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Symptomatology and Range of the Blood Disease Bacterium A2 HR MARDI Strain (*Ralstonia syzygii* subsp. *celebensis*) on Selected Hosts

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Abstract: *Bacterial wilt disease is one of the major diseases in banana. In Malaysia, banana blood disease (BBD) is caused by the blood disease bacterium (BDB) A2 HR MARDI (Ralstonia syzygii subsp. celebensis). This disease bears similarities in symptomatology with Moko disease which caused by Ralstonia solanacearum and BBD in Indonesia, which caused by BDB R229. To determine the symptoms and host range of BDB, a pathogenicity test and host range study were carried out. In this study, there are four stages of external and internal symptoms which were observed. The pathogenicity of the bacterium cultures was then tested on banana, tomato and heliconia plantlets to determine the host range for BDB. To reconfirm that the banana was infected with BDB, re-isolation of BDB from the infected banana plants and Koch's postulates test were performed. The results showed that there were symptoms of wilting and yellowing of leaves, which eventually caused plants death in the banana plantlets but no symptoms appeared in tomato and heliconia. The results indicate that BDB A2 HR MARDI is host-specific pathogen, only infecting banana similar to BDB R229 and is not as a broad range pathogen as R. solanacearum.*

Keywords: *Banana, Blood Disease Bacterium, Host Range, Ralstonia syzygii subsp. celebensis, and Symptomatology*

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I. Introduction

Banana has numerous cultivars, which are planted and consumed globally. It is among the world's most important foods and commodities. In 2014, world production was 114 million metric tons, with a gross production value of US$40.6 billion [1]. Malaysia has a huge variety of banana species, at around 50 types, including commercial varieties, such as Berangan, Rastali, Nangka and Abu Nipah, which belong to three genome types from the genus Musa (AAA, AAB and ABB) [2]. In Malaysia, banana is listed as the sixth highest-value non-seasonal tropical fruit crop, alongside papaya, pineapple, rockmelon, starfruit and jackfruit, under the Entry Point Project of the National Key Economic Area for premium production [3]. Banana is also placed fourth as a vital fruit crop, as banana provides food for millions of people in Southeast Asia.

Although banana ranks as one of the major planted fruit crops in Malaysia, occupying 28,036 ha and with a total production of 309,508 metric tonnes per year [4], it has faced a serious disease problem, caused by a bacterial pathogen. In 2007, a critical epidemic of bacterial wilt occurred in Pontian, Johor, Malaysia due to a massive flood that hit the state in that year [5]. This outbreak of bacterial wilt disease caused a decline in production from more than 500,000 tonnes in 2000 to 280,000 tonnes in 2007. Banana blood disease (BBD) caused by the blood disease bacterium (BDB) A2 HR MARDI, was first detected in Perak in 2013, from where it spread to Selangor [6]. Banana bacterial wilt disease is usually related to Moko disease [7], caused by *Ralstonia solanacearum*. It then spread to other states in the country, which resulted in decreased banana production. A previous study [6] reported that Moko disease and BBD likely occurred separately, as they were detected separately in Peninsular Malaysia; however, these two diseases do produce identical typical symptoms [8].

BBD is a bacterial wilt disease that has affected banana plantations in Indonesia since 1921 [9,10] and is caused by BDB R229. BDB A2 HR MARDI and BDB R229 have significant biological differences [10]. BDB strains from Indonesia have been reported as a single-host pathogen, only infecting banana [9]. The infected plants are unable to produce edible fruits and, thus, the percentage losses due to the disease have a linear correlation with disease incidence [9]. The symptoms include wilting and yellowing of leaves, vascular

discolouration, bacterial ooze and reddish-brown fruit rot [10]. This study was undertaken in order to determine and reconfirm the symptomatology of infected banana plants and the host range of the pathogen BDB A2 HR MARDI.

