IDENTIFICATION AND PATHOGENICITY OF *Pantoea* spp. IN LEAF BLIGHT DISEASE OF *Oryza sativa* L. LOCATED AT IADA BARAT LAUT SELANGOR

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KUALA LUMPUR

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IDENTIFICATION AND PATHOGENICITY OF *Pantoea* spp. IN LEAF BLIGHT DISEASE OF *Oryza sativa* L. LOCATED AT IADA BARAT LAUT SELANGOR

ABSTRACT

Leaf blight is one of the major constraints to rice production and this disease is commonly associated with bacterial species *Xanthomonas oryzae* pv. *oryzae* (Xoo). However, in recent years, bacteria belonging to the genus *Pantoea* had also been reported to cause leaf blight disease in rice-growing areas worldwide. The objectives of this study were to determine leaf blight disease infestation in rice fields using aerial monitoring, to identify leaf blight disease-causing agent in IADA Barat Laut Selangor rice fields, and to evaluate the pathogenicity of the leaf blight disease-causing agent on locally cultivated rice. A series of surveys were conducted in several localities in IADA Barat Laut Selangor rice fields from October to December 2018 using unmanned aerial vehicle (UAV) to monitor and identify areas with leaf blight disease infestation. Symptomatic leaves from the areas were collected, surface-sterilised and subjected to bacterial isolation on Trypticase soy agar (TSA) medium. The morphological characteristics of the isolates were determined. The isolates were then purified for biochemical assays including Gram-testing, catalase, oxidase, KOH, starch hydrolysis, and anaerobic tests. The isolates were subjected to a preliminary pathogenicity test against local rice cultivar, MR220 using clipping method. The isolates showing leaf blight disease symptoms were further analysed for molecular identification using 16SrRNA markers. The identified causing agents were confirmed with a second pathogenicity test against another local rice cultivar, MR220CL2. Aerial monitoring of rice fields identified 15 plots infested with leaf blight disease. The infestation coverage ranged from 6.6 to 48.2%. The isolates from the field yielded mild, yellow-pigmented colonies with undulated margin, raised elevation, and circular to irregular forms. The biochemical tests of the isolates displayed inconclusive

determination of putative causative agents for leaf blight disease. The preliminary pathogenicity test showed five isolates displayed symptoms of leaf blight disease. Molecular analysis identified SK09 and SK15 isolates as *Pantoea agglomerans* and *P. wallisii*, respectively. The second pathogenicity test confirmed these two putative agents to cause leaf blight disease to cultivated rice, MR220CL2 similar to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). This study proposes the usage of UAV as one of the monitoring tools in rice fields especially for the application of disease surveillance in rice granaries. This study also identified *Pantoea agglomerans* and *Pantoea wallisii* as new causative agents for new leaf blight disease for rice in Malaysia. An understanding of this newly emerged pathogen should lead to guidelines for rice breeders and agricultural practitioners for developing sustainable management strategies for dealing with outbreaks of this disease in the future.

Keywords: Leaf blight disease, precision agriculture, *Pantoea* spp., plant pathogen, rice disease.

PENGENALPASTIAN DAN KEPATOGENAN *Pantoea* spp. DALAM PENYAKIT HAWAR DAUN BAKTERIA UNTUK *Oryza sativa* L. DI IADA BARAT LAUT SELANGOR

