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CHARACTERIZATION AND PATHOGENICITY OF Pantoea stewartii SUBSPECIES stewartii CAUSING BRONZING DISEASE OF JACKFRUIT (Artocarpus heterophyllus Lam.) IN PENINSULAR MALAYSIA

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Jackfruit (*Artocarpus heterophyllus* Lam.) is one of the most popular fruit. In Malaysia, it is important to keep the fruit healthy as it provides revenue to the local and export markets and enhancing economic contribution towards the gross domestic product (GDP) in Malaysia. However, a disease known as jackfruit-bronzing is frequently found in the jackfruit crop. The reddish discolouration, and rusty specks or bronzing with yellowish-orange discolouration symptoms affected pulps and rags of the fruit. Jackfruit-bronzing is suspected to be caused by *Pantoea stewartii* subspecies *stewartii* (*P. stewartii* subsp. *stewartii*). The objectives of the study were (i) To identify the bacterial strains associated with bronzing disease of jackfruit in Peninsular Malaysia using phenotypic and molecular characteristics; (ii) To determine the pathogenicity of *P. stewartii* subsp. *stewartii* on jackfruits and host range of *P. stewartii* subsp. *stewartii* on sweetcorn, cucumber and pineapple and (iii) To assess and determine the genetic diversity of *P. stewartii* subsp. *stewartii* strains associated with bronzing disease of jackfruit using multilocus sequence analysis (MLSA). Twenty-eight pure bacterial isolates obtained from samples with typical jackfruit-bronzing symptoms from four different collection area in Peninsular Malaysia, comprises of Jenderam from Selangor state, Maran and Muadzam Shah from Pahang state and Ipoh from Perak state) were studied. Phenotypic verification, molecular verification, pathogenicity test on different jackfruit varieties, host range test on sweetcorn, cucumber and pineapple as well as the genetic diversity were performed in this study. Phenotypic characterization revealed positive *P. stewartii* subsp. *stewartii* verification based on the eleven phenotypic tests and hypersensitivity test. Molecular characterization performed via the 16S-23S Internally Transcribed Spacer (ITS) yielded the expected amplicons (0.92-kb fragment). Phylogenetic analyses confirmed that all of the 28 bacterial strains of

P. stewartii subsp*. stewartii* were clustered to reference strains of Stewart's wilt causing disease in the USA (DC283) and jackfruit-bronzing strains studied earlier in Malaysia (S3 and W1). Pathogenicity tests on jackfruit varieties all produced symptoms thus fulfilling Koch's postulate, except for jackfruit variety J39 (no symptom was observed). Furthermore, the host range testing on sweetcorn (nine-week-old seedlings), cucumber and pineapple also produced symptoms. A significant difference ($p < 0.05$) in the disease severity was exhibited by jackfruit varieties J33, J34 and J39. Statistical analyses based on pathogenicity tests on jackfruits and host range test on sweetcorn, pineapple and cucumber (period of 14 days) on its collection area showed consistency with significant difference ($p < 0.05$) for the strains collected, where Jenderam showed the highest disease severity (%) among all the strains, except in the host range test on cucumber. There was also consistency with significant difference ($p < 0.05$) for the strains collected from Jenderam and Maran with the strains collected from Muadzam Shah and Ipoh (period of 14 days). The total disease rating on the jackfruit varieties, sweetcorn, cucumber and pineapple on day 14 revealed that the JEN-14 strain demonstrated the highest severity or aggressiveness. Multilocus sequence analysis (MLSA) was performed using four housekeeping genes (*gyr*B, *rpo*B, *atp*D and *inf*B) on the 28 bacterial strains*.* Single *gyr*B, *rpo*B, *atp*D and *inf*B and concatenated genes phylogenetic trees of this study showed the bootstrap value of 99-100% between the 28 bacterial strains with *P. stewartii* subsp. *stewartii* reference strains and *P. stewartii* subsp. *indologenes* reference strains. Moreover, the phylogenetic analysis based on the concatenated genes were able to distinguish that the strains were more closely related *to P. stewartii* subsp. *stewartii* (99 similarities) from its close relative *P. stewartii* subsp. *indologenes*, although sequence similarity between the subspecies was high (up to 100%). All the strains collected from the four collection areas clustered together, showing no variation between the collection area of the bacterial strains. These findings point no correlation between the disease severity and MLSA's genetic cluster of the 28 bacterial strains. This study would be a major platform on generating details documentation on bronzing disease of jackfruit and its causal pathogen, *P. stewartii subsp. stewartii* in Malaysia, since jackfruit has been identified as one of the most important commercial fruit crops with high economic value in this country.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PENCIRIAN DAN KEPATOGENAN KE ATAS *Pantoea stewartii* SUBSPESIS *stewartii* PENYEBAB PENYAKIT KARAT NANGKA (*Artocarpus heterophyllus* Lam.) DI SEMENANJUNG MALAYSIA

