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Identification and Characterization of The Causal Agent of Infected Iceberg Lettuce (*Lactuca Sativa* L.) in Perak, Malaysia

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ABSTRACT

Lack of knowledge about the causal agent of infected crops lead farmers to use wrong treatment on their crops, which is causing negative effects on environment and human's health. This study was conducted to identify and characterize the causal agent from the infected iceberg lettuce (*Lactuca sativa* L.) in Perak, Malaysia. The infected iceberg lettuces were sampling from Perak area and the causal agent was isolated by direct plating technique on a nutrient medium. The isolated bacteria were identified by molecular method and biochemical test. Molecular identification was carried out using 16S rRNA sequence and then, several tests such as amylase, catalase, oxidase and others were done for identification of bacterial species based on their biochemical activities. The obtained results showed that *Serratia marcescenes* and *Stenotrophomonas* sp. were identified as the causal agents. The pathogenicity has been confirmed by the establishment of Koch's Postulate, which showed 100% disease infection on them. The environmental effects also have been tested on the bacteria to study the ability of bacteria to grow in different environmental conditions based on different in pH, salinity, and temperature. All the bacterial sample showed that they able to growth on the pH between 4.0 to 9.0, at the temperature 10 °C to 45 °C and at the addition salinity to the medium between 2.5 %, 5.0% and 7.5 %.

Keywords: *Stenotrophomonas* sp., *Serratia* sp., lettuce, lettuce disease

INTRODUCTION

Lactuca sativa var. capitata L. family Asteraceae or known as iceberg (crisphead) lettuce is the major important crops in the group of leafy vegetables. Normally, people will eat them as fresh salad or cooked them first (Lebeda et al., 2007). Iceberg lettuce not only high in water content, but there are also some health benefits. This type of lettuce may help in reduce or maintain weight because it contains low calories. It also aids in prevention of birth defect. This vegetables contains some amount of folate that essential to prevent birth defect and really important for pregnant woman. Besides, even iceberg lettuce has low amount of vitamin A and C, but it still have considerable amount of both vitamin that give good side effect to the body. Like vitamin A, it helps in eye maintenance and the prevention of macular degeneration, the leading cause of age-related blindness, while for vitamin C, it aids in increase the immune system through their antioxidant properties (Kim et al., 2016).

Lack of knowledge about the causal agent cause most farmers to use wrong treatment on their crops. This problem leads the farmers tend to use general pesticide or general bio-pesticide without have specific target pests. Pesticides have slowly come to be used regularly as final decision for every problems arise, rather than be functioning as a curative means to control pest and pathogen outbreaks (Lamine et al., 2010). This practice affected the functional diversity within food webs for pest regulations (Moonen & Barberi, 2008), thus decreasing the sustainability. Furthermore, if the applications of the pesticide were prolonged, it will cause pesticide–resistant pests.

This research was conducted to determine the causal agent that caused disease on the iceberg lettuce in Perak and determine the environmental effects (salinity, pH and temperature) on bacterial growth. By knowing the causal agent of the disease, the farmer can take an appropriate action before the disease spread throughout the fields and getting worse.

MATERIALS AND METHODS

Isolation of Disease Causing Microorganisms.

Direct Plating Technique

The samples for infected iceberg lettuce were taken from Perak and proceed for direct plating method (Verdier et al. 2014). The surface of the infected parts were disinfected by using 70% of ethanol (Khalilian et al., 2015) for 30-60 seconds and followed by washed with sterile distilled water for 60 seconds. The infected part was cut by using a sterilized scalpel at about 1-2 cm along from the healthy part.

Then, the infected part was dried on the sterile filter paper and immediately placed on Nutrient Agar (NA) using direct plating technique. All the NA plates were incubated in the incubator at 30°C aerobically for 16-24 hours. All steps were performed followed by the aseptic techniques protocol. The mixed colonies then were re-streaked on NA until pure colonies were obtained.

Molecular Identification of Isolated Bacteria

DNA Extraction from Bacteria

Genomic DNA of isolated bacteria was extracted using Wizard Genomic DNA Purification kit (Promega). One mililitre 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 16SR1 : 5'- GAC TAC HVG GGT ATC TAA TCC -3' for the reverse primer (Yang & Li, 2017). PCR amplications were performed in 40 µl volumes containing ~100 ng of template DNA, PCR MasterMix (contain $MgCl_2$ Tris-HCl, KCl, dNTPs & Taq Polymerase), deionized distilled water, 0.5 µM of forward and reverse primer. The condition of the PCR that needs to amplify the targeted DNA followed these steps; the initial denaturation step 2 mins with 95 °C; followed by 40 cycles of denaturation for 30 s in 95 °C; annealing at 50 °C for 1 min (Yang & Li, 2017); elongation at 72 °C for 2 min; and final elongation at 72 °C for 10 min. The DNA samples were sent for sequencing and the data obtained were analysed using Basic Local Alignment Search Tool (BLAST) with the database from National Centre of Biotechnology Information (NCBI).