ABSTRAK

Hawar bakteria merupakan salah satu punca utama pengurangan hasil padi dan dikaitkan dengan spesis bakteria *Xanthomonas oryzae* pv. *oryzae* (Xoo). Walau bagaimanapun, mutakhir ini, bakteria dari genus *Pantoea* telah dilapor menyebabkan hawar daun bakteria di beberapa kawasan penanaman padi di seluruh dunia. Objektif bagi penyelidikan ini adalah untuk menentukan serangan penyakit hawar daun di kawasan sawah padi melalui pemantauan dari udara, untuk mengenalpasti ejen penyebab penyakit hawar daun di jelapang padi IADA Barat Laut Selangor, dan menguji kepatogenan penyakit hawar daun bakteria ke atas varieti padi tempatan. Beberapa siri kajian lapangan telah dijalankan di kawasan Sawah Sempadan di IADA Barat Laut Selangor dari Oktober hingga Disember 2018 dengan menggunakan kenderaan udara tanpa pemandu (UAV) bagi memantau dan mengenalpasti kawasan sawah yang dijangkiti penyakit hawar daun. Daun yang bergejala telah diambil sebagai sampel, disterilkan permukaan dan diproses untuk pengasingan dan pembiakan bakteria di atas media soya triptikas. Ciri-ciri morfologi asingan tersebut telah ditentukan. Selanjutnya, asingan-asingan tersebut telah ditulenkan untuk ujian biokimia seperti ujian Gram, ujian pemangkin, ujian oksidase, ujian kalium hidrosida dan ujian hidrolisis kanji. Asingan yang terpilih dilanjutkan kepada ujian kepatogenan awal terhadap anak pokok padi tempatan, MR220 dengan menggunakan kaedah klipan daun. Asingan yang menunjukkan simptom hawar daun selanjutnya dianalisis untuk pengenalpastian molekul menggunakan jujukan 16sRNA. Ejen penyebab penyakit disahkan dengan ujian kepatogenan kedua dengan varieti padi tempatan, MR220CL2. Pemantauan udara telah mengenalpasti 15 plot telah dijangkiti dengan penyakit hawar daun. Kawasan yang diserang penyakit ini sekitar 6.6 hingga 48.2%. Asingan dari kawasan yang diserang penyakit ini menghasilkan koloni kuning sederhana, bentuk sisi

beralun, ketinggian menaik, dan bentuk bulat serta tidak teratur. Ujian biokimia ke atas asingan ini tidak dapat menetapkan ejen penyebab penyakit hawar daun ini. Ujian kepatogenan awal menunjukkan 5 asingan memberi gejala penyakit hawar daun kepada padi. Analisis molekul mengenalpasti asingan SK09 dan asingan SK15 sebagai *Pantoea agglomerans* dan *Pantoea wallisii*. Ujian kepatogenan kedua mengesahkan bahawa dua ejen ini sebagai penyebab penyakit hawar daun kepada varieti padi MR 220 CL2, seperti *Xanthomonas oryzae* pv. *oryzae* (Xoo). Penyelidikan ini mengesyorkan penggunaan UAV sebagai salah satu alat pemantauan di kawasan penanaman padi terutamanya untuk pemantauan penyakit padi di kawasan jelapang. Penyelidikan ini juga mengenalpasti *Pantoea agglomerans* dan *Pantoea wallisii* sebagai salah satu penyebab penyakit hawar daun yang baru bagi tanaman padi di Malaysia. Dengan pemahaman kemunculan patogen yang baru, garis panduan yang terbaharu perlu diolah oleh pembiak padi dan pengamal pertanian bagi mengembangkan lagi stratergi pengurusan mampan bagi menguruskan wabak ini pada masa hadapan.

Kata kunci: Penyakit hawar daun, pertanian jitu, *Pantoea* spp., patogen tumbuhan, penyakit padi.

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In the name of God, the Most Gracious and The Most Merciful.

About three years to complete this master research and now writing this note of thanks is the finishing touch on my dissertation. Writing this dissertation has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout the progress of my final year project.

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LIST OF SYMBOLS AND ABBREVIATIONS

16srRNA	:	16S Ribosomal ribonucleic acid
API20E	:	Analytical profile index 20E
ASM	:	acibenzolar-S-methyl
atpD	:	Adenosine triphosphate dehydrogenase
ATR	:	Acid tolerance response
BLAST	:	Basic Local Alignment Search Tool
BLB	:	Bacterial leaf blight
carA	:	Chimeric antigen receptor A
CFU	:	Colony forming units
DJI	:	Da-Jiang Innovations
DNA	:	Deoxyribonucleic acid
DOA	:	Department of Agriculture
EDTA (TBE)	:	Tris-borate ethylenediaminetetraacetic acid
EMBL	:	European Molecular Biology Laboratory
EPS	:	Extracellular polymeric substances
EtB"Out"	:	Ethidium bromide "Out" Nucleic Acid Staining Solution
GB Buffer	:	Galbraith's buffer
gDNA	:	Genomic deoxyribonucleic acid
GT Buffer	:	Guanidinium thiocyanate buffer
HR	:	hypersensitive reaction
IAA	:	Indole-3-Acetic Acid
IADA	:	Integrated Agricultural Development Authority
ISO	:	camera's sensitivity to light

