



Dissemination pattern of bacterial heart rot (BHR) disease and screening of the disease resistance among commercial pineapple varieties in Malaysia

Azlan Azizi Muhamad Nor^{1*}, Rozlaily Zainol², Raziyah Abdullah³, Nur Sulastris Jaffar², Muhammad Zamir Abdul Rasid⁴, Rozeita Laboh⁵, Norsyuhaida Ahmad Shafawi⁶ and Noor Baiti Abdul Aziz⁶

¹Soil, Water and Fertilizer Research Centre, MARDI Cameron highlands, P.O. Box 19, 39000, Cameron Highland, Pahang, Malaysia.

²Horticulture Research Centre, MARDI Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia.

³Animal Science Research Centre, MARDI Kluang Station, P.O. Box 525, 86009, Kluang, Johor, Malaysia.

⁴Soil, Water and Fertilizer Research Centre, MARDI Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia.

⁵Agrobiodiversity and Environment Research Centre, MARDI Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia.

⁶Horticulture Research Centre, MARDI Kluang Station, P.O. Box 525, 86009, Kluang, Johor, Malaysia.

Email: azlanmn@mardi.gov.my

ABSTRACT

Aims: Bacterial heart rot (BHR) disease caused by *Erwinia chrysanthemi* or the new nomenclature *Dickeya zae* was identified as the lethal disease of pineapple and caused massive losses for the farmers due to non-satisfactory solutions. Thus, this study aims to understand the disease dissemination pattern and screen for tolerance pineapple variety prior to establishment of disease management strategies.

Methodology and results: Dissemination of BHR disease was observed visually in 2 study plots consisting 200 plants in each plot. Single plant inoculation of the pathogen was done in each plot namely Plot A at the edge and Plot B at the middle. Disease incidence was recorded at weekly interval for 12 weeks. The pattern of disease spreading in both plots was then mapped based on the results. Separately, 8 commercial pineapple varieties (Maspine, N36, MD2, Morris, Sarawak, Kristal, Gandul and Josapine) were screened for their resistance towards BHR. The varieties screening study was carried out using complete randomized block design. Overall, disease incidence (DI) was observed lower in plot A compared to Plot B. Percentage of DI in Plot A increased continuously from week 1 to 12, but in plot B the DI was stagnant starting from week 3 onwards. This study revealed that there is highly significant difference in percentage of infection between varieties tested. Josapine and MD2 were the most infected varieties based on lesion on plant. Both were found susceptible to BHR. Besides that, Chrystal Honey, Maspine and Sarawak varieties were less infected and classified as moderately resistance compared to other varieties.

Conclusion, significance and impact of study: Inoculum source was recognized as determinant factor for dissemination of BHR. Aggregation pattern was observed, and disease spreading was severe when disease started from the edge of the plot compared to in the middle. These findings will help farmers to choose the varieties of interest and plan for disease control measure based on first observed disease symptom in their field. This study is also important to researchers and plant breeders for varietal improvement in the future.

Keywords: Bacterial heart rot, *Erwinia chrysanthemi*, pineapple, Josapine, MD2, *Dickeya* sp.

INTRODUCTION

Pineapple (*Ananas comosus* var *comosus*) is one of the most popular tropical fruit crops in family of Bromeliaceae and classified under the order of Bromeliales (Coppens d'Eeckenbrugge and Leal, 2003). Pineapple was reported as the third most important commercial fruit crops in the world with approximately 30 cultivars grown for commercial purpose (Karnataka, 2007). In Malaysia, pineapple industry was recognized as source of wealth for the nation. Pineapple is the oldest industrial crops in Malaysia and started more than 100 years ago. This plant

can provide a good income, especially with the high-density cultivation that can provide stable revenue, such as breed of Gandul for cans and Moris for fresh consumption (Selamat, 1996). However, pineapple industry particularly in Malaysia has been badly affected by disease tribulation. Various diseases caused by bacterial infection have been reported and the most severe is bacterial heart rot (BHR) caused by *Erwinia chrysanthemi* or newly known as *Dickeya zae* (Marrero *et al.*, 2013). BHR was first reported in Malaysia by Johnston in year 1957 (Lim, 1985; Toth *et al.*, 2011). According to Ramachandran *et al.* (2005), it is one of the

