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Jackfruit Bronzing Disease Surveillance Study in Selected Orchards of Peninsular Malaysia

H.Y. Gan[1], N. Govender[2], and M.Y. Wong[3*]

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ABSTRACT

Purpose: *IIn Malaysia, jackfruit (variety Tekam Yellow) significantly contributes to the nation's economy. The valuable fruit production is affected by jackfruit bronzing disease (JBD). Information on JBD occurrence in Peninsular Malaysia is necessary to develop effective disease control strategies. Hence, this study aimed to survey JBD occurrence and identify the corresponding causative pathogens in selected jackfruit orchards distributed in Peninsular Malaysia.*

Research Method: *A total of eight jackfruit orchards from five states in Peninsular Malaysia were selected for JBD sampling. Fruit samples were described for characteristic symptoms and disease incidence was expressed in percentage. The causative pathogens were identified using morphological observation and molecular methods while the pathogenicity test was done using Koch's Postulates.*

Findings: *JBD incidence in Peninsular Malaysia ranged between 20.13-70.25%. JBD-causal pathogen isolation showed that the bacterium was gram-negative, oxidase-negative, and non-capsulated straight rod shape with an average size of 0.35 x 1.31 µm (facultative anaerobic). A DNA sequence analysis on the 16S rRNA gene indicated that 15 putative isolates were unique to P. stewartii and one to P. dispersal at a 99-100% similarity index. Pathogenicity test of the P. stewartii isolates showed JBD characteristic symptoms suggesting that JBD occurrence caused by P. stewartii is common in Peninsular Malaysia and the pathogen could co-exist with P. dispersa.*

Research Limitations: *The number of orchards sampled in this study is low and therefore, the findings pose biases to a certain extent.*

Originality/Value: *JBD occurrence is common in small jackfruit orchards in Peninsular Malaysia. JBD is primarily caused by P. stewartii while both P. stewartii and P. dispersa could co-exist in JBD pathosystem.*

Keywords: *Jackfruit, Jackfruit bronzing disease, Jackfruit orchards in Peninsular Malaysia*

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lam.) is a flex crop extensively cultivated in the Asia-Pacific region for food, fuel, timber fodder and a range of medicinal and industrial products (APAARI, 2012; Wangchu *et al.*, 2012). The fruit grows well in tropical South and Southeast Asian countries such as Bangladesh, India, Myanmar, China, Indonesia, Malaysia, and Sri Lanka (Acedo *et al.*, 2000; Soepadmo *et al.*, 1999). The tree belongs to the Moraceae family and produces about 700 fruits annually with each weighing between 0.5-50 kg (Merrill, 1976). The fruit consists of starchy seeds enclosed in the sweet and aromatic edible pulp. With an average life span of 60-70 years, the jackfruit tree attains maturity after a long juvenile phase, spanning about 7-8 years.

[1]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Malaysia
[2]Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, Bangi, Malaysia
[3]Institute of Plantation Studies, Universiti Putra Malaysia, Serdang, Malaysia

*muiyun@upm.edu.my

https://orcid.org/0000-0002-6944-4860

The Indo-Malayan region serves as the center of origin for jackfruit (Harlan, 1987) and to date, Asia has been a leading jackfruit producer with India, Bangladesh, Vietnam and Myanmar, ranked among the top contributors.

In the past decade, worldwide jackfruit consumption increased tremendously. The meaty texture of the fruit is noticed and employed to produce plant-based mock-meat products such as curry, pasta, noodles, burger patties and nuggets (Zhou *et al.*, 2021). In Europe, Britain and the US, an increasing number of vegan eateries are catering to jackfruit-based meals. The high demand for jackfruit export is credited to consumers' preferences (Safi, 2019). Jackfruit and its close relative breadfruit are named superfoods owing to its nutritious rich source of vitamins (A, C, thiamine, riboflavin, and niacin) and minerals (calcium, potassium, iron, sodium, and zinc). The highly enriched dietary fiber acts as a colon-protecting roughage that binds to the colon mucous membrane and alleviates depositions of cancer-causing chemicals (Govender *et al.*, 2023; Konsue *et al.*, 2023). In India, jackfruit consumption has been recommended to diabetic patients as a substitute for rice, the staple food. One cup of jackfruit has 40% less carbohydrate content as compared to a similar amount of rice (Azad, 2000; Haq *et al.*, 2006; Swamy *et al.*, 2012).