KADA	:	Kemubu Agricultural Development Authority
KB	:	King's B
MADA	:	Muda Agricultural Development Authority
MAMPs	:	microbe-associated molecular patterns
MWS	:	Maize white spot
NA	:	Nutrien Agar
NCBI	:	National Centre for Biotechnology Information
NDVI	:	Normalized difference vegetation index
NIR	:	Near-infrared
NSCV-In	:	Natural Single Chromosome Vibrip-In
OEM	:	Onion extract media
PCR	:	Polymerase chain reaction
PGSA	:	*Pantoea* genus-specific agar
PR5	:	pathogenesis-related protein 5
proteinase K	:	serine protease
PR-proteins	:	pathogenesis-related proteins
recA	:	Recombinase A
RH	:	Relative humidity
ROS	:	reactive oxygen species
rRNA	:	Ribosomal ribonucleic acid
SAR	:	Systemic acquired resistance
SSL	:	Self-sufficient level
SSWM	:	Site-specific weed management
T3SSs	:	Type III Secretion Systems

TSA	:	Trypticase soy agar
TSB	:	Tryptic soy broth
UAV	:	Unmanned aerial vehicles
WAI	:	Week after inoculation
Xoo	:	*Xanthomonas oryzae* pv. *Oryzae*

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Rice is an important commodity crop which is a vital part of food security in Malaysia. Moreover, 4,443 kg/ha of average rice production was recorded in 2018, which yields 19% more production compared to the previous year. Despite the increase in rice production, self-sufficient level (SSL) is not enough to fulfil the needs of the population in Malaysia. Till now, the SSL is only at 72% and Malaysia is still depending on the imported rice (Kankyakumari 2017). For a decade, the production of rice in Malaysia was stagnant and declining.

The major rice growing granaries in Malaysia include MADA (1,063,247 ha), KADA (248,172 ha), IADA Barat Laut Selangor (222,033 ha), IADA Kerian (165,027 ha) and IADA Pulau Pinang (148,297 ha). Rice planting in Malaysia is mainly grown twice per year, where rainfall precipitation and temperature regime are available throughout the year. The utilization of high yield and herbicide-resistant varieties had boost rice production for a while, started with the introduction of rice varieties, MR220 CL1 and MR220 CL2. These two varieties are able to withstand imidazolinone-herbicide which is purposely made to control the weedy rice infestation problem.

Nevertheless, one of the threats that could suppress rice production in Malaysia is a wide broad of rice diseases including bacterial leaf blight (BLB). Moreover, there is no local rice variety that is fully resistant to BLB and continuous exploitation of the same susceptible-

moderate resistant variety for several planting seasons could give huge problems to rice farmers. According to Saad and Habibuddin (2010), rice yield can be reduced up to 50% by the infestation of BLB. The first BLB incidence in Malaysia was observed in the early 1980s and became widespread due to large scale utilization of susceptible variety MR84 which leads to approximately RM50 million loss of rice production (Saad 1994). The BLB disease is caused by the Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo). Infestation of BLB is difficult to control due to the rapid resistant build-up by the bacterium against bactericides and resistant varieties.

Recently, BLB has been reported to be caused by the bacteria belonging to the genus *Pantoea* in various rice growing regions. The new emergence of bacteria could disrupt disease management since Xoo has been widely identified as the only BLB's pathogen. The emergence of *Pantoea* species as causative agent for BLB was first discovered in Venezuela in 2002 (González *et al.,* 2015). Since then, numerous reports also stated the occurrence of *Pantoea* spp. as BLB-pathogen in rice agrosystem worldwide.

Generally, *Pantoea* spp. belongs to the group of *Gammaproteobacteria* and family *Enterobacteriaceae.* The genus *Pantoea* is a highly diverse group and can be found in a multitude of environments. According to Walterson and Stavrinides, (2015), isolates of *Pantoea* have been isolated from various sources including different plant hosts, soil and animals. At the present time, there are 20 species of bacteria belong to genus *Pantoea* and most of them are associated with various plant diseases. Briefly, Pantoea-belonging species are Gram-negative, having rod-shaped, yellow-pigmented, facultative anaerobic and movement facilitated by the presence of peritrichous flagella (Doni *et al.,* 2019).

The capability of *Pantoea* species to be phytopathogenic is not new. Different species have been found to infect various crops, including dicotyledonous and monocotyledonous plants. The reported *Pantoea*-infected crops include onion, eucalypts, maize and watermelon (Coutinho *et al.,* 2002; Yumiko *et al.,* 2005; Goszczynska *et al.,* 2007; Walcott *et al.,* 2003). The symptoms showed by the infected crops are various, depending on plant host. For instance, *Pantoea ananatis* able to cause leaf blight, seed stalk rot and bulb decay on onion crops as well as causing cause fruitlet rot (rot fruitlet) on pineapple. Another species reported is *Pantoea stewartii* which is commonly associated with Stewart's wilt on maize.