II. Materials And Methods

2.1 Pure Culture

BDB (A2 HR MARDI) was isolate by MARDI researchers from an infected banana plant sample from Kuala Kangsar, Perak. A pure culture (seven days old) was obtained from the Crop Science and Pathology Laboratory, MARDI, Serdang, Selangor. Bacteria were grown on tetrazolium chloride (TZC) plates, and stored at 4°C prior to use.

2.2 Preparation of BDB Suspension

A single colony of BDB from the TZC plate [11] was picked, using a loop, and was grown on a casamino acid-peptone-glucose (CPG) medium overnight at 28°C and at 200 rpm in an incubator shaker, until an optical density of 1.0 at 600 nm wavelength (using spectrophotometer model 6405UV/VIS) was reached. The culture was adjusted to obtain ~108 cfu/ml bacteria.

2.3 Pathogenicity tests

A pathogenicity test of BDB was carried out under greenhouse conditions. Two-month-old banana plantlets (Berangan variety) were used. The banana plantlets were obtained from the MARDI station located in Klang, Selangor. A total of 20 replicates of banana plantlets were used. The banana plantlets were inoculated with a BDB suspension, and sterile, distilled water was used as a negative control treatment. A Pure culture of BDB was grown on CPG medium overnight at 28ºC, in an incubator shaker at 200 rpm, until an optical density of 1.0 at 600 nm wavelength (using spectrophotometer model 6405UV/VIS) was reached. A culture of ~108 cfu/ml was obtained after adjustment. Inoculation was carried out by injecting 5 ml of the bacterial suspension into the pseudostems of the banana plantlets using a syringe with a needle [12]. Wilting and changes in leaf colour in the inoculated plantlets were observed for 14 days. All the plantlets were harvested 14 days after inoculation (DAI).

2.4 BDB re-isolation from inoculated plantlets

Isolation of the bacteria was performed following the procedure described by [13], with some modifications. The infected pseudostems and roots of the banana plants were cut into 1 cm lengths and their surfaces were sterilised with 10% Clorox. These were left to dry in a laminar flow. The samples were then immersed in 10 ml sterilised distilled water before being macerated. Tenfold dilution series were performed on the macerated plant-water samples; the diluted samples were labelled 10-1,10-2,10-3,10-4, 10-5, 10-6, 10-7,10-8,10-9 and 10-10. Aliquots of 10 µl from each dilution were then spread on a semi-selective medium (Selecta-MEDIA, South Africa – SMSA) in order to obtain a single colony [14]. After a single colony of BDB was obtained, it was restreaked onto TZC medium and kept at 28°C before use.

2.5 Hosts Range Study

A host range study was conducted by inoculating two-month-old banana, tomato and heliconia plantlets with a pure culture of BDB. Inoculation was carried out based on the method described above. In this experiment, 20 plantlets of each type were used. Inoculated seedlings were kept in an infection house. The plants were observed daily for the development of wilt symptoms over 14 days.

2.6 Experimental Design and Data Analysis

A completely randomised design was used for this experiment, with 20 replicates of each host plant; a total of 80 plants were tested. A disease severity index was calculated using a formula described in [15], based on five disease scales. The means of various plant values were separated using one-way ANOVA (Duncan's multiple range test – DMRT) at $P \leq 0.05$, from SAS software (version 9.4), to determine differences between the plants.

2.7 Disease Severity

Disease severity was assessed using a scale from 0–5, where 0 corresponded to healthy or symptomless plants and 5 to severe wilting symptoms. Disease severity relates to the proportion of the plant area that is infected. The calculation of disease severity was to determine how severe the bacterial infection was to the components of the inoculated plants. It was also important to know the prevalence of, and damage caused by, the disease. The severity of infection in the individual inoculated plantlets was evaluated. Grading for disease severity was performed based on symptoms observed in the inoculated plantlets.