Oleh

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Nangka (*Artocarpus heterophyllus* Lam.) adalah salah satu buah yang popular. Memastikan kesihatan buah-buahan di Malaysia adalah sangat penting kerana ia memberi hasil kepada pasaran tempatan dan eksport serta meningkatkan sumbangan ekonomi ke arah keluaran dalam negara kasar (KDNK) di Malaysia. Walaubagaimanapun, penyakit yang dikenali sebagai karat nangka sering dijumpai dalam tanaman nangka. Gejala karat dan perubahan warna kekuningan-jingga dan kemerahan telah menjejaskan pulpa buah. Serangan buah nangka disyaki disebabkan oleh bakteria *Pantoea stewartii* subspesies *stewartii* (*P. stewartii* subsp. *stewartii*). Objektif kajian ini adalah (i) Untuk mengenal pasti strain bakteria yang berkaitan dengan penyakit karat nangka di Semenanjung Malaysia menggunakan ciri fenotipik dan molekul; (ii) Untuk menentukan kepatogenan *P. stewartii* subsp. *stewartii* pada nangka dan ujian julat perumah *P. stewartii* subsp. *stewartii* pada jagung manis, timun dan nenas dan (iii) Untuk mengakses dan menentukan patogenik diversiti *P. stewartii* subsp. *stewartii* strain yang berkaitan dengan penyakit karat nangka menggunakan analisis urutan *multilokus* (MLSA). Sebanyak 28 isolat bakteria telah diperolehi dari sampel yang mempunyai simptom, diambil dari ladang-ladang terjejas dari empat kawasan di Semenanjung Malaysia (Jenderam dari negeri Selangor, Maran dan Muadzam Shah dari negeri Pahang dan Ipoh dari negeri Perak). Kajian ini dijalankan dengan pengesahan fenotip, pengesahan molekul, ujian patogenik pada pelbagai varieti nangka, ujian julat perumah pada jagung manis, timun dan nenas dan juga kepelbagaian genetik. Berdasarkan sebelas ujian fenotip dan ujian hipersensitiviti, ia menunjukkan positif pengesahan kepada *P. stewartii* subsp*. stewarti*. Pencirian molekul yang dilakukan melalui 16S-23S Penjarak Transkripsi Dalaman (*ITS*) menghasilkan amplikon (0.92-kb cebisan). Analisis filogenetik mengesahkan 28 *P. stewartii* subsp. *stewartii* strain telah dikelompokkan bersama strain rujukan yang menyebabkan penyakit layu Stewart di Amerika Syarikat (DC283) dan juga

strain yang menyebabkan karat nangka, yang diteliti lebih awal di Malaysia (S3 dan W1). Ujian kepatogenan pada pelabagai jenis nangka menghasilkan simptom sehingga memenuhi Postulat *Koch*, kecuali untuk varieti nangka J39 (tiada symptom). Manakala, ujian julat perumah pada jagung manis (berumur sembilan minggu), timun dan nenas menghasilkan simptom. Keseriusan penyakit di antara nangka J33, J34 dan J39 menunjukkan perbezaan yang signifikan ($p < 0.05$). Analisis statistik dari ujian patogenik pada nangka dan ujian julat perumah pada jagung manis, timun dan nenas (tempoh 14 hari) berdasarkan tempat diperoleh menunjukkan konsistensi dengan perbezaan yang signifikan ($p < 0.05$) untuk strain, di mana strain yang dikumpulkan dari Jenderam mempunyai keparahan penyakit yang sangat tinggi (%) untuk semua inokulasi kecuali pada timun. Strain yang dikumpulkan dari Jenderam dan Maran juga menunjukkan konsistensi dengan perbezaan yang signifikan ($p < 0.05$) dengan strain yg dikumpulkan dari Muadzam Shah dan Ipoh (tempoh 14 hari). Pengiraan keseriusan penyakit pada jenis nangka, jagung manis, timun dan nenas pada hari ke-14 mendedahkan strain JEN-14 yang paling teruk dan mempunyai keparahan tertinggi. Analisis rangkaian *multilocus* (MLSA) dilakukan dengan menggunakan empat *housekeeping* gen (*gyr*B, *rpo*B, *atp*D dan *inf*B) pada kesemua 28 strain bakteria. Kajian filogenetik berdasarkan setiap satu dan penggabungan *gyr*B, *rpo*B, *atp*D dan *inf*B gen dalam kajian ini menunjukkan nilai *bootstrap* 99-100% diantara kesemua 28 strain dengan strain rujukan *P. stewartii* subsp. *stewartii* dan *P. stewartii* subsp. *indologenes*. Selain itu, analisis filogenetik berdasarkan penggabungan gen dapat membezakan bahawa kesemua 28 strain lebih dekat dengan *P. stewartii* subsp. *stewartii* (99 persamaan) daripada *P. stewartii* subsp. *indologenes*, walaupun persamaan antara subspesies adalah tinggi (sehingga 100%). Semua strain yang dikumpulkan dari empat kawasan berkelompok di dalam satu kluster, menunjukkan tiada variasi di antara kawasan pengumpulan strain bakteria. Oleh kerana nangka telah dikenal pasti sebagai salah satu tanaman buah komersial yang paling penting dengan nilai ekonomi yang tinggi di negara ini, kajian ini akan menjadi platform utama untuk menghasilkan dokumentasi terperinci mengenai penyakit karat nangka dan patogen penyebabnya, *P. stewartii* subsp. *stewartii* di Malaysia.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 8 October 2020

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%	percent
°C	degree Celsius
β	beta
bp	base pair
DNA	deoxyribonucleic acid
g	gram
h	hour
kb	kilobase pair
kg	kilogram
L	litre
mL	millilitre
M	molar
min	minutes
ml	millilitre
M	molar
min	minutes
ml	millilitre
mt	metric tonne
nm	nanometre
CFU	colony-forming unit
OD	optical density
PCR	polymerase chain reaction
rpm	rotation per minute
s	seconds
TBE	*Tris-Borate-EDTA*
Taq	*Thermus aquaticus*
TM	melting temperature
U	Unit
V	voltan / volt
v/v	volume per volume
w/v	weight per volume
µg	Microgram
µg/mL	microgram per millilitre
µl	Microliter
µM	micromolar µm micrometre
g L^{-1}	gram per litre
CFU/mL	colony-forming unit per millilitre

INTRODUCTION

1.1 Background of the study

Artocarpus heterophyllus Lam. (Jackfruit) comes from the mulberry family (Moraceae) is an important and popular fruit grown worldwide mainly for pulps and rags can be consumed directly, cooked as well as processed as a different form of food. Jackfruit cultivation widespread throughout tropical and subtropical regions worldwide, including Malaysia. Jackfruit is an important crop in Malaysian agriculture and considered as a fruit that helps in enhancing economic-driven contribution towards the gross domestic product (GDP) in Malaysia domestically and international market. Pahang, Sarawak and Negeri Sembilan are topmost jackfruit producing Malaysian states (DOA, 2017; Baliga, Shivashankara, Haniadka, Dsouza and Bhat, 2011). Fifteen varieties of jackfruit cultivated in Malaysia, but the well-known varieties are Tekam Yellow (J33), Mantin (J32) and Mastura (J35) (KADA, 2018; Ismail and Kaur, 2013).

Bronzing disease of jackfruit, caused by an aerobic, Gram-negative bacterium *Pantoea stewartii* subspecies *stewartii* (*P. stewartii* subsp. *stewartii*) are frequently found in the jackfruit crop (Gapasin, Garcia, Christine, Cruz and Borines, 2014). Areas of jackfruit plantations infected were reported in the Philippines, Mexico and Malaysia (Hernández-Morales, Pérez-Casillas, Soria-Guerra, Velázquez-Fernández and Arvizu-Gómez, 2017; Zulperi, Manaf, Ismail, Karam and Yusof, 2017; Gapasin *et al*., 2014). Currently, jackfruit-bronzing is a threatening disease in the jackfruit industries in Malaysia. Examination and inspection records on the status of jackfruit-bronzing currently is essential to keep high quality and ensures the security of the food supply of local jackfruit. The causal agent, *Pantoea stewartii* subsp. *stewartii* is also well known causing Stewart's wilt in corn and maize (Liu, 2010; Michener, Pataky and White, 2002). Stewart's wilt of maize was the first case of a plant disease ever reported among the genus *Pantoea* and the discovery led to major losses in crop yield (EPPO, 2016). Other hosts infected by *Pantoea stewartii* subsp. *stewartii* include *Dracaena sanderiana* (Cameroon's native ornamental plant) and *Oryza sativa* (rice) (Jeger, Bragard, Candresse, Chatzivassiliou, Dehnen-Schmutz *et al*., 2018). Until today, this pathogen has spread and present in America, Africa, Asia (including Malaysia) and Europe.