*Corresponding author

major pathogens threatening pineapple industries in Malaysia. To date, there are no definite solutions to overcome this problem. Although conventional techniques were used in the control of BHR disease but none of them are satisfactory due to the resistant strains and effect on environment caused by the chemical treatments (Abdenbi *et al.*, 2010). He also reported that once the pathogen invades the inner parts of the plants, the conventional chemical products such as copper may not provide adequate control for the disease. To solve this problem, many scientists have focused on alternative solutions such as biological control agents and developing resistance variety as a substitute for ineffective chemical control. Hence, this study was implemented to understand the spread, epidemiology, management strategies for BHR disease and also screen for tolerance pineapple variety towards BHR.

MATERIALS AND METHODS

Isolation of the pathogen

Bacterial causing agent of the disease was isolated from symptomatic samples of infected pineapple in Pontian, Johor. The disease samples were processed within 24 h. after sampling. A small piece from the edge of infected lesion was cut, rinsed with 10% Clorox, and followed by 3 times of sterilized distilled water. The piece was then crushed in sterile mortar with 1 mL of sterilized distilled water. The suspension was subjected to serial dilution with sterile water and a loopful of the suspension from dilution was streaked on a selective agar media, Luria-Bertani (LB). The plates were incubated at 28 °C ± 2 °C and bacterial growth was observed from 24 to 36 h. Purified bacterial colonies were multiplied by streaking onto nutrient agar (NA) and stored at -80°C in 20% aqueous glycerol for further use.

Confirmation of bacteria ID

DNA extraction

Genomic DNA was extracted and purified by using a commercial kit, DNeasy Blood and Tissue Kit (QIAGEN, Germany). Extraction method was according to the manufacturer's instruction. DNA was stored at -20 °C until required.

Detection of bacteria by species-specific primer

Detection of bacteria was done by using a pair of species-specific primer for *D. zeae*. This primer pair, ADE1 (5'-GATCAGAAAGCCCGCAGCCAGAT-3') and ADE2 (5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3'), was expected to amplify a 420-bp fragment. The DNA was amplified in 50 µL reaction volumes containing PCR buffer (25 µL PCR Master Mix) by Thermo Scientific-DreamTaq Green PCR Master Mix (2x), 1 µM or each primer and 1 µg of DNA template. The PCR was run for 5 min at 94 °C in the first cycle, 25 cycles of 1 min at 94 °C,

2 min at 72 °C and for the final step, 8 min at 72 °C (Nassar *et al.*, 1996) in MyCycler™ Thermal Cycler (Bio-Rad). All PCR products were analyzed on a 1% agarose gel at 80V for 90 min in Tris-borate buffer. Gel stained with Floresafe DNA stain and visualized by Compact Digimage System UVDI (Major Science), together with 100 bp ladder set (Thermo Scientific).

PCR amplification and sequencing of 16S rRNA

The 16S rRNA were amplified by using universal primers fD1 and rP2 (Weisburg *et al.*, 1991). Amplification reactions were prepared in a total volume of 50 µL that contained PCR buffer (25 µL PCR Master Mix) by Thermo Scientific- DreamTaq Green PCR Master Mix (2x), 1 µM or each primer and 1 µg of DNA template. Amplification conditions were as follows: Initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 4 min, annealing at 58 °C for 1 min, and extension at 72 °C for 3 min and followed by final extension at 72 °C for 10 min. DNA sequencing was performed at First Base Laboratories, Seri Kembangan, Selangor, Malaysia.

Sequence analysis

The 16S rRNA nucleotide sequences obtained in this study were aligned with ClustalW multiple alignment programme using MEGA software, version 6.0 and compared with other known sequences of BDB strains in GenBank using BLAST (Basic Local Alignment Search Tool) search programme of National Institute of Biotechnology Information (<http://blast.ncbi.nlm.nih.gov>). The 16S rRNA sequences which we determined have been deposited in the GenBank database.