In Malaysia, jackfruit peak production dates from June to December (Othman *et al.*, 1995). The Department of Agriculture (DOA), Malaysia has around 42 registered jackfruit varieties since its establishment in the 1960s and these commercial planting materials have a relatively short maturity period of 3-4 years. Jackfruit planting materials and their corresponding growth performance are area-specific and vary to geographical regions, soil and climatic conditions. For instance, in Johor (Southern Peninsular Malaysia) the Uncle Hong and Crystal varieties are widely cultivated (Tahir and Jamaluddin, 2008). The Tekam Yellow cultivar (J33 clone), also known as "nangka madu" (syn. honey jackfruit) is highly preferred by consumers for its crunchy and sweet taste. On a periodic evaluation, the planting acreage of Tekam Yellow has shown a significant increment of up to 17.08% between 2014-2018 (Mijin *et al.*, 2020). Tekam Yellow is mainly exported to Japan, New Zealand, Singapore, Taiwan, the USA, Canada, Vietnam, China, Hong Kong and Indonesia (DOA, 2016). In 2014, under the Malaysian Agriculture National Key Economic Areas (NKEA), jackfruit export was successfully expanded to Middle Eastern and European countries and such international ties continued to foster the growth of Malaysia's jackfruit industry and other Asian countries alike (ETP, 2014; Mijin *et al.*, 2020).

Jackfruit cultivation and subsequent yield performance are challenged by a range of diseases such as soft rot, dieback, leaf spot, rust and fruit bronzing (ITFN, 2012). Amongst them, the bronzing disease is particularly challenging as symptoms are only realized upon harvest. In Malaysia, the first symptom of jackfruit bronzing disease (JBD) was observed at Bukit Goh, Pahang whereby the fruit pulps, rags and seed membrane showed rusty specks or yellowish-to-orange bronzing (Horst, 2013). The disease affects various parts of the fruit such as pulp, skin and rags. Fruits affected by JBD are asymptomatic on the outer surface (exocarp) whilst the pulps confer an unpleasant taste (DOA, 2017). Early reports have indicated *Pantoea stewartii subsp. stewartii* bacterium as the causal agent of JBD (Zulperi *et al.*, 2017; Abidin *et al.*, 2020; Abidin *et al.*, 2021). The *Pantoea* spp. have been described as JBD causal agents in the Philippines (Gapasin *et al.*, 2014), Mexico (Hernandez-Moraleset *et al.*, 2017), Malaysia (Zulperi *et al.*, 2017; Ibrahim *et al.*, 2020) and China (Zhao *et al.*, 2023).

In Malaysia, the Tekam Yellow variety is described as JBD-susceptible whilst other commercial varieties such as the Mastura and Mantin are tolerant (DOA, 2018). Research on JBD remains very limited and the disease identification is often presented based on visual observations performed at the post-harvest stage (Punan *et al.*, 2000). This study aimed to survey the occurrence of JBD in jackfruit orchards in Peninsular Malaysia and identify the JBD causative pathogens using morphological observation, and molecular methods while the pathogenicity test was done using Koch's Postulates test.

MATERIALS AND METHODS

Jackfruit Bronzing Disease (JBD) Assessment in Small Orchards

The jackfruit bronzing disease (JBD) incidence was recorded at small orchards located in five different states in Peninsular Malaysia: Perak, Negeri Sembilan, Pahang, Johor, and Selangor. A total of 8 orchards were evaluated and about 10-12 trees (5-year-old) from the Tekam Yellow variety were selected at each orchard for fruit sampling. Three mature fruits were harvested from each tree and the cut-open fruits were screened for the following JBD symptoms: reddish-to-brownish discoloration at the rags and fruit surface. Each tree with at least 2 fruits showing characteristic symptoms of JBD was scored as an infected plant. Disease incidence (DI) was calculated by using the following

equation (Cooke, 2006): Disease incidence (DI%) = Number of infected plants/Total number of plants * 100. The JBD assessment was conducted thrice in the same jackfruit orchards from January 2014 to August 2016 (JBD assessment 1; January-February 2014, JBD assessment 2; March-April 2015 and JBD assessment 3; July-August 2016). The DI values are presented as the average of three independent evaluations of JBD.