1.2 Problem statement

In Malaysia, the first suspected outbreak of jackfruit-bronzing disease was found in severely affected jackfruit plantations in Negeri Sembilan and Pahang 2010 (Karam, 2017). In 2016, the infected Tekam Yellow (J33) jackfruits variety were collected from Muadzam Shah, Pahang Malaysia, examined in the laboratory and the bacterial strains were confirmed as *P. stewartii* subsp. *stewartii* (Zulperi *et al*., 2017). The infected fruits downgraded the quality from the fresh-market sale to processing, can cause a major threat in financial losses to consumers

and processors (Zulperi *et al*., 2017). The humidity and warmth of Malaysia's tropical condition enhance the growth of the pathogen and promotes the development of disease (Correa, Majerczak, Ammar, Merighi, Pratt *et al*., 2012; EPPO, 2006; Azad, Holmes and Cooksey, 2000). The growth and dispersal of the pathogen blooming with the favourable tropical regions of Malaysia, especially the study of severe diseases of crop plants (Dow, An and O'Connell, 2017).

The occurrences of bronzing disease have further diminished little enthusiasm of farmers in jackfruit industry, as it can cause major financial losses to consumers and processors when the infected fruit is downgraded from fresh-market sale to processing as well as impacting the jackfruit growth sector. Malaysian government has implemented National Key Economic Areas (NKEA) EPP7, which is under Economic Transformation Programme (ETP), where jackfruit is recognized as one of the agriculture premium fruits (PEMANDU, 2012). Therefore, the constant occurrences of the bronzing disease on jackfruit have been very crucial and considered as a major constraints to jackfruit production and industry in Malaysia as it leads to loss of jackfruit production (DOA, 2017; Gapasin *et al*., 2014; Leonberger, Jackson, Smith and Gauthier, 2013).

1.3 Significance of the study

As jackfruit remains as one of the most important economic-driven fruit crops in Malaysia, for both local and export markets, scrutinizing records on the status of jackfruit-bronzing disease is significantly important. Up to this point, very limited documentation of jackfruit-bronzing disease in Malaysia were recorded since the first suspected outbreak in 2017. The outcome from this study would be a major platform on generating details documentation of jackfruit-bronzing disease and its causal pathogen *P. stewartii* subsp. *stewartii* in jackfruit fruit crops in Malaysia by using the combination of phenotypic characterization and molecular phylogenetic approaches.

1.4 Objectives of the study

This study was carried out with the following objectives:

(i) To identify the bacterial strains associated with bronzing disease of jackfruit in Peninsular Malaysia using phenotypic and molecular characteristics.

(ii) To determine the pathogenicity of *P. stewartii* subsp. *stewartii* on jackfruits and host range of *P. stewartii* subsp. *stewartii* on sweetcorn, cucumber and pineapple

(iii) To assess and to determine the genetic diversity of *P. stewartii* subsp. *stewartii* strains associated with bronzing disease of jackfruit using multilocus sequence analysis (MLSA).

The output and data obtained from this study will be useful for quarantine purposes and suppression of jackfruit-bronzing disease spread, thus improving the jackfruit industry in Malaysia. Once the disease is properly diagnosed, management options can be deployed to mitigate the disease impact. As the research of jackfruit-bronzing in Malaysia is still ongoing, this would be a proper reference in the diagnosis and documentation on bronzing disease of jackfruit and its causal pathogen, *P. stewartii* subsp. *stewartii*.

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LIST OF PUBLICATION

Research article

Abidin, N., Ismail, S. I., Vadamalai, G., Yusof, M. T., Hakiman, M., Karam, D. S, Ismail-Suhaimy, N. W., Ibrahim, R., & Zulperi, D. (2020). Genetic diversity of *Pantoea stewartii* subspecies *stewartii* causing jackfruit-bronzing disease in Malaysia. *PLOS ONE*, 15(6), e0234350. doi: 10.1371/journal.pone.0234350

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Abidin, N., Ismail, S. I., Yusof, M. T., Ismail-Suhaimy, N. W., Hakiman, M., Karam, D. S., & Zulperi, D. (2018). Diagnostic approach and genetic diversity of jackfruit bronzing bacterium in Malaysia. *Asian Journal of Plant Pathology*, 12(1), 46-55. doi: 10.3923/ajppaj.2018

Poster and Proceeding

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Awards

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