Disease dissemination pattern study

Two observation plots were prepared for disease dissemination study. Two hundred (200) plants of Josapine pineapple variety were planted in each plot. One plant from each plot was selected as disease infection source. The plant was then inoculated with *E. chrysanthemi* inoculum. In Plot A the inoculated plant was set at the edge of the plot while in Plot B in the middle. Disease incidence (DI) was evaluated and mapped for 12 weeks.

Screening of pineapple varieties for its resistance towards BHR.

BHR field inoculation procedures

Erwinia chrysanthemi inoculum was prepared in laboratory and Josapine pineapple variety was used as a control. The plants were inoculated with bacterial suspension at 1×10⁸ CFU per mL into 4-month-old pineapple plants (Ramachandran *et al.*, 2015). Ten milliliters of bacterial suspension inoculated into each test plants.

Evaluation of lesion development incidence

Eight varieties of pineapple (Maspine, N36, MD2, Morris, Sarawak, Chrystal honey, Gandul and Josapine) were planted in the field using standard agricultural practices and arranged in Random Complete Block Design (RCBD). Percentage of lesion and leaf infected were recorded and classified based on disease observation scale index (Figure 1).

Scale	Definition	Presence of Lesion	No. leaf infected per plant
0	Resistant	No lesion on leaves.	No leaves infected.
1	Moderately resistant	Slight lesion on leaves. (Less than 5%)	Less than 10% leaves infected.
2	Moderately susceptible	Slight lesion on leaves. (over 5% to 10%)	10-20% of leaves infected.
3	Susceptible	Lesion on leaves. (over 10% to 20%)	20-30% of leaves infected.
4	Very susceptible	Lesion on leaves. (21% and above)	40% and above leaves infected.

Figure 1: Scale descriptions of pineapple leaves' physical parameters.

Statistical data analysis

Field experiment was performed using a randomized complete block design with four replications. Percentage of lesion and leaf infected were recorded and classified based on disease observation scale. Data was then analysed using analysis of variance (ANOVA) followed by comparison of means separation using Duncan multiple range test (DMRT).

RESULTS AND DISCUSSION

Confirmation of bacteria ID

An approximately 420-bp amplified fragment of the ubiquitous was obtained from bacterial strains isolated from the study (Figure 2a). The species specific- PCR test with ADE1 and ADE2 primers used were able to accurately detect and identify the *D. zeae* isolated from pineapple. This 420-bp fragment is part of the pectate lyase-encoding pel gene belonging to the pelADE family in *D. zeae*. Approximately 1500-bp product (Figure 2b) was obtained for the primer pair fD1/rP2. The partial 16S rRNA gene sequences obtained was then compared with gene sequences of known strains in Genbank database. Tested strain had highest homology (95 to 96%) to the 16S rDNA of *D. zeae* CFBP 2052 (NR041923). The partial 16S rDNA sequence of the isolated strain was deposited into the Genbank database with accession number MK874911. This strain was later used as a working culture inoculated in the field test.

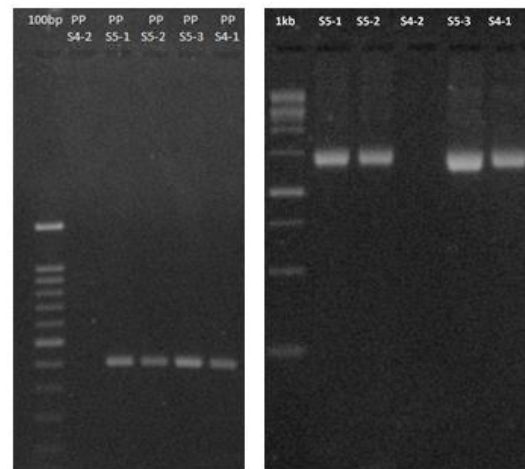


Figure 2 (a)

Figure 2 (b)

Figure 2: Amplification of DNA. Figure 2(a), Amplification of DNA from strains isolated from symptomatic pineapple plant in Pontian, Johor using ADE1/ADE2 primers, displaying the 420-bp amplification product. Figure 2(b), PCR amplification of DNA from strains isolated from symptomatic pineapple plant in Pontian, Johor using fD1/rP2 primers for 16S rRNA sequence, displaying the 1500-bp amplification product.