In the case of rice leaf blight, *Pantoea* species can be considered as a new emerging pathogen. Few reports were stated in the last few years regarding the species and their association with rice crops. Even though the bacteria are not a common causative agent for leaf blight, it has been reported to cause palea browning, grain discoloration and stem necrosis on rice (Azegami 1983; Tabei *et al.,* 1988; Cother *et al*., 2004). Considering the importance of rice leaf blight caused by *Pantoea* spp, there is an urge to explore the pathogenicity and identify the exact species of rice leaf blight-causing pathogen.

1.2 Research Question

Primarily, the rice leaf blight disease is caused by bacteria *Xanthomonas oryzae* pv. *oryzae*. Recently, *Pantoea* spp. was described as one of the causal agents for many crop diseases including rice worldwide. This suggested that there are other species capable to cause the same rice disease as Xoo. Therefore, is there any potential for other bacteria to cause rice leaf blight disease in Malaysian rice agrosystem?

1.3 General Objectives

The general goal of this study is to investigate the morphological, biochemical, disease pattern and pathogenicity of the collected pathogenic bacteria found in rice leaf, *Pantoea* spp in IADA Barat Laut Selangor.

1.4 Specific Objectives

This study is designed to achieve the following objectives:

1. To determine the leaf blight disease infestation areas using aerial monitoring.
2. To identify leaf blight disease causing bacteria in IADA Barat Laut Selangor.
3. To evaluate the pathogenicity of the identified bacterial isolates on locally cultivated rice in glasshouse condition.

1.5 Hypothesis

1. The Unmanned Aerial Vehicle (UAV) will increase the human capacity to detect leaf blight infestation in the field.
2. There is a new causative agent of leaf blight disease in Malaysian rice granaries.
3. The new causative leaf blight agent possesses similar properties as well as disease symptoms as *Xanthomonas oryzae* pv. *oryzae*.

CHAPTER 2: LITERATURE REVIEW

2.1 Biotic and abiotic stress in rice production

Rice is the second most widely grown crop in the world which is consumed by more than half of the world population. This crop is rich in carbohydrates and proteins that provides essential nutrient for people. Rice does not only act as food crop for people but also contribute in people employment and become a vital part against world hunger and global poverty, especially in developing countries (Siwar *et al*., 2014). However, rice, like other major crops, is vulnerable to climate change and the production might deteriorate in the future.

Climate change will affect the surrounding temperature, rainfall variability, accessible of standing water and the migration of insect either pest or pollinator. Mishra *et al*., in 2014 stated that rainfall variability and high temperature greatly reduced rice production in Bangladesh and the application of flood-tolerant rice is needed to enhance the yield. Water availability influences the growth of pest such as rice root-knot nematode, *Melodogyne graminicola* and the absence of water in certain area could increase nematode population in rice granaries in Asia (Cabasan *et al*., 2012).

In 2008, the mass migration of rice pest, small brown planthopper (SBPH) *Laodelphax striatellus* occurred at rice field in western Japan and had proven that this species also caused the outbreak of rice stripe disease in that province (Otuka, 2013). By using meteorological data and generic process-based infection model, the raising of global temperature will initially increase the infestation of both fungal pathogen *Bipolaris oryzae* and *pyricularia*

oryzae and both are expected to give some pressure against rice production in all rice producing countries in Europe from 2030 to 2050 (Bregaglio *et al*., 2013).

As a rice growing country, Malaysia (particularly in peninsular region of Malaysia) also will be affected with climate change, which mainly grown as irrigated lowland crop which comprises eight major granaries including in Kedah, Perak, Selangor, and Kelantan. As a rainfed crop, Malaysian rice is highly dependent on water availability. Thus, global temperature rise, uncertain rainfall and new emergence of dominant pest-pathogen are the factors concerned by the Malaysian rice farmers.

2.2 Use of Unmanned Aerial Vehicle (UAV) in agriculture

The application of Unmanned Aerial Vehicles (UAV) has emerged globally in the last decade. By definition, UAV is an aircraft without any human pilot that is controlled autonomously by a ground-based controller and commercially known as a drone. Drones have been found to achieve a multitude of aims in agriculture. Their applications ranging from growth monitoring, biomass, yield estimation of fertilizer, weeds and pest management, water stress assessment, and etc.