Isolation of JBD Causal Pathogen

Mature fruits randomly picked from the jackfruit plants were harvested and cut-open fruits were screened for JBD characteristic symptoms. Briefly, fruits were horizontally sectioned into halves, and each half was further cut into eight pieces. In the presence of the JBD symptoms, the disease tissues (rag, fruit flesh and seed membrane) were collected and cut into small pieces (1x1 cm). Disease tissues were surface sterilized (immersed in 70% ethanol for 5 min followed by sterile distilled water for 1 min) before performing the JBD causal pathogen isolation (Sanders, 2002; Cooke et al., 2006). The sterilized disease tissue was excised to weigh about 5 mg. The sample was placed in a stomacher storage bag containing 25 mL of RINGER Solution. The samples were homogenized by a laboratory blender for 60 sec and then, left standing at room temperature (24±2°C) for 30 min. About 100 µL of the suspension was inoculated on a nutrient agar plate and incubated at 37°C for 24 h. Plates with visible bacterial-like colonies at 4-7 days post-inoculation were subjected to morphological characterizations.

Morphological Characterization of JBD Causative Pathogen

Plates with visible bacterial colonies were carefully screened and grouped. Bacterial colonies from each plate were subjected to a 3% potassium hydroxide (KOH) test to differentiate the gram-negative from the gram-positive colonies. For the identification of oxidase-negative bacterial colonies, an oxidase test was conducted using a Microbiology Bactident Oxidase strip on the gram-negative colonies only (Wilson, 1997; Bochner *et al.*, 2009). The oxidase-negative colonies were observed under a stereomicroscope (Olympus SZ61-CCD, Japan) coupled to an Olympus DP25 Camera. The form, elevation and margin of the bacterial colonies were recorded by the Cell^{B} software (Olympus, Japan).

Microscopy Observation of JBD Causative Pathogen

Bacterial isolates from the most occurring species were selected for morphological analysis using a scanning electron microscope (SEM) JEOL JSM 6400 attached to EDX (JEOL-USA, Inc., USA). Briefly, the bacterial colonies cultured on a nutrient agar plate were cut into approximately 1 cm^3 cubes and fixed in 2.5% glutaraldehyde for 6 h at 4°C. The samples were washed with 0.1 M sodium cacodylate buffer for 3 subsequent changes at each 10-minute interval. Samples were post-fixed in 1% osmium tetroxide for 2 h at 4°C and washed again with 0.1 M sodium cacodylate buffer as described previously. The sample was dehydrated using a series of acetone solutions with variable concentrations (35, 50, 75, 95 and 100%), dried under a critical point dryer (CPD 030, BAL-TEC AG, Blazers, Liechtenstein) and fixed on a gold-coated sputter (SCD 005 BAL-TEC AG, Liechtenstein) before observation at 5 000X magnification, set at 15 kV.

Molecular Analyses of JBD Causative Pathogen: DNA Isolation, PCR and Sequencing

The pure cultures of JBD putative bacterial isolates were subjected to DNA extraction using the SDS method (Kim *et al.*, 2015). Briefly, 500 µL of a cocktail mixture described as following was added into the bacterial pellet: 467 µL TE Buffer, 30 µL SDS and 3 µL Proteinase K (20 mg/µL). The suspension was incubated at 60°C for 2 h in a rotary incubator. Following incubation, an equal volume of phenol-chloroform was added to the sample, mixed, and then centrifuged. The clear upper layer was transferred into a new tube and an equal volume of phenol-chloroform was added. Centrifugation under similar conditions was repeated. The supernatant (referred to as parts) was transferred into a new tube and 1:10 parts of 3M sodium acetate and 1:2 parts of isopropanol were added, mixed gently and centrifuged. The air-dried pellet was re-suspended in 100 µL TE buffer (Roper *et al.*, 2011). The quality and quantity of the isolated DNA were determined by Nanodrop. Good quality DNA (abs 260/abs 280=1.7-2.0) was used as the template sequence for PCR amplification of the 16S ribosomal RNA sequence (Kini *et al.*, 2018; Nechwatal *et al.*, 2018). Primers were designed according to Turner *et al.* (1999).

A PCR cocktail with a final volume of 25 µL was prepared as following:1x PCR buffer (Promega, USA), 1.5 mM of MgCl2, 0.2 mM of dNTP, 0.2 µM of primer B27F, 0.2 µM of primer U1492R, 0.02 U/µL of Taq DNA Polymerase and 50 ng DNA template. The amplification was carried out according to Lane, 1991 using the BIORAD T100 (BIO-RAD USA) PCR system with initial duration at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, extension at

72°C for 1 min and final extension at 72°C for 10 min. The resultant PCR products were sequenced by MyTACG Sdn. Bhd. Sequencing service provider, Malaysia. The DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) and pairwise sequence alignment (LALIGN), open-source tools. Evolutionary analysis was conducted using the MEGA7 software and the distances were computed by the Maximum Composite Likelihood method.

JBD Causal Pathogen Confirmation by Koch's Postulates

A total of 20 ripe jackfruits from the "Tekam Yellow" variety that weigh about 3-5 kg each were obtained. Fifteen fruits were used as the host for artificial inoculation whilst the remains were treated as negative controls (non-inoculated). Three different tissues were collected separately from the host for inoculation with putative causal agents of JBD. Three different isolates cultured and purified from this study were selected to carry out Koch's postulates. For inoculum preparation, the pure cultures maintained in Luria-Bertani broth were regenerated as follows: a loopful of primary culture was inoculated onto nutrient agar broth (100 mL) and was incubated on a shaker (300 rpm) overnight at 37 °C. The concentration of inoculum was adjusted to an optical density (OD) of 600 nm=0.5-0.6 (concentration of 108 colony forming units/mL) using a spectrophotometer (CECIL 2041, Aurius, UK) and a hemocytometer. Host fruit tissues were injected with 10 mL of bacterial inoculum suspension and sterile distilled water (negative control) using a 20 mL sterile syringe. All samples (inoculated and non-inoculated) were incubated at 28°C, with a relative humidity of 80% for 6 days. Symptom development was observed and recorded on days 1-to-7 post-inoculation. Samples with bronzing symptoms were re-isolated on nutrient agar and pathogen identity was confirmed by molecular analyses as described previously.

Jackfruit Bronzing Disease (JBD) Occurrence in Selected Orchards of Peninsular Malaysia

Jackfruit farms in Malaysia span about 9000 hectares and fruit production in recent years (2015-2020) has shown a gradual increment. The highest number of orchards are found distributed in the east and southern regions of Peninsular Malaysia, which includes Pahang, Negeri Sembilan and Johor (www.met.gov.my). The average temperature of Peninsular Malaysia is 21-33°C and relative humidity (RH)=80-88%. This region experiences plenty of rainfall throughout the year with an annual rainfall of 1800-2540 mm, even during the dry season. The dry season has an average temperature of >32°C low rainfall (below 100 mm per month) and RH=> 80% (www.mydata.gov.my).

Jackfruit is a non-seasonal fruit crop widely cultivated in Peninsular Malaysia at small (orchard) scales (ETP, 2014). Generally, the average size of an orchard is about 4-12 hectares. In early 2008, the occurrence of jackfruit bronzing disease (JBD) was first reported by DOA, Malaysia. Over the years, JBD in Malaysia has become more prevalent and continues to affect the fruit quality and taste (DOA, 2017). JBD is completely asymptomatic throughout the disease development, and it could only be confirmed at the post-harvest stage with the occurrence of orange-to-rust color specks observed on virtually the inner parts of the fruit, leaving farmers completely absurd with the unforeseen yield losses (EPPO Global Database, 2023). As a result, the local farmers practice fungicide and pesticide applications for JBD prevention, although no proven evidence has demonstrated the efficacy of chemical pesticides for JBD management (EPPO Global Database, 2016).

Jackfruit orchards located in five different states in Peninsular Malaysia showed JBD incidence at 20-70% throughout three independent evaluations conducted from January 2014 to August 2016. The number of trees sampled (10-15 trees) at each evaluation varied as trees bearing mature fruits (at least 3 fruits) showed an inconsistent number of ripe fruits at each independent evaluation. Cut opened infected fruits showed JBD characteristic symptoms (rusty specks and streaks) in the pulp, bulb, rind, seed membrane, rag, and core region (Fig. 01). Disease incidence scored for each orchard showed that JBD incidence was highest at Negeri Sembilan (DI= 70.25%), followed by Pahang (DI=61.42%), Perak (DI=40.71%), Selangor (36.78%) and Johor (20.13%) (Table 01).

In this pilot investigation, JBD showed a non-uniform occurrence (20-70%) in Peninsular Malaysia. In Negri Sembilan and Pahang, regions with short, but fairly regular dry seasons, the JBD incidence was highest at 61-70%. Contrarily, in regions without a regular dry season (Selangor, Johor, Perak) JBD incidence ranged from 20-40%. Isolation of 120 colonies showing characteristic visible dark yellow pigments from JBD-infected fruit tissues suggests that the disease is caused by a bacterium. A total of 16 bacterial isolates were confirmed gram-negative by the positive gram staining test. The results corroborated with the Pantoea spp. description (Deletoile *et al.* , 2009; Ibrahim *et al.* , 2019).

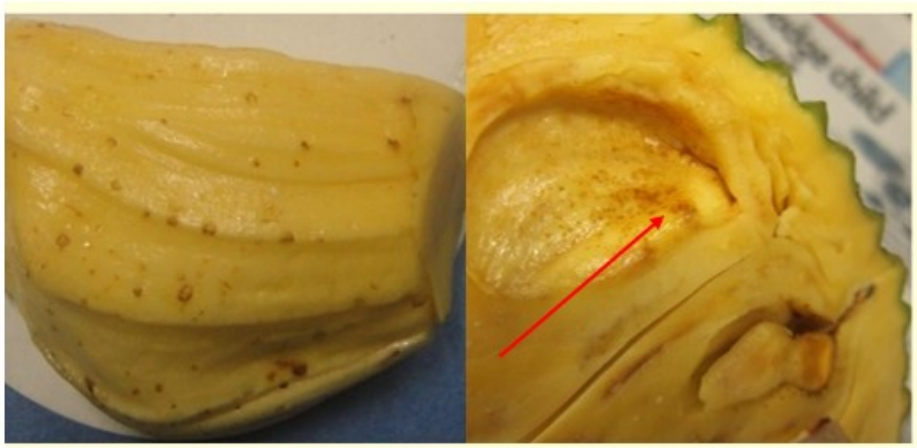

Figure 1: Characteristic symptoms of the jackfruit bronzing disease (JBD).JBD-infected fruits showing rusty specks (A) and bronze streaks (B) are indicated by red arrows.

Table 1: Jackfruit bronzing disease (JBD) incidence among Tekam Yellow jackfruit variety small orchards distributed in Peninsular Malaysia.

Location	Agro-ecological Features	Disease Incidence (%)*
Temerloh, Pahang	Short and fairly regular dry season	61.42 ± 8.24
Rawang, Selangor	Irregular dry season	36.78 ± 7.33
Jelebu, Negri Sembilan	Short and fairly regular dry season	70.25 ± 3.21
Kluang, Johor	Irregular dry season	20.13 ± 10.30
Bidor, Perak	Irregular dry season	40.71 ± 7.75

*The numerical value in each column represents the average mean of three independent evaluations $\pm$ standard deviation.

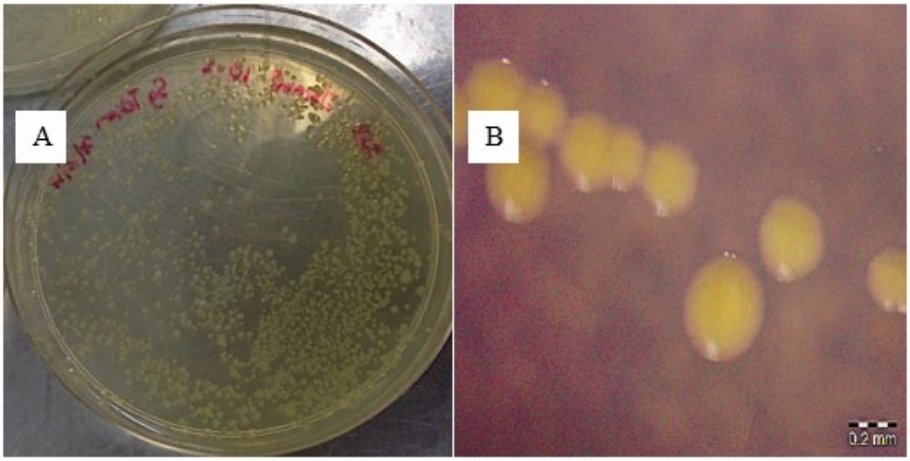

Figure 2: Bacterial pathogen isolated from jackfruit bronzing disease (JBD) infected fruit tissues. Axenic culture at day 7 post-inoculation (A). Yellow color single colonies with an average diameter of 0.2 mm (B).

Characterization of JBD Causative Pathogen

Isolation of bacterial pathogens from JBD-infected fruit tissues showed the formation of dark yellow colonies on the nutrient plate. Generally, the dark yellow bacterial colonies were uniform and circular. The bacterial colonies emerged at 8 hours post-inoculation and the diameter ranged between 0.15-0.2 mm (Fig. 02). Negative control samples from healthy fruit tissues did not show any visible colonies. A total of 38 yellow-pigmented bacterial isolates were subjected to gram staining and oxidase-negative tests. Gram staining with 3% potassium hydroxide (KOH) dissolved the cell wall of gram-negative bacteria to form a visible viscous mixture. Isolates that were negative oxidase remained colorless upon treatment with 1% tetramethyl-p-phenylenediamine dihydrochloride while the positive control sample (Pseudomonas aeruginosa UPMP3) changed into purple under similar conditions. A total of 16 gram-negative and oxidase-negative isolates

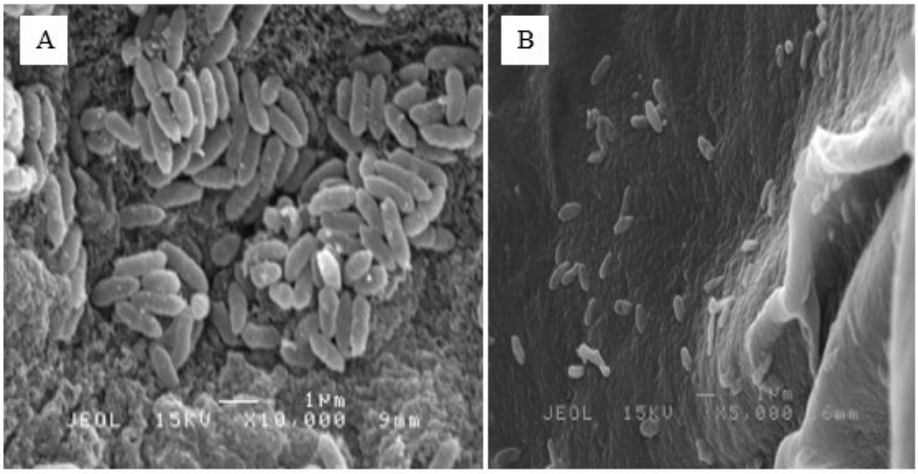

Figure 3: Scanning electron micrographs of putative jackfruit bronzing disease causal bacterium. Isolate A at 10,000X magnification (A) and isolate B at 5000X magnification (B). All bacterial cells were inoculated on nutrient agar and incubated for 24 hours before a 100X dilution. The bar indicates 1 µm.

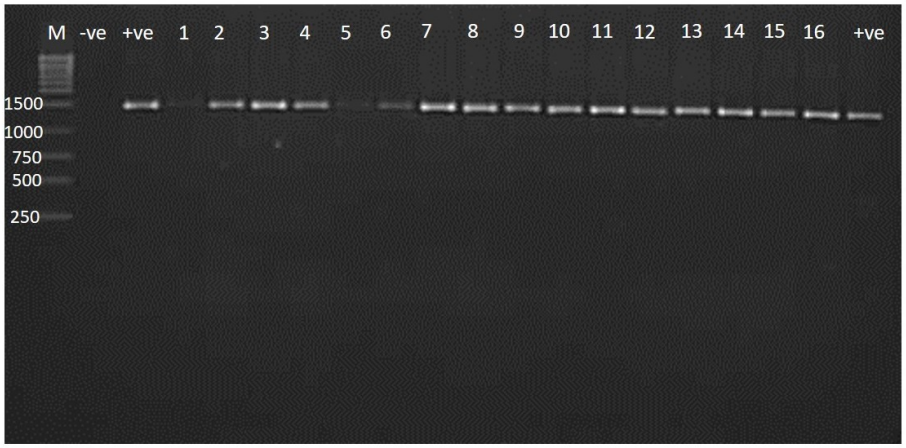

Figure 4: PCR amplicons of the 16S rRNA gene (15000 bp) viewed on 1% (w/v) agarose gel. Lane M: GeneRuler 1.5kb DNA ladder; Lane -ve: negative control; Lane +ve: positive control; Lane 1: N01M225; Lane 2: N02F292; Lane 3: P01F304; Lane 4: P01R305; Lane 5: P01S303; Lane 6: P02R264; Lane 7: P02F308; Lane 8: P06F290; Lane 9: P06F291; Lane 10: P08F328; Lane 11: S01F314; Lane 12: S02C325; Lane 13: S02F321; Lane 14: S03F319; Lane 15: S03F326; Lane 16: S03R323. The numbered lanes represent a unique isolate.