Disease dissemination pattern

Dissemination pattern showed that BHR spreading was aggregated in both plots and can be observed clearly in Plot A (Figure 3 and 4). Disease incidence (DI) was found higher (6.0%) in plot A where disease source was at the edge of the plot compared to plot B where source was in the middle (2.0%). Percentage of DI in Plot A was gradually increasing from week 1 to 12. However, for Plot B, it was stagnant from week 3 to 12 as showed in the Figure 5. The dissemination pattern in plot A, showed that DI spreading from the edge towards the center of the plot. This pattern might be influenced by wind direction due to the source of inoculum located at the edge of plot easily blown by the wind compared to the Plot B which the inoculum source in the middle of plot covered by the surrounding plants. It was supported by Rohrbach and Johnson (2003) whom reported that wind and soil splash are mode of disease dissemination in field. Aggregated pattern of disease spreading showed immobility characteristic which resulted in the higher concentration of infected plants in the same area compared to random spreading normally occurred caused by the spreading due to insects. Previously study conducted by Sueno *et al.* (2014) also reported random pattern of symptomatic plant was observed in field and suggested the disease occurrence was triggered due to the infected planting materials.

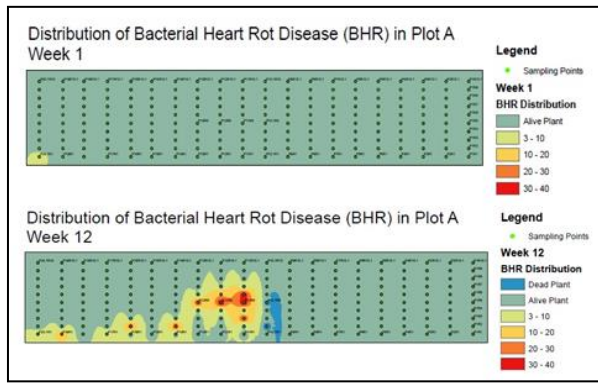


Figure 3: Disease dissemination patterns in Plot A (Observation done on week 1 to week 12 after inoculation of *E. chrysanthemi*).

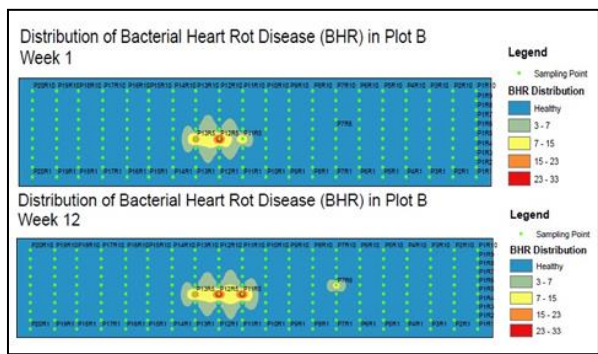


Figure 4: Disease dissemination patterns pattern in Plot B (Observation done on week 1 to week 12 after inoculation of *E. chrysanthemi*).

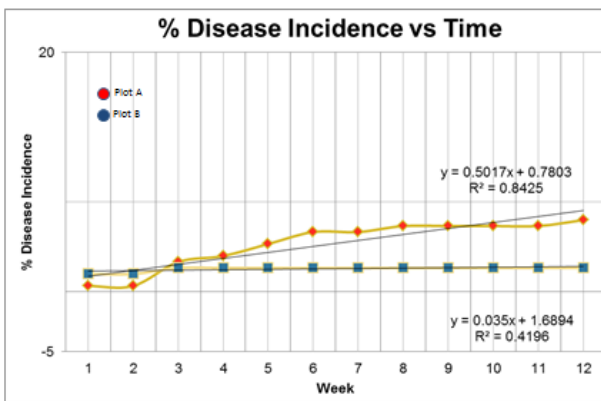


Figure 5: Percentage of Disease Incidence (DI) in Plot A and Plot B.