For instance, in conventional farming, herbicides are sprayed randomly in the field even in weed-free area. By deploying UAV as a spraying tool, herbicides can be used efficiently by spraying at the particular weed area, resulting in less unnecessary waste of resources. This practice is known as Site-Specific Weed Management (SSWM). SSWM can be defined as spatial variable application of herbicides (Tsouros *et al.,* 2019). To obtain accurate application, UAV will be used to gather data (images of field) which can be used as a weed

mapping for site-specific herbicide application and also for the crop health monitoring. Crop health is influenced by many factors including diseases and conventionally, done by human experts, yet it is arduous and time consuming.

UAV application which is based on crop imaging can be used to identify the changes on plant health. For instance, by detecting the slight changes of the plants especially their colour saturation, potential diseases can be detected. The current UAVs are mostly equipped with RGB sensors which is able to process aerial images into orthomosaic map and maintain the image quality as authentic as they are on the field. This criterion is important in crop disease monitoring. Application of UAV-based RGB imagery system can enhance disease surveillance of banana's diseases with high accuracy in Congo and Benin (Selvaraj *et al.,* 2020). The advancement of the crop disease surveillance is influenced by UAVs that are able to fulfill the temporal, spatial and spectral requirement to detect disease symptoms. This can be assisted by integrating UAVs with more advance sensors such as Near-infrared (NIR) sensor. This sensor is able to process Normalized Difference Vegetation Index (NDVI) images which has better accuracy of crop health over time. By deploying all these technologies, agricultural activities are becoming more advance and evolve to the next level.

2.3 Bacterial Leaf Blight

Bacterial Leaf Blight (BLB) is one of the major constraints in rice production. BLB is a vascular disease, caused by gram-negative bacteria, *Xanthomonas oryzae* pv. *oryzae* (Xoo). BLB becomes a major problem in rice agro-system by reducing 70 percent of rice production in South Asia (Naqvi *et al*., 2016). This high reduction is attributed from the reduction of leaf surface area which lead to low grain production. In fact, Pakistan and Tropical Asia

recorded high loss of rice production due to BLB which range from 2% to 74% and were highly affected due to local climatic conditions, crop age and rice cultivars (Manzoor *et al.,* 2017). In Malaysia, BLB was first detected in the Peninsular's rice granaries in early 1980's (Saad, 1994; Sankaran *et al.,* 2010). Furthermore, there is no systemic data that shows the diversity and effective control measures for this phyto-pathogen. The severity of this disease in South Asia might be caused by the heavy rainfalls throughout the year (Naqvi *et al.,* 2016). The pathogen can be transmitted through windblown and rain splash from irrigation to rice plant. For plant-to-plant infection, contaminated tools that were used in transplanting, fertilizing and weeding are able to maximise the transmission of pathogen.

2.3.1 Symptoms of BLB in rice

The symptoms that caused BLB in rice is conspicuous on the tip of the leaf. It causes browning and scorching pattern and eventually will lead to the death of the leaf cell (Figure 2.1). It is also known as systemic infection because the pathogen will spread throughout the plant system and disrupt the growth and physiology of the plant. Reduction of the leaf area will affect photosynthesis process as the chlorophyll in the cell cannot be synthesized and eventually will lead to low quality and production of the rice grains. Bacterial leaf blight can occur at different rice stages. Infection during seedling is known as '*kresek'* in which is typically due to the wound during transplanting. This syndrome is very devastating as the rice could be destroyed if the infection occurs in early stage. Other than that, rice yield was also reduced significantly due to leaf blight infection at panicle formation stage (Noh *et al*. 2006).

Figure 2.1: Brown and dried disease lesions on rice leaf indicating infection of bacterial leaf blight

2.3.2 *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae is a gram-negative bacterium that caused vascular disease in rice (Gnanamanickam, 1999). This bacterium is a slime-producing bacterium and normally appears as yellowish colonies on the agar plate. Xoo infects the host plant via two sites, either by hydathodes or wounds. Wounded area is a preferable and successful entrance for Xoo compared to hydathodes and other natural openings (Gnanamanickam *et al.,* 1990). This pathogenic bacterium chiefly enters hydathodes through connecting tissue called epitheme

before they multiply and grow until the xylem is blocked (Shen & Ronald, 2002). Appropriate and suitable inoculation method is required to ensure the bacteria enter the host plant. Leaf clipping method or dipping of non-leaf parts of rice with bacterial suspension can be used (Mew, 1984). These methods are reliable to induce injury for bacterial entrance to the host plant's leaf by cutting off a bit leaf tips with scissors that is dipped with Xoo suspension (Yinggen *et al.,* 2017).