were selected for microscopy analysis. All isolates showed the presence of non-spore-forming and straight rod-shaped non-encapsulated cells. The average size (length by width) of the isolates was 1.05x0.35 µm (Fig. 03). A total of 16 pure isolates validated through morphological and biochemical analyses were selected for the molecular identification of the Jackfruit Bronzing Disease (JBD) causal pathogen. The PCR amplification of the *16S rRNA* fragment in all isolates yielded a 1500 bp amplicon. Gel electrophoresis indicated the presence of the target amplicon at various band intensities (Fig. 04). The consensus sequences of the isolates were unique to *Pantoea stewartii* and *P. dispersa* at a 99–100% similarity index.

Based on the sequence similarity search, a total of 15 isolates were identified as *P. stewartii*, and one isolate was identified as *P. dispersa*. The evolutionary analysis formed a phylogenetic tree with two closely related clusters (a and b) and an outlier. All 16 isolates (16S rRNA gene sequence) comprised of *P. stewartii* and *P. dispersa* collectively formed a cluster. The *P. ananatis* was found in cluster b. The outlier species was represented by *P. agglomerans* (Fig. 05). Phylogenetic analysis represented the 16 isolates into two clusters: i) Major-consist of two groups [an (i) *P. stewartii*] and group [an (ii) *P. dispersal* and *P. ananatis*], and ii) minor-consist of outlier spp. The result of the NJ tree corresponded with BLAST results obtained at a maximum identity between 99-100%. The genus *Pantoea* is highly diverse and often varies at the species level (Coplin *et al.*, 2002; Ibrahim *et al.*, 2019). Although two distinct *Pantoea* spp. were isolated and characterized from JBD-infected fruits, the results of the pathogenicity test were contradictory.

The pathogenicity test for JBD was positive for 15

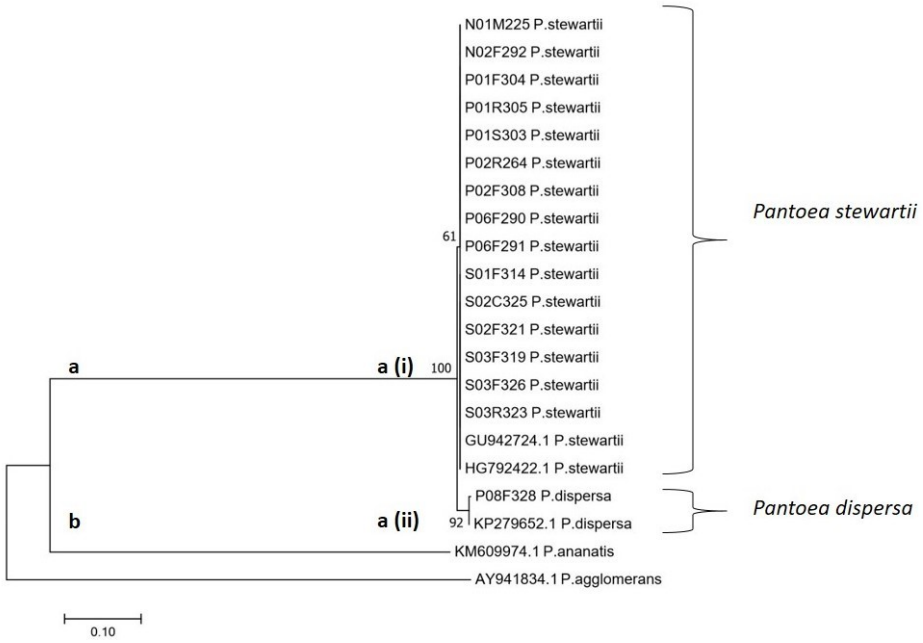

Figure 5: Phylogenetic tree derived from Neighbor-joining method showing the genetic distances of 21 bacterium isolates comprised of *P. stewartii, P. dispersa, P. ananatis* and *P. agglomerans*, the putative causal agents of jackfruit bronzing disease.