Screening of pineapple varieties

Observation of lesion development on each plant varieties (Table 1) indicated that there is highly significant difference between pineapples varieties observed. Based

on developed lesion scale, scored from 0 to 4, highest score was recorded in Josapine variety (3.09), which is over 10-20% of lesion was recorded. Low percentages of lesion (less than 5%) were recorded in 3 varieties (Chrystal Honey, Maspine and Sarawak) which their scales range from 1.41 to 1.46. This indicated that, Josapine variety is the most susceptible variety with the highest percentage of lesion while chrystal honey, maspine and Sarawak showed tolerant ability towards BHR. Study also found that, there is highly significant difference between lesion and days of observation. Study recorded that as day after planting increased; percentages of lesion on plant also increased.

Table 1: Observation of lesion scale with different pineapple varieties and time of observation.

Variables	Lesion scale
Pineapple varieties	
Maspine	1.46d
N36	1.66cd
Md2	2.49b
Morris	2.36b
Sarawak	1.56d
Chrystal honey	1.41d
Gandul	1.87c
Josapine	3.09a
F-test	**
Observation days	
5 days	
8 days	
11 days	
14 days	
17 days	
20 days	
23 days	
F-test	**
Interaction	Ns

Notes: Non-significant (NS), significant (*) and highly significant (**) at $P \leq 0.05$, respectively by DMRT.

The study on leaf infected number also found that there is highly significant difference between pineapple varieties tested (Table 2). Based on developed leaf infected scale (from 0 to 4), the highest percentage of leaf infected recorded in two varieties of pineapple which is MD2 and Josapine with scoring 1.52 and 1.43 respectively. Besides that, the lowest percentage of leaf infected found in Chrystal Honey variety with scale 1.04. Although there is a difference in ANOVA group, all the varieties are in the similar scoring group which is score 1, with slight difference in the percentage of infected leaves (less than 10%). This disease normally affects the young leaf which is located in the heart (core) of pineapple plant. Study also found that there is no significant difference between leaf infected and different observation days.

Although the highest percentage of lesion observed in scale 3 (over 10-20% lesion) and percentage of leaf infected only less than 10% in individual plant, this disease has the potential to spread easily, especially

during raining season where the drainage and sanitation in the field is not in good order.

Although the highest percentage of lesion observed in scale 3 (over 10-20% lesion) and percentage of leaf infected only less than 10% in individual plant, this disease has the potential to spread easily, especially during raining season where the drainage and sanitation in the field is not in good order.

Table 2: Observation on number of leaves infected between pineapple varieties and time of observation.

Variables	Leaf infected scale
Pineapple varieties	
Maspine	1.09 _{cd}
N36	1.11 _{cd}
Md2	1.52 _a
Morris	1.24 _b
Sarawak	1.18 _{bc}
Chrystal honey	1.04 _d
Gandul	1.21 _{bc}
Josapine	1.43 _a
F-test	**
Observation days	
5 days	
8 days	
11 days	
14 days	
17 days	
20 days	
23 days	
F-test	Ns
Interaction	Ns

Notes: Non-significant (NS), significant (*) and highly significant (**) at $P \leq 0.05$, respectively by DMRT.

CONCLUSION

Disease dissemination pattern of BHR disease in field was found to be aggregated. Initial inoculum source spot was observed as the determinant factor for disease development and spreading. Thus, disease monitoring, early detection, removing and destroying of infected plant is essential as the best management strategy in controlling the spreading of BHR in the field. Chrystal Honey, Maspine and Sarawak varieties were recommended based on their tolerant to BHR. However, for farmers who choose to plant other varieties, frequent disease surveillance is very important as prevention of disease infection. This finding is also important especially for plant breeders for developed of new varieties, identification of the potential varieties and prospecting for resistance gene for varietal improvement in the future.

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