2.3.3 Factors contribute the BLB infestation

The BLB infestation can be caused by several factors such as harsh weather and excessive fertilizer. The severity of disease infestation is affected by the amount of fertilizer, especially nitrogen. Early study conducted in Korea reported that rice field with high amount of fertilizer shown higher infestation of BLB (Cha *et al.,* 1982). Both deficiency and excessive nitrogen could increase the susceptibility of the rice to the leaf blight disease (Manzoor *et al.,* 2017). Excessive application of nitrogen would make plant fresher and watery in which will expose the plant to the microbial infection. The low rate of nitrogen will induce susceptibility of the plant against pathogens. BLB is observed more severe on the susceptible rice varieties when nitrogen was applied in low amount. Nitrogen deficiency altered and reduced morphological and physiological characteristics of the crop limitation of growth, leaf number and leaf area. This factor highlighted on the role of nitrogen fertilization for the degree of disease infestation. Similar with the other fertilizer, prolong use of nitrogen fertilizer exposes crop to various disease, including bacterial leaf blight.

2.3.4 Management of Rice Bacterial Leaf Blight

Current control for BLB is by using chemical control but it is not impracticable due to the absence of specific bactericide that are able to suppress the infestation of *Xanthomonas oryzae* (Saad & Habibuddin, 2010). According to Tagami & Mizukami (1962), BLB can be controlled by spraying copper-oxychloride and streptomycin solution to the infected crops. Low concentration of dicarbamoylacetylin is able to supress the growth of *Xanthomonas oryzae* in liquid form (Okimoto and Misato, 1963). Chlorinating the irrigation water by stable bleaching powder is also recommended to prevent the outbreak of the disease (Gnanamanickam, 1990) but chemical controls cannot be sustained for a long time. For biological control, *Xanthomonas oryzae* can be suppressed by using the combination of antagonistic bacteria and *Pseudomonas fluorescens* (Gnanamanickam, 1990). The control measure of BLB is becoming more complicated as the pathogen able to adapt rapidly with the applied chemical and surrounding environment. The ability to adapt may be due to the variability and diversity of the population of the pathogen. The most suitable and sustainable way to control the disease is by plant breeding approach. Sustainable agricultural system can be defined as the use of infinite renewable resources in agriculture and can be sustained for a long period of time (Gerber 1992). With discovery and unlimited access of desirable alleles, disease resistance crop can be improved. According to Bradshaw in 2017, utilizing desirable alleles from wild/elite germplasms in crop breeding is important for crop improvement, including disease resistance (Bradshaw et al. 2017).

2.4 *Pantoea*, newly emerging bacteria of rice leaf blight

2.4.1 *Classification of Pantoea spp.*

Generally, members of the genus *Pantoea* are non-encapsulated, non-spore-forming Gram-negative bacteria from Enterobacteriaceae family and can be isolated from diverse environments (Tambong et al. 2019). The enterobacterial plant pathogen is considered diverse, versatile species and have been collected and isolated from numerous ecological niches. According to Walterson and Stavrinides in 2015, bacterial genus *Pantoea* was described 25 years ago, there are approximately 20 species that have been identified from various environments. In 1929, all *Enterobacteriacea*-pathogenic bacteria were included in genus *Erwinia*, based on similarity of gram-negative, non-spore forming, fermentative and morphological characteristics (Winslow *et al.,* 1920). The genus *Pantoea* was first used and proposed by Gavini *et al.,* in 1989 to merge the species that belong to the same DNA hybridization group which is known as *Pantoea agglomerans*. Since then, more species have been described in this genus including *P. dispera* (Gavini *et al.,* 1989), *P. punctata, P. citrea, P. terrea* (Kageyama *et al*., 1992), *P. stewartii*, *P. stewartia* ssp. *indologenes, P. stewartia* ssp. *Stewartia and P. ananatis* (Mergaert *et al*., 1993).

Table 2.1 Reports of infected commodity crops by *Pantoea* spp.