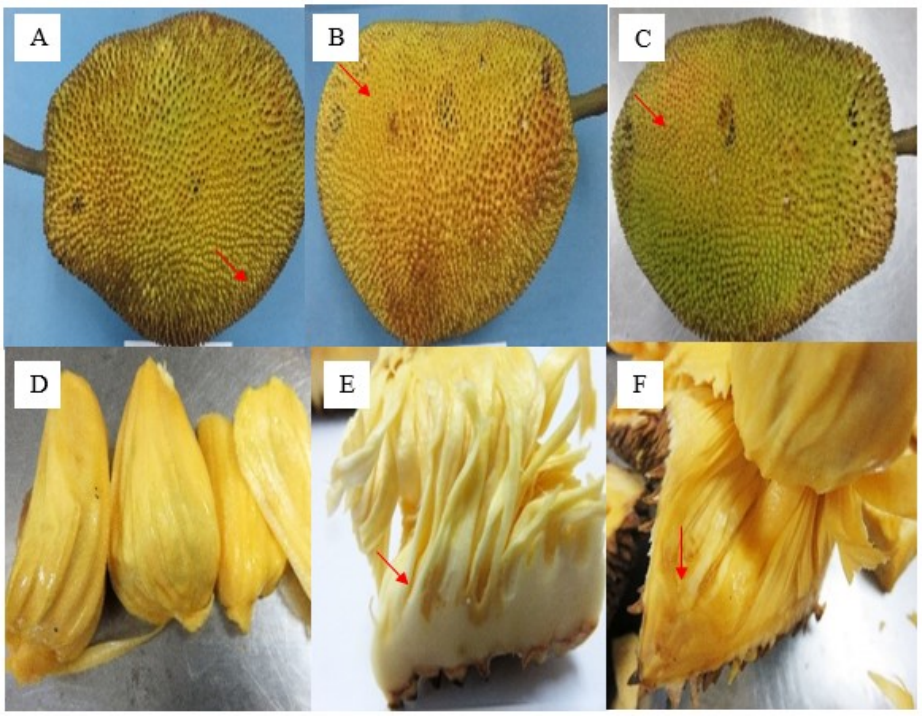

Figure 6: Jackfruit inoculated with *P. dispersa* and *P. stewartia* isolates at day 6 post-inoculation.(Top) Jackfruits inoculated with *P. dispersa* P05F328, *P. stewartii* isolate UPM 001 and *P. stewartii* isolate UPM 002 (from A to C). Red arrows indicate the point of inoculation. (Bottom) Open-cut jackfruits inoculated with *P. dispersa* P05F328 (no symptoms), inoculated with *P. stewartii* isolate UPM 001 and inoculated with *P. stewartii* isolate UPM 002 (from D to F). Red arrows indicate discoloration of rigs and membranes.

P. stewartii isolates and negative for *P. dispersa*. The jackfruit infected with *P. dispersa* did not show any visible disease symptoms throughout an incubation period of 2 weeks. The jackfruit artificially infected with the *P. stewartii* isolates showed JBD characteristic symptoms with variable severity at day 6-12 post-inoculation (Fig. 06). The *P. stewartii* and *P. dispersa* isolate yielded a PCR 16S rRNA gene amplicon (1500 bp) and a subsequent sequencing of the PCR amplicon revealed that each sequence was unique to *P. stewartii* and *P. dispersa* at 99% similarity index. Artificial infection with *P. stewartii* inoculum

on fruits showed characteristic symptoms of JBD whilst a similar experiment with *P. dispersa* inoculum showed no visible symptoms of JBD. Interestingly, both species could co-exist in the JBD pathosystem and JBD development was attributed to P. stewartii only.

CONCLUSION

Our findings suggest that jackfruit orchards in Peninsular Malaysia are prone to JBD at a disease occurrence of 20.13-70.25%. The jackfruit orchards distributed within the southern region are much more susceptible to JBD as compared to the northern region. For the first time, this study showed the co-existence of *P. stewartii* and *P. dispersa* as the causative pathogens of JBD. In light of our findings, robust studies on JBD etiology and large-scale field assessments are required to monitor JBD patterns and occurrence, ultimately to develop successful management strategies in the future.

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Conflicts of Interest

The authors declare no conflict of interest.

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