Table 1: Symptoms and scales of Banana Blood Disease

Disease symptom	Scale
I) No symptom (No symptoms appeared to the plantlets)	0
II) Wilted leaves (Wilting leaves of the plantlets)	1
III) Initial yellowing (Leaves started to yellowing)	2
IV) Two to three chlorotic leaves	3
V) Four or more chlorotic leaves	4
VI) Plant death	5

The following formula was adopted to calculate percentage of disease severity:

$$\textbf{Disease severity} = \frac{\text{Sum of all disease rating}}{\text{Total number of rating x Maximum disease grade}} \times 100\%$$

The data for disease severity were recorded and analysed according to the analysis of variance (ANOVA). Means at various treatments were separated using one way ANOVA (Duncan new multiple range test) at $P \leq 0.05$ from SAS software (version 9.4) to compute the difference between the infected host plants.

III. Results

3. Symptomatology of Banana Blood Disease

A pure culture of BDB A2 HR MARDI isolates was confirmed, either it host specific pathogen and symptomatology study results were based on the observation on disease symptoms in infected banana plantlets after inoculation. Symptoms in the inoculated plantlets were recorded at the time when they first appeared on the leaves, which was 6 DAI. Based on the symptoms observed, the BBD development could be categorised into four stages, leading up to plant death. The non-inoculated (control) seedlings remained healthy (Figure 3A[figures should be referred to and presented in numerical order -]). The initial external symptom (first stage) was slight yellowing and wilting of the lower leaves and loss of turgidity at 6 DAI (Figure 3B). The second stage began when the leaves showed intense yellowing and wilting, at 9 DAI (Figure 3C). The third stage was when the infected plant showed severe wilting symptoms, discolouration of the leaves and a change in colour of the pseudostem from green to brown, at 12 DAI (Figure 3D). The final stage was when the infected plants became severely wilted, the leaves turned from yellow to brownish, with vascular discolouration and rotting, leading to plant death (Figure 3E) at 14 DAI.

Figure 3.: Symptomatology of BBD on banana plantlets after inoculation with BDB showing (A) Healthy seedling (control) (B) first stage (C) second stage (D) third stage and (E) four stage of BBD infection.

In terms of internal symptoms, the first stage was indicated by the initial discolouration of vessel systems (xylem tissue) due to bacterial colonisation in the pseudostem (Figure 3.1A) and roots (Figure 3.1B), in which the tissues turned from whitish to brownish at 6 DAI. In the second stage, the pseudostem (Figure 3.1C) and root (Figure 3.1D) xylem vessels were intensely discoloured due to prominent colonisation by BDB at 9 DAI. In the third stage, both pseudostem and roots started to rot, eventually becoming slimy, at 12 DAI (Figures 3.1E, F). In the fourth stage, plantlets showed severe rot symptoms on the pseudostem (Figure 3.1G) and roots (Figure 3.1H), with the tissues turning black at 14 DAI.

Figure 3.1: The first stage of internal symptom of BBD at 6 DAI (A and B), second stage at 9 DAI (C and D), third stage at 12 DAI (E and F) and fourth stage at 14 DAI (G and H).

3.2 Re-Isolation of BDB from infected plants

Suspected bacterial colonies appeared on the SMSA plate at 3 days after incubation. The isolated BDB colony is shown in Figure 3.2A. After being subcultured onto the TZC medium, the BDB colony appeared as shown in Figure 3.2B. This BDB A2 HR MARDI strain showed colonies that were irregularly round in outline, creamy white, sticky and with red centres.

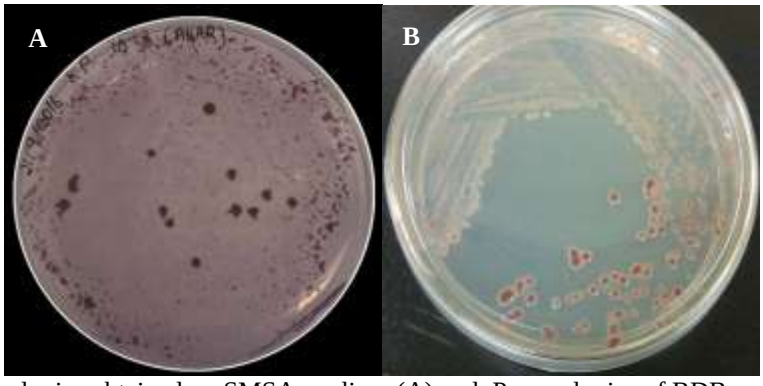

Figure 3.2: Bacteria colonies obtained on SMSA medium (A) and Pure colonies of BDB on TZC plate (B).