Biochemical Test

Gram-staining

A Glass slide with the heat fixed bacteria was flooded with crystal violet for 30 seconds. The slides were gently rinsed with run tap water. Then, the slides were flooded with iodine for 1 minute and washed with tap water. After that, the slides had been flooded again with decolorizing agent (95% alcohol) for 10 to 15 seconds. Lastly, safranin was added onto the slide for 1 minute and washed with tap water. After air-drying, the slides were observed under the light microscope with oil immersion at 100x magnification (Beveridge, 2001).

Amylase Test

Isolated bacteria were streaked on the sterile starch sugar plate for 48 hours at 30 °C. The plates were flooded with iodine and excess iodine was drained off. Plates were examined for the zone of clearance around the growth for each organism (Hemraj et al., 2013).

Catalase Test

A drop of reagent 3% H2O2 was put on a clean slide and transferred bacterial colonies into it. The effervescence formation then was observed. Air bubbles would form for a positive result but, if there were no oxygen bubble means that was a negative result. (Hemraj et al., 2013).

Oxidase Test

About 1-2 drops of 1% oxidase reagent were dropped on a piece of filter paper. A small colony of the bacteria sample was taken using a sterile loop and transferred onto the filter paper. If the color turned into a purple color after 5-10 seconds, that was indicated as a positive result (Hemraj et al., 2013).

Triple Sugar Iron (TSI) Test

The inoculated bacteria was stabbed using a sterilized wire needle into the middle of TSI agar and the culture had been streaked gently onto the surface of the agar slant. The tubes were incubated at 30 °C for 24 hrs (Shraddha et al., 2011). The slant colour and production of gas were observed.

Sulphide Indole Motility Medium (SIM)

A well-isolated colony was taken from a pure culture plate and was picked at the centre using a wire needle. Then, it was inoculated by stabbing the middle of the tube about ⅔ the depth of the medium. The tubes were incubated aerobically at 30°C. The tubes then were examined after 18 to 24 hours of incubation. For the indole

test, four drops of Kovac's Reagent was added and the results were observed within 1 minute and the reagent should remain at the medium surface (Shraddha et al., 2011).

Establish Koch's Postulate

The seed of iceberg lettuce were planting by using a hydroponic system. Then after the iceberg lettuce starts to grow, Koch's Postulate was established. Healthy iceberg lettuce were undergoes surface sterilization using ethanol 70% for 30 seconds and rinsed thrice in sterile distilled water. The isolated bacteria were inoculated into a healthy iceberg lettuce plant. The pathogen was re-isolated if the plant developed the same symptoms and signs with the infected plants to confirm the original inoculated bacteria (Neville et al., 2018). The percentage for disease incidence was calculated using this formula:

$$\text{Disease Incidence} = \frac{\text{Number of plant infected}}{\text{Number of plant inoculated}} \text{ X } 100\%$$

Environmental Effects

Salinity test

A loopful of fresh bacterial culture (~0.5OD at 600nm) was streaked in NA agar at different additional NaCl concentration which were 2.5%, 5.0% and 7.5% and 10.0%. The agar plates were incubated at 30 °C for 72 hours and the bacterial growth was observed daily (Yaish et al., 2015).

Temperature

Isolated bacteria were sub-cultured on the petri dish containing NA agar. An axis will be drawn outside the petri dish and sealed with parafilm. The petri dish was incubated at different temperature of 10 °C, 20 °C, 25°C, 30°C, 37 °C, 45 °C, 50 °C, and 60°C.

pH

Isolated bacteria were cultured on the petri dish containing NA agar medium in pH 4, 5, 6, 7, 8 and 9 (Bach et al., 2016). The pH was adjusted by using 0.1M HCl and 0.1M NaOH. All the petri dish were incubated at 30 °C for 48 hrs. The axis was drawn outside the petri dish and monitored daily.

RESULTS AND DISCUSSION

Bacterial Isolation and Identification

Bacteria pure culture was isolated from the infected parts of the iceberg lettuce (*Lactuca sativa L.*) which were from the root area and leaves part. Then, all the infected parts were growth on the NA plates by direct plating methods. After three days of isolation, ten types of pure bacterial cultures which consistently obtained after a few subcultures. The pure culture obtained were labelled as S1, S2, B1 (1), B1 (2), B2 (1), B2 (2), B2 (3), B2 (3), B2 (4), and B3 (1). S1 and S2 were from the leaves part, while B1, B2, and B3 from the bottom part of different iceberg lettuce (Fig. 1).