Commodity Crops	Diseases	Bacterial Pathogen	References
Rice	Leaf Blight	*P. agglomerans*	González *et al*., (2015)
		P. ananatis	Mondal *et al*., (2011)
		P. agglomerans	Lee *et al*., (2010)
		P. ananatis	Egorova *et al*., (2015)
		P. ananatis and *P. stewartii*	Kini *et al*., (2017a)
		P. ananatis and *P. stewartii*	Kini *et al*., (2017b)
		P. agglomerans and *P. ananatis*	Aksoy and Boluk (2019a, b)
		P. ananatis, *P. dispersa* and *P. stewartia*	Toh *et al*., (2019), Azizi *et al*., (2019)
Onion	Center Rot	*P. ananatis*	Walcott *et al*., (2002)
		P. ananatis	Gigaitis *et al*., (2003)
		P. allii	Brady *et al,.* (2011)
		P. ananatis and *P. agglomerans*	Dutta *et al*., (2014)
		P. stewartii	Stumpf *et al*., (2018)
Corn	Stewart's Wilt	*P. stewartii*	Freeman and Pataki (2001)
		P. stewartii	Pataki (2003)
		P. stewartii	Herrera *et al*., (2008)
Cotton	Boll Rot	*P. agglomerans*	Ren *et al*., (2008)
		P. agglomerans	Yaqin *et al*., (2008)
		P. agglomerans	Ehetisham *et al*., (2014)
Beet	Gall Formation	*P. agglomerans*	Manulis and Barash (2003)
		P. agglomerans	Nissan *et al*., (2019)

Phylogenetic relationships information and relatedness among *Pantoea* species is vital for their proper identification. Based on three protein-coding genes *atpD*, *carA*, and *recA, P. agglomerans, P. ananatis, and P. stewartii* were analysed and considered as close-related species by using 16s rRNA analysis (Alexis *et al.,* 2009). *Pantoea*-belonging bacteria were isolated from various sources including plants, seeds, water, human's blood and urine. The genus *Pantoea* can be classified under class Proteobacteria in which comprises of mostly single-celled bacteria. This genus also belonging to the family *Erwiniaceae* and division of Gracilicutes (Agrios, 2006). Bacterial leaf blight commonly appeared on the leaf plant. It will change the coloration, reduce pigment chlorophyll and surface area for photosynthesis. Similar to common pathogen for rice leaf blight *Xanthomonas oryzae*, bacteria belonging to the genus *Pantoea* also shows same leaf blight symptoms. Till now, in total, there are about 25 identified species of *Pantoea* that retrieved from various sources including water, soil and plants. Newly, seven of the species have been classified into two genera, namely genus *Tatumella* and *Mixta* (Brady et al. 2010; Palmer et al. 2018).

2.4.2 Characteristics of Pantoea spp.

The bacteria are capable to proliferate in a wide niche of environment. The rate of infection and adaptability to surrounding environment are largely determined by the movement of pathogen within host plant. The bacteria are able to move fast due to having flagellum as their swimming mobility. Thus, motility is an essential phenotype for plant pathogenic bacteria. Most of the *Pantoea* strains are motile with peritrichous flagella that appeared all over the surface with counterclockwise rotation movement. Pathogenic strain of *Pantoea ananatis* was found to have flagellar secretion system which assist in attachment on the host cell and marked obvious symptoms on onion leaves (Weller-Stuart *et al*., 2017). The genus *Pantoea* in the

Enterobacteriaceae typically will form yellow colonies, rod-shaped and possess gram-negative cell wall. Vinodhini *et al*. (2017) and Carrerfilho *et al*. (2017) reported that collected *Pantoea* isolates from blight-infected rice were gram-negative, yellow pigmented with smooth margin, non-sporogenic, has translucent colonies and exhibit facultative anaerobes. Isolated pathogen that caused necrotic stem lesion of rice was off-white to yellow bacterial colony on King's Medium B (KB) medium (Cother *et al*., 2004).

Common pathogenic bacteria will possess special characteristics such as biofilm production. According to Centre of Disease Control, 60% of the bacterial infection are caused by biofilm-producing bacteria. Biofilm production is important for microbial as they act as a defense mechanism against antimicrobial agents. Other than that, it is also important for microbial aggregation once the bacteria attached to the host. *Pantoea stewartii* that caused Stewart's vascular wilt and maize leaf blight, was identified to have biofilm-producing ability which plays significant role for host colonization (Herrera *et al*., 2008). This phyto-pathogen attach to the plant xylem by surface-adherent biofilm and surface swarming to form bacterial aggregation. Hasson *et al*., (2018) reported the occurrence of biofilm-producing bacteria from isolated sample from human urine which then has been identified as *Pantoea* sp. and *Serratia fonticola.* The information was supported by the detection of SmaI and EsaI, two quorum sensing genes that responsible for strong multi-drug resistance in these bacteria.