3.3 Hosts Range Study

The host range study showed that no disease symptoms appeared in the tomato (Figures 3.4A–D) or heliconia (Figures 3.4E–H) plantlets, but that severe symptoms were observed in banana plantlets at 14 DAI (Figure 3.3). Generally, the disease symptoms in banana were similar to those reported above. Figure 3.3A shows the control banana plantlets that were inoculated with sterile, distilled water; these did not showed any signs of wilt at 14 DAI. Figure 3.3B shows that the banana plantlets, 9 DAI, started to wilt and lose turgidity. Figure 3.3C shows that the banana plantlets at 12 DAI showed yellowing of leaves and increase intensity of wilting. Figure 3.3D shows banana plantlets at 14 DAI that had experienced changes in leaf colour, from yellow to brown, severe wilting, vascular discolouration and plant death. The disease severity in banana Berangan was 10%, 38%, 80% and 98%, respectively (Table 2). There were null results for disease severity in the tomato and heliconia due to a lack of symptoms produced after inoculation. This suggests that the BDB are only pathogenic to a single host – banana. The DMRT showed that there was a significant difference between banana and the other selected hosts. No significant differences were indicated between the heliconia and tomato plantlets.

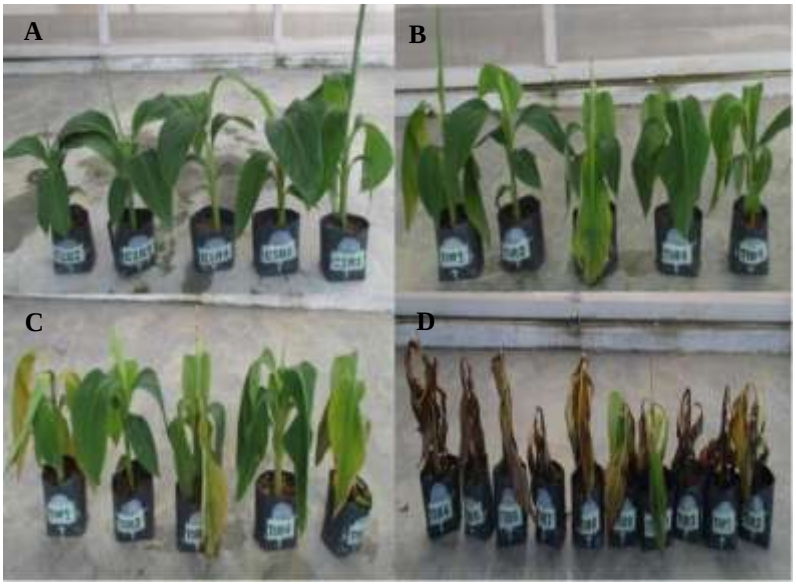

Figure 3.3: Banana plantlets variety (Berangan) served as control on 14 DAI (A). Infected banana plantlets on 9 DAI (B). Infected banana plantlets on 12 DAI (C) . Infected banana plantlets on 14 DAI (D).

Table 2.: Disease severity (%) of banana, tomato and heliconia after inoculated with BDB

Hosts plant	6 DAI (%)	9 DAI (%)	12DAI (%)	15 DAI (%)	Mean DSI (%)	DMRT
Banana	10	38	80	98	56.6	B
Tomato	0	0	0	0	0	A
Heliconia	0	0	0	0	0	A

Note: All data analyzed using Duncan Multiple Range Test (DMRT). The, means within the column with the same letters indicated no significance difference (P=0.05).