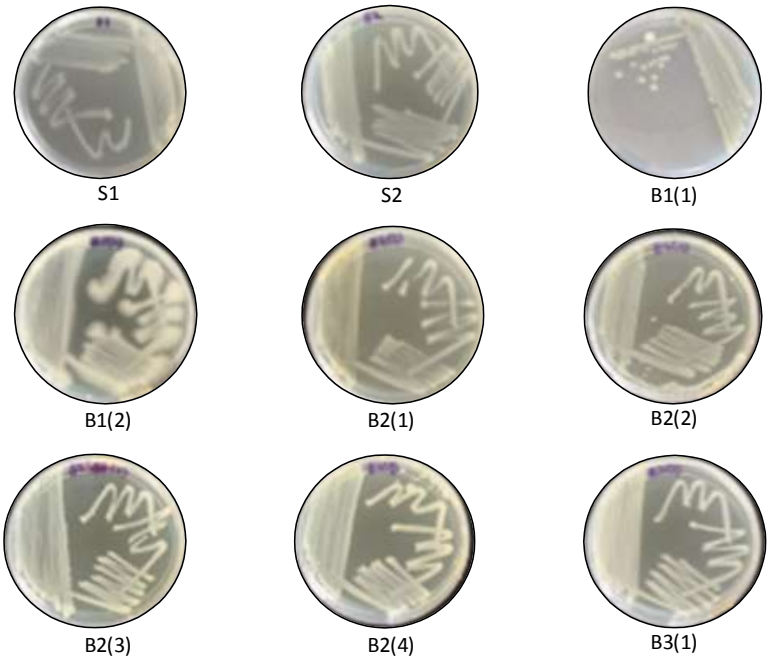

Fig. 1. The pure sample culture obtained through primary screening.

Molecular Identification of Bacterial Isolates

Four species of bacteria was successfully identified by molecular method as shown in Table 1 using 16S rRNA primer. Only one sample was identified as *Serratia marcescenes* and other 8 samples were identified under *Stenotrophomonas* genus. *Serratia marcescenes* has been identified as causal agent of Cucurbit Yellow Vine Disease (CYVD) (Besler and Little, 2017), soft rot pepper (Gillis et al., 2014) and whorl rot in corn (Wang et al., 2015). While, *Stenotrophomonas* sp. was recognized as pathogen or opportunistic pathogen in butternut root (Adegoke and Okoh, 2015), potato roots (Dawam et al, 2013), maize roots (Pereira et al., 2011) and sunflower roots (Ambrosini et al., 2012). The identified samples were proceed for Koch's postulate for the confirmation of causal agent.

Table 1. DNA sequencing result

Bacteria Samples	Bacteria Species	Similarity (%)
S1	*Serratia marcescenes*	99.60
S2/B1(1)/B1(2)/B2(3)/B3(1)	*Stenotrophomonas* sp.	99.87
B2(1)/B2(2)	*S. maltophilia*	98.73
B2(3)/B2(4)	*S. rhizophila*	99.60

Biochemical Test

Firstly, Gram staining was carried out to differentiate the bacteria by physical and also chemical properties of their cell wall. Cell wall was detected by the presences of peptidoglycan, which is present only in Gram-positive bacteria. The Gram negative bacteria showed pink colonies when been observed under microscope while Gram

positive bacteria showed purple in color. Based on Table 2, all the bacterial samples were Gram negative bacteria but with different shapes such as coccus or bacillus.

Table 2. Gram staining result of different bacteria species

Bacteria species	Gram	Shape
Serratia marcescenes	Negative	Bacillus
Stenotrophomonas sp.	Negative	Bacillus/Coccus
S. maltophilia	Negative	Bacillus
S. rhizophila	Negative	Bacillus

Based on the results showed in Table 3, bacterial species that are positive results in catalase test can produce enzyme catalase and they were able to catalyse hydrogen peroxide into oxygen and water. Therefore, the bubbles were produced. The reaction has shown negative results for oxidase test where the samples were not able to produce cytochrome c. oxidase which catalyses the reaction of cytochrome and oxygen which shows the results in no production of purple color when we added the oxidase reagent and left them for about 20 seconds. The bacteria samples were all motile.

All the bacteria samples have shown negative results for indole production which also indicated them do not possess tryptophanase which will degrade tryptophan into indole, ammonia and pyruvic acid. As results, there were nor red or pink bands produced on top of the medium. In amylase test if the results shows negative results, means that the bacterial sample unable to breakdown starch into sugar and no clear zone produced around the streak area.

Table 3. Biochemical test result of different bacteria species

Biochemical test	**Bacterial sample**			
	Serratia marcescenes	*Stenotrophomonas* sp.	*S. maltophilia*	*S. rhizophila*
Amylase	-	+/-	-	+
Catalase	+	+	+	+
Oxidase	-	+	-	-
Motility	Motile	Motile	Motile	Motile
H_2S production	-	-	-	-
TSI (slant/blant)	A/A,G	K,A/K,A,G	A/A	K/K
Mac Conkey (ability to ferment lactose)	Unable	Unable	Able	Unable

+ = Positive - =Negative A= Acid, K= Alkaline, G= Gas produced

Sulphide production showed negative results for all the bacteria samples as there was no production of ferrous ammonium sulphate and sodium thiosulphate which can usually be detected when the surface of media turns black. Triple sugar iron test indicated test for fermentation of glucose, lactose and sucrose. Based on the results, when the slant and but was both red in color that was alkaline, means only peptone catabolized. If the results shows both yellow in color and have gas produced means acidic, glucose and lactose and/or sucrose fermentation with gas produced. If the results have red slant and yellow butt, alkaline and acidic, means only glucose fermentation only and peptone catabolized.

All the samples were grew on Mac Conkey agar which indicated the Gram negative bacteria. The Gram-negative bacteria that grow on MacConkey Agar appeared as colourless colonies or in their natural color means there were unable to ferment lactose. Only *S. maltophilia* was observed as lactose fermenter.

Koch's postulate

For Koch's postulate establishment, the inoculation was conducted in several parts of the iceberg lettuce with the overnight bacterial broth. The overnight bacterial broth was added with Tween 20 before sprayed and applied on the healthy iceberg lettuce to make sure that all the bacteria stayed in the broth and not attached to the wall of the flask and separate evenly. All the treatments showed 100% disease symptoms same as the sample of infected iceberg lettuce as shown in Table 4 and Fig. 2.

Table 4. Percentage of disease incidence of different bacteria

Bacteria	Disease incidence (%)
Serratia marcescenes	100
Stenotrophomonas sp.	100
S. maltophilia	100
S. rhizophila	100

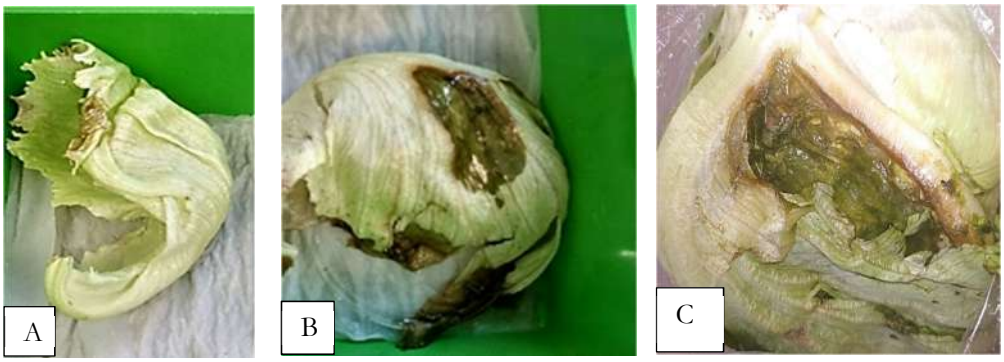

Fig. 2. The results of Koch's postulate after 5 days treatment; (A) Control (B) Treated with *S. marcescenes* (C) Infected iceberg lettuce sample.

Environmental Effects

Table 5. Environmental effects test on different species of bacteria.

Bacterial species	Environmental Test		
	pH	Salinity (% NaCl)	Temperature
Serratia marcescenes	4.0 – 9.0	2.5 - 7.5	10.0 - 45.0
Stenotrophomonas sp.	4.0 – 9.0	2.5 - 7.5	10.0 - 45.0
S. maltophilia	4.0 – 9.0	2.5 - 7.5	10.0 - 45.0
S. rhizophila	4.0 – 9.0	2.5 - 5.0	10.0 - 45.0

Based on Table 5, all the bacterial species able to grow at the pH 4.0 until pH 9.0 even the colonies were fewer at pH 4.0. They also can tolerate with the salinity until 7.5% except *S. rhizophila* that can survive only in 5% NaCl. The range of temperature they able to grow were 10.0 °C until 45.0 °C. They are considered as mesophile bacteria as they able to live at moderate temperature.

CONCLUSION

In conclusion, the causal agents for a disease that infected the iceberg lettuce (*Lactuca sativa* L.) were isolated and being identified as *Serratia Marcescenes, Stenotrophomonas sp., S. rhizophila* and *S. maltophilia* as they showed 100% disease incidence. Apart from that, biochemical characterizations were done to support findings in order to classify bacteria according to their biochemical activities and their enzymatic similarities. All the bacterial species were Gram negative bacteria with different morphological characteristics. All the bacteria were motile, indole negative and negative in sulphide production. All the bacteria preferred environmental conditions with the pH range 4.0, to 9.0, salinity between ranges 2.5% to 7.5%, and temperature at the ranges 10.0°C to 45.0 °C. Further research about on the causal agent treatment should be investigated for the better crop management.

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