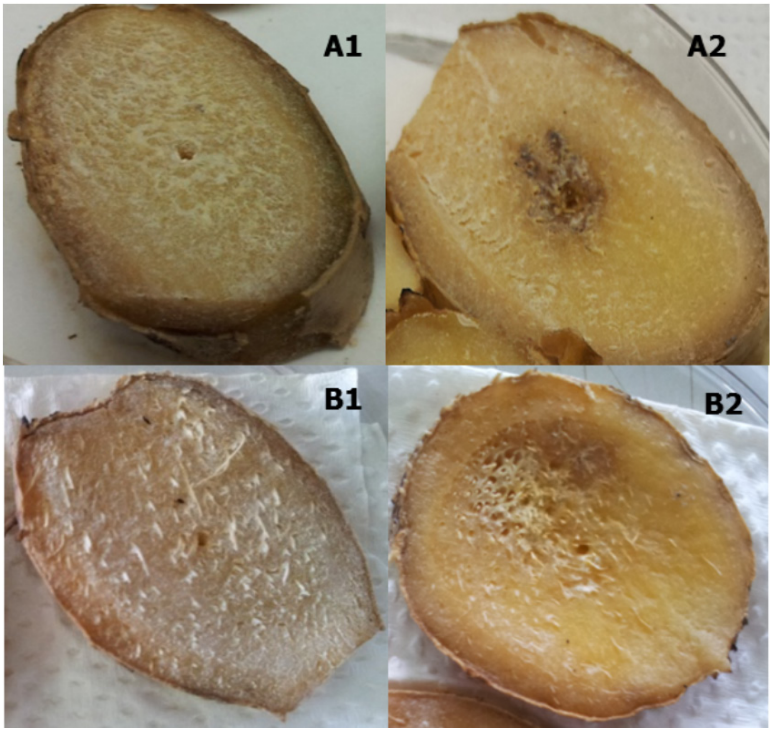

Figure 2. Symptoms produce by *Enterobacter cloacae* on rhizome slices. A: Rhizome slices inoculated with bacteria using toothpick (A1: Control inoculated with sterile distilled water, A2: Infected rhizome showing decaying symptoms). B: Rhizome slices inoculated with 10^{11} CFU/ml bacteria using pipette (B1: Control inoculated with sterile distilled water, B2: Infected rhizome with discoloration which is a mild rotting symptoms)

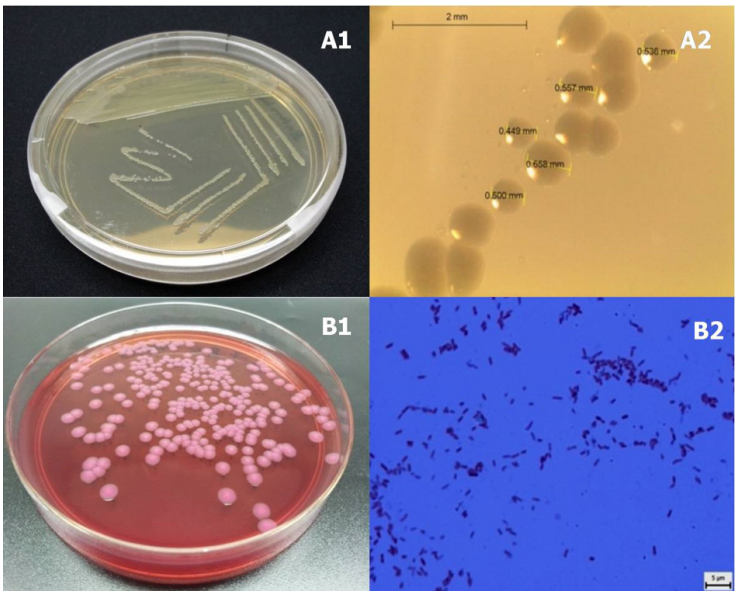

Figure 3. *Enterobacter cloacae* colony characteristics. A: Nutrient agar (A1: Colony form in nutrient agar; A2: Colony view under stereo microscope). B: MacConkey agar (B1: Colony form in MacConkey agar, B2: Microscope view showed Gram-negative and rod shape bacteria, 1.4 um at 100x magnification)

selective media agar for *E. cloacae* (i.e., MacConkey agar). The isolated bacteria were indeed *E. cloacae* because they were able to grow in the selective agar and confirmed by MALDI-TOF analysis. The *E. cloacae* colonies were cream-colored in nutrient agar, and pink in MacConkey agar, irregular in form with raised elevations and an entire margin size of 0.45 to 0.66 mm. The bacteria were Gram-negative and rod-shaped with a size of 1.0 to 2.0 µm (Figure 3).

CONCLUSION

The bacterial pathogen isolated from the ginger plants with yellowing and wilting symptoms had been identified up to species level as a species of *Enterobacter cloacae* complex instead of *Ralstonia solanacearum,* as expected. However, further studies on the interaction of *E. cloacae* with other isolated species are required to confirm the causes of ginger wilt disease in Sabah, Malaysia.

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