Figure 3.4: Tomato plantlets serve as control on 14 DAI (A). Tomato plantlets after inoculated with BDB on 9, 12, and 15 DAI respectively (B,C and D). Heliconia plantlets serve as control on 14 DAI (E). Heliconia plantlets after inoculated with BDB on 9, 12, and 14 DAI, respectively (B,C and D).

IV. Discussions

Bacterial wilt disease in banana is caused by a number of bacterial pathogens that are grouped into the *R. solanacearum* species complex. *R. solanacearum* is a soil-borne pathogen that has a broad host range and unusual genetic diversity [8]. *Ralstonia* spp.cause bacterial wilt in more than 200 species, including

commercially important crops such as potato, tomato and banana [16]. *R. solanacearum* is a complex species with a large, heterogeneous group of related strains. It has been subdivided into five races based on host range, and five biovars based on biochemical properties [16]. The *R. solanacearum* species complex includes *R. solanacearum, R. syzygii* and BDB [10].

BDB R229 is a bacterial strain that is believed to cause BBD in Indonesia, while BDB A2 HR MARDI has been isolated from an infected banana plantation in Kuala Kangsar, Malaysia. A host range study was performed to determine the range of BDB A2 HR MARDI isolates. Based on a previous study, *R. solanacearum* race II affects the triploid banana (Moko disease) and Heliconia, while BDB has been reported to be a single-host pathogen, limited to banana species [16]. This data is pertinent in distinguishing BDB from *R. solanacearum,* which causes Moko disease in Malaysia. Another report has shown that *R. solanacearum* can infect triploid banana, heliconia, tomato and other ornamental Musaceae plants [16], and can cause Moko disease. Moko disease-causing strains and BDB are believed to have independently evolved pathogenicity in wild Heliconia spp. in Central America and wild Musa spp. in Indonesia, respectively. The *R. solanacearum* species complex causes wilt disease by colonising plant xylem vessels, as with BDB, and the symptoms appear identical, although they could be differentiated by the host range study, as they have significant biological differences [10].

There is a significant difference between the whole genomes of these strains, these differences being in the chromosomes, megaplasmid lengths and guanine and cytosine content (GC%) of the bacteria. Results from a genome study on BDB A2 HR MARDI [17] revealed that it has longer sequences – 3,603,619 bp compared to 3,574,388 bp in BDB R229. In the megaplasmids, BDB R229 has longer sequences,(584,610 bp) than BDB A2 HR MARDI (1,486,041 bp[correct?]). The GC% is 66.4% and 66.5% for BDB A2 HR MARDI and BDB R229, respectively. This study aimed to determine whether there were differences in the symptomatology and host ranges of the BDB strains from Malaysia.

The symptomatology study had findings that were similar to those in [7], who reported that the midrib and petiole of the banana leaves wilted and turned yellow, and eventually the entire plant wilted and died. The symptoms of banana plants infected with BDB are wilting and the yellowing of leaves, vascular discolouration, bacterial ooze and reddish brown fruit rot [10].

The re-isolation of BDB from infected plants indicated that the banana plantlets were infected by BDB A2 HR MARDI, based on Koch's postulates test results. The morphology of BDB was observed. Bacterial colonies with irregularly round outlines, creamy white and with dark red centres were isolated from infected plants. This morphology is similar to that found in the study by [18]. Other than that, this bacterium is slow to grow, and is grouped with the Gram-negative bacteria [18].

Reported by [19] there is no resistance to BBD in over 100 banana varieties. The results of the present study are similar to those in [6], who also found that there were no symptoms in heliconia or tomato plants two weeks after inoculation. According to [9], BDB is a single-host pathogen, only infecting banana, unlike other Ralstonia spp. complex, which has a broad range of hosts.

V. Conclusion

This symptomatology study confirmed that the Banana Blood Disease caused BDB A2 HR MARDI strain isolated from banana plantation in Kuala Kangsar, Perak, Malaysia produce similar symptoms (external and internal) as BDB strain from Indonesia and Moko disease caused by *R. solanacearum.* While host range study identified that BDB A2 HR MARDI identified as single host pathogenic which only infected banana and not to tomato and heliconia plant.

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