2.4.3 Pantoea spp. isolated in agricultural ecosystem

Pantoea sp has diverse roles which can be as plant pathogen, cause human disease, plant growth promoter and biological control to other plant pathogen. Few bacterial strains from genus Pantoea were discovered for their unique microbial potential that can enhance plant growth. *Pantoea alhagi* was identified to possess various plant growth promoting abilities, including mineral phosphate solubilization, production of Indole-3-acetic acid (IAA), siderophores, protease and ammonia (Chen *et al*., 2017). Isolated *Pantoea ananatis* from root

and rice leaf was identified to have growth promoting properties such as production of siderophores, auxins and cellulase (Megías *et al*., 2017). One of the Pantoea strain was found to enhance the oxidizing environment in rice rhizosphere by improving root expansion and Fe uptake (Lakshmanan *et al*., 2015). *Pantoea* is also known as epiphytic and endophytic bacteria which are able to colonize external and internal part of the plant.

2.4.4 Pantoea spp. as phytopathogens

Newly emerging pathogens have a significant effect on the economy, especially in agriculture sector. Leaf blight is widely known as a destructive disease on paddy crops. The causative agent that is responsible for this vascular disease is *Xanthomonas oryzae*, however, there are a few reports stating that this disease is caused by other phyllo sphere bacteria recently. Several species from genus *Pantoea* are reported causing leaf blight on several crops, including rice. Various species from this genus are capable to cause galls, wilting, soft rot, and necrosis in rice (Walterson & Stavrinides, 2015). On the other side, *Pantoea*-belonging species are also a pathogen for several crops.

The plant-pathogen association of *Pantoea* species with gramineous plant has been reported in Japan (Azegami *et al.,* 2013) and in Australia (Cother *et al.,* 2004). *Pantoea ananatis* is found to cause palea browning of rice in Japan which spread during flowering stage and degrade the quality of grain (Azegami *et al.,* 2013). Stem of rice that is infected by stem necrosis which is caused by *Pantoea ananas* was observed weaker and brown in color in Australia (Cother *et al.,* 2004). Water-soaked lesions and necrotic were observed on maize leaf due to maize white spot (MWS) which caused by *Pantoea ananatis* in Brazil (Sauer *et al.,* 2015). Maize leaf will appear straw in colour, form white leaf spots and eventually the entire leaf will dry. Meanwhile, *Pantoea stewartii* that commonly infected maize, was found to cause Stewart's wilt on maize crop in Argentina (Orio *et al.,* 2012). Brown and dried streaks with wavy margin

was observed on the leaf blade of maize and lead to the death of the leaf. Several reported cases stated that the leaf is the common infected site for typical blight disease.

Rice leaf blight in Korea clearly appeared on the upper leaf blight as water-soaked lesions and usually occurred on post-flowering (Lee *et al*., 2010). Infected palea or lemma of rice showed water-soaked lesions which later turned brown and consequently produced spoiled grains (Egovora *et al*., 2015). Yellowing to brown stripes appeared on both halves of the leaf margin and turned to grayish-brown when the disease is getting severe (Kini *et al*., 2016). Rice leaf was observed to have brown-to-slightly reddish spots on the upper leaf blade after being infected by *Pantoea agglomerans* (Aksoy & Boluk, 2018). Based on leaf blight survey in Malaysian rice granaries, 80% disease incidence was reported with water-soaked lesions and brown stripes on the upper part of leaf (Azizi *et al*., 2018). Toh *et al.* (2019) stated that the healthy green rice leaf turned brownish stripes and become pale and dry after being inoculated with *Pantoea* strains.

Other than rice leaf blight, *Pantoea*-belonging bacteria also have been reported to cause various plant diseases and have wide host range for both monocotyledonous and dicotyledonous plant. This genus has important role as phyto-pathogen. As stated by Coutinho *et al*., 2009, *Pantoea ananatis* able to cause blotches, spots, die-back and bulb rot. In addition, this species also has been reported to cause brown spots in honeydew melons which occurred during post-harvest stage (Bruton *et al*. ,1991). The host range of *Pantoea* spp. is quite ubiquitous and fairly wide especially for agricultural crops which stated in Table 2.4. Common crops that usually infected by Pantoea spp are rice, onion and corn.

However, non-agricultural plants are also found to have epiphytic relationship with the bacteria. At least 25 asymptomatic weed species were found to be potential seed-borne inoculum for *Pantoea ananatis* including crabgrass, wild radish, yellