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Identification and Characterization of The Causal Agent of Infected Cucumber (*Cucumis sativus*) in Perak, Malaysia.

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Cucumis sativus or known as cucumber is the creeping plant, widely cultivated for its edible fruit. Nowadays, cucumbers are cultivated in most likely all countries in temperate zones for fresh consumption, or as pickling cucumber. This study aimed to identify and characterize the causal agent of infected cucumber from Perak, Malaysia. Leaves showing typical symptom of leaf spot disease were isolated to identify the possible pathogenic microbes. By direct-plating technique, nine type of pathogens obtained were included seven bacteria strain and two types of fungi. Bacterial were isolated and identify through gram-staining, biochemical test and molecular identification. While fungi were confirm to their genus based on their conidial morphology and molecular identification. Polymerase Chain Reaction (PCR) was then carried out to study genotypic identification of the fungus using universal primer set; 16SF2 and 16SR2 for bacteria while LROR and LR7 for fungi by using 100 bp DNA ladder. For further confirmation on the symptom of disease Koch's Postulate had been applied. The disease incidence was determined by measuring the number of plant affected over the number of plant inoculated. Koch's Postulate proved that sample C4 and C5(1) were pathogenic to cucumber since they showed highly similar symptom to natural infection at the field. DNA sequencing shows that *Pseudomonas gessardii* and *P. azotoformans* were identified as pathogenic fungi that caused the disease

Keywords: cucumber, cucumber disease, causal agent, *Pseudomonas sp.*

INTRODUCTION

Cucumber commonly known as cucurbits or gourds belong to the Cucurbitaceae family includes a number of cultivated species of global or local economic importance (Robinson and Decker, 1997). The cucumber or its scientific name, *Cucumis sativus* are warm season plants and grow best between 18 °C to 23 °C (Dalgleish et al., 2007; FDA, 2015). These creeping plant, widely cultivated for its edible fruit. Low in the nutritional value, not hinder the delicate flavour that makes it popular for salads and relishes (Paul, 2017). In order to control disease of plant from spreading, farmers need to diagnose the plant problems. Nowadays, farmers basically rely on chemical pesticides as control method for controlling crop diseases and infestation due to the lack of information or wrongly diagnosed the infected plant. These resulted of the excessive use of the toxic chemicals. There is now

enormous evidence that excessive use of these chemicals do pose a negative effect to humans and other life forms and unwanted potential risk to the environment (Jeyaratnam, 1985; Igbedioh, 1991). This study was conducted to identify and characterize the causal agent for the diseases infestation on *C. sativus*. By knowing the causal agent of the disease, it is easier for farmers to take appropriate action to control the diseases from spreading away. Besides, this research can educates farmers more about the diseases. The results derived from this study could educate farmers and other people about cucumber disease and actions to control them. The objectives for the study were to identify and characterize the causal agent of *C. sativus* in Perak, Malaysia.

MATERIALS AND METHODS

Isolation of Infested Pathogen

The infected leaves parts were obtained from Cucumber (*C. sativus*) in Perak, Malaysia. The infected parts were cut into 1-2 cm pieces, and they were soaked into 10% sodium hypochlorite for 30 sec, 70% ethanol for 30-60 sec and washed with distilled water for 60 sec. Then, it was dried with sterile filter paper and was placed on NA (Kadir and Charudattan, 2000) in 10 petri plates each with three pieces a plate. The plate was incubate at 30 °C for 48 hours, and was observed for any growth of the pathogen. The presence of bacteria growth was observed in the plate. Mix colonies of bacteria were isolated then re-isolated again to obtain pure culture for each plate. There were seven total number of isolated pathogen obtained.

Phenotypic bacteria identification

Gram Staining

Slide that contains the streaked bacteria was placed with heat fixed smear on staining stray. The smear was stained with crystal violet (1 minute), The slide was gently rinsed with distilled water. The slide flooded again with Iodine (1 minute), 95 % ethanol (5 seconds) and safranin counterstain (1 minute). The slide was rinse with distilled water and blotted dry. Lastly, the slide was observed under light microscope (Forbes, 2007).

TSI Test

A sterile needle was used for inoculation where the center of the medium was stabbed and then streaking on the surface of the agar slant. Then, it was incubated with caps loosened at 30 °C (Forbes, 2007).

Oxidase Test

Bacteria colonies was picked and transferred to the glass slide pick where oxidase reagent was dropped into the streaked colonies. The colour changed was observed (Reynolds, 2018).

Catalase Test

A loop was used to transfer a small amount of colony growth in the surface of a clean, dry glass slide. A drop of 3 % Hydrogen Peroxide (H2O2) was placed on the glass slide (Reynolds, 2011).

Amylase Test

According to Sanders (2012), pathogen was inoculated on starch agar plate and incubated at 30 ºC for 24 hours. Amylase production will be detected as a colourless zone on surrounding of colony on addition of iodine (Ebby, 2011).

SIM Test

Well-isolated colony was taken using a straight inoculating needle. It was inoculate through stabbing the middle of the tube 2/3 (two-third) of the medium and was incubate at 30 °C (Reller, 1975).

Mac Conkey

Pathogen was inoculated on Mac Conkey agar plate and incubated at 30 ºC for 24 hours (Reller,1975).

Molecular Identification of the Isolated Bacteria

DNA Extraction from Bacteria

Single colony from the bacteria culture was transferred into NB and left overnight at 30 ºC. Wizard Genomic DNA Purification kit (Promega) was used for DNA extraction of bacteria. 1 ml of an overnight culture was used as starter material and finally, diluted with 30 µl of DNA Rehydration Solution. The DNA was then stored at 4 °C until used. DNA concentration and quality was evaluated by agarose gel electrophoresis and spectrophotometer.

Polymerase Chain Reaction (PCR)

PCR was used to amplify the targeted DNA strands. PCR was carried out by using primer pairs of 16S, which are 16SF2 : 5'- GAG TTT GAT CCT GGC TCA -3' for forward primer and 16SR2 : 5'- ACG GCT AAC TTG TTA CGA CT -3' for the

reverse primer. PCR amplications were performed in 40 µl volumes containing 5 µl of template DNA, 20 µl of MasterMix (contain $MgCl_2$ Tris-HCl, KCl, dNTPs & Taq Polymerase), 11 µl of deionized distilled water, 2 µl of forward and reverse primer.The conditions for PCR amplification was conducted as follows, initial denaturation at 95 ºC for 2 minutes, denaturation at 95 ºC for 30 seconds, annealing temperature at 50 ºC for 1 minutes, extension at 72 ºC for 2 minutes and final extension at 72ºC for 10 minutes. PCR run for 30 cycles to reach sufficient amplification. The PCR product was separated by 1% of agarose gel and stained with ethidium bromide to be visualized which was carried out in 1X TAE electrophoresis buffer. Computer-image analysis of photographs of the gel-red stained agarose gels was carried out by Gel-Doc system.

Environmental Factors Influence Bacteria Growth

Effect of Salinity on Bacteria Growth

Isolated bacteria was inoculated into NA media with different concentration of Sodium Chloride (NaCl) which are 2.5, 5, 7.5, and 10 % and was incubated at 30 °C for 24 hours and the bacteria growth was observed and recorded. As NA media contains already 0.5 % of NaCl, the addition of NaCl was calculated and weighed.

Effect of Temperature on Bacteria Growth

Isolated pathogen was inoculated into NA media and incubated at seven different temperature levels 10, 25, 30, 37, 45, 50 and 60 °C for 24 hours. The bacteria growth was observed and recorded.

Effect of pH on Bacteria Growth

The isolated pathogen was transferred on the center of NA media varying pH 4, 5, 6, 7, 8, and 9. The final pH of NA media was 6.8 ± 0.2 so the pH adjusted accordingly by using HCl and NaOH. The bacteria growth was observed after incubated at 30 ºC for 24 hours.

Establishing Koch's Postulate

Three healthy and uninjured leaves were undergoes surface sterilized by using 70% ethanol for thirty seconds and rinsed thrice in sterile distilled water. A sterile cotton swap of about 5.0 square cm was dipped in the suspension (containing bacteria culture and 2 drops of Tween 20) was then swabbed over the leaves surface of the plant. The inoculated plants were watered twice to maintain humidity for pathogen symptom pop out. The disease incidence was recorded 72 hours after inoculation (Hailmi, 2006).

The disease assessment can be done by determining the disease incidence of the plant. The disease incidence can be obtained by calculating the number of plant affected out of the number of plant inoculated as follow:

$$\text{Disease incidence} = \frac{\text{The number of plant affected}}{\text{The number of plant inoculated}} \times 100\%$$

RESULTS

Isolation of Infested Pathogen

Primary Isolation

Through direct plating technique, there were seven types of bacteria strains obtained. Pure cultures of bacteria were obtained after undergoing several series of streaking. As shown Figure 1 below, colonies isolation produces by bacteria varied from white, yellow and pink.

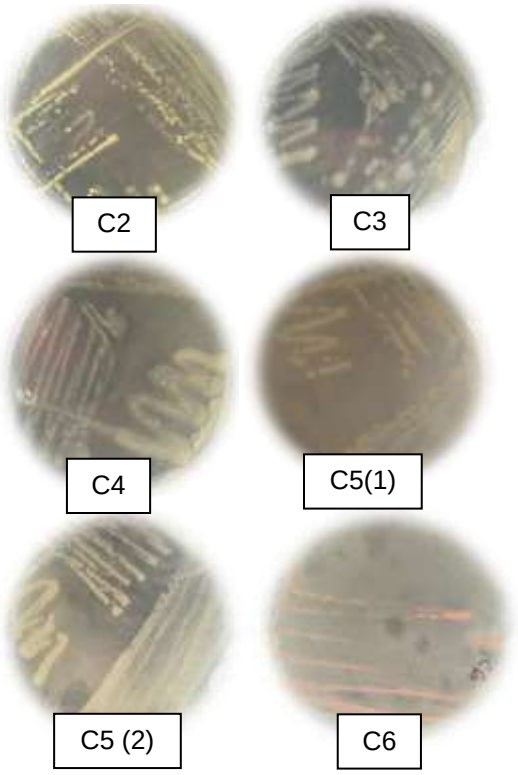

Figure 1: The bacterial strains after two days incubation.

Bacterial identification

The biochemical characteristics of seven strains of bacteria were compared with those of type strains of other bacteria species. Biochemical test results were shown in Table 2. Further investigation by molecular identification confirmed that the bacteria been isolated were varied. DNA sequencing results shows in the Table 1.

Establishing Koch's Postulate

Two out of seven bacteria isolates (C4 and C5(1)) from cucumber cultivars were pathogenic when they were inoculated onto leaves of the plants. Figure 2 shows the results of Koch's Postulate along with the disease incidence. These two bacteria strains scored 100% disease incidence.

Environmental Factors Influence Bacteria Growth

Bacteria isolate shows varied result on analysis of environmental test. Table 3 shows the environmental test results of the bacteria strains isolated.

Table 1: The molecular identification results of isolated bacteria.

Bacteria	ID Name	Similarity
C2	*Pantoea agglomerans*	97.05 %
C3	*Enterobacter cloacae*	99.82 %
C4	*Pseudomonas gessardii*	98.45 %
C5(1)	*P. azotoformans*	97.52 %
C5(2)	*Bacillus cereus*	99.25 %
C6	*Methylobacterium sp.*	83.51 %
C8	*Pseudomonas libanensis*	98. 34 %

DISCUSSION

Cucumber cultivation has been carried out in Kinta Highland and almost all the cultivated area increase in disease with 'leaf spot' symptom becomes a serious problem nowadays. Therefore, this was conducted to determine the causal agent that affecting cucumber in Perak, Malaysia. During isolation of the samples, there are seven bacteria isolates include C2, C3, C4, C5(1), C5(2), C6 and C8. The pathogenicity of C4 and C5(1) identified as *Pseudomonas gessardii* and *P. azotoformans* were shown pathogen on cucumber leaves wiith 100 % diseases incidence. According to Shankar et al. (2014), *Pseudomonas sp.* Is one of the most important causal agent of bacterial disease in cucumber.

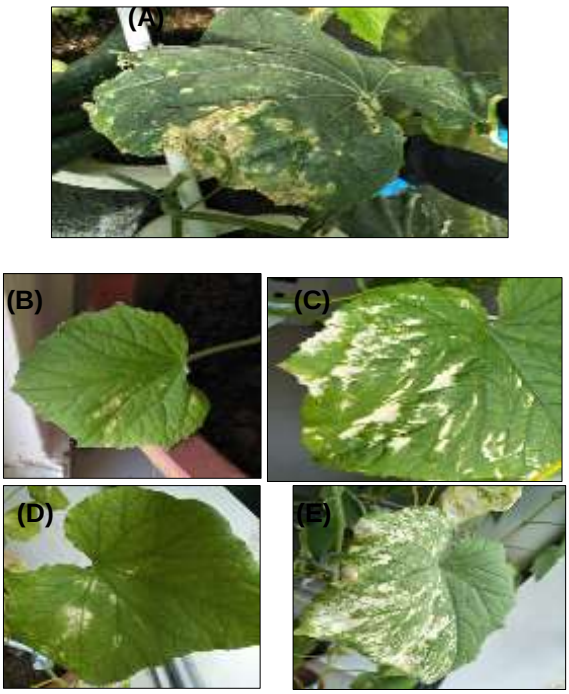

Figure 2: Cucumber leaves showing typical symptom of 'leaf spot' disease; (A) natural infection at field (B) affected plant by C4 at early stage (C) affected plant by C4 at final stage (D) affected plant at early stage of C5(1) (E) affected plant by C5(1) at final stage.

The biochemical characteristics of the bacteria strains indicate that these bacteria are vary in genus and species. Only one bacteria strain were Gram positive include C5(2) while others were Gram negative. All the isolates tested were negative in gas, H_2S and indole production. They were presented motile and non-lactose fermentation. Results in oxidase, catalase and amylase test vary according to the strains. Oxidase negative only presented on C2 and C5(1) strain, while for catalase and amylase test indicate

that only C5(1) show negative result and C6 shows positive result respectively.

A C2 strain showed similar characteristics of *Pantoea agglomerans* according to Delétoile et al., (2009) and Mergaert et al., (1993). According to Michael, (1981), C3 strains show similar characteristics of strain *Enterobacter cloacae.* As for C4, C5(1) and C8 were identified in *Pseudomonas* genus after being compared in previous study according to Shivangi et al. (2017). C5(2) and C6 were similar in characteristics of *Bacillus cereus* and *Methylobacterium sp*. observed by Liu et al., (2017) and Lidstrom et al., (2002).

In this experiment, the optimum temperature, pH and salinity required by each bacterium to grow have been determined. It is important when doing further research different places will indicate different type of pathogens and their metabolism

and their activity would be different (Bruslind, 2017). All of the samples have been incubated at different temperature; 10, 25, 30, 37, 45, 50 and 60 °C (optimum temperature). The samples were incubated with different additional concentration of NaCl which were 0, 2.5, 5, 7.5, and 10 % (optimum salinity). Lastly, the samples were inoculated on NA medium with varying pH 4, 5, 6, 7, 8, and 9 before incubated at incubator. Table 3 shows the results on temperature, salinity and pH.

The results showed that, all bacteria sample were able to grow on temperature range from 10 to 45 °C but no growth observed on temperature higher than 50 °C. As for pH results indicates that only bacteria sample C2 able to grow in pH range 4 while other bacteria unable to grow in pH range 4. Higher percentages of NaCl (10% of NaCl) inhibited growth of all the bacteria samples. Strain C6 showed that at 5 to 7.5 % of NaCl, only small colony manage to growth while on 7.5 % NaCl only C3 abled to grow well. All bacteria inoculated in NA media contain 2.5 and 5 % of NaCl shows the ability to growth under that condition. The results obtained can be used as control condition for the plant disease prevention.

Table 2 : Biochemical test for bacterial identification.

Strain	Gram Staining (-/+)	TSI		Oxidase	Catalase	Amylase	SIM		Lactose fermenter
		Slant/ Butt	Gas/H_2S Production				Indole	Motility	
C2	-	Yellow/ Yellow	-/-	-	+	-	-	+	-
C3	-	Yellow/ Yellow	-/-	+	+	-	-	+	-
C4	-	Red/ Yellow	-/-	+	+	-	-	+	-
C5(1)	-	Red/ Red	-/-	-	-	-	-	+	-
C5(2)	+	Yellow/ Yellow	-/-	+	+	-	-	+	-
C6	+	Yellow/ Yellow	-/-	+	+	+	-	+	-
C8	-	Yellow/ Yellow	-/-	+	+	-	-	+	-

+:Positive, -:Negative

Table 3: The environmental test results of the bacteria strains isolated.

Bacteria	Environment Test																
	Salinity, %				pH						Temperature, °C						
	2.5	5.0	7.5	10.0	4	5	6	7	8	9	10	25	30	37	45	50	60
C2	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-
C3	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
C4	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
C5(1)	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
C5(2)	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
C6	+	±	±	-	-	+	+	+	+	+	+	+	+	+	+	-	-
C8	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-

+:Positive test, -:Negative test, ±:Slightly grow

CONCLUSION

In conclusion, several methods had been carried out in this study to identify the possible causal agents that causes disease on leaves of *C. sativus*. Based on the study, the disease causal agents of *C. sativus* were identified as *Pseudomonas gessardii and P. azotoformans* via molecular and biochemical test method. The causal agents then were characterized by using analysis on different environmental effects on bacteria growth which include different temperature, pH and salinity. It had been further confirmed when Koch's Postulate had been established and proved.

There were few suggestions that can be highlighted for continuing this study. Next, study on how to control these pathogens should be carried out by applying biological control or chemical control as treatment on the plants at the fields.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NAB devised the project, the main conceptual ideas and proof outline. NAS contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript, with help from NAZ, SIK, ASK and MHS. NY and MNL helped supervise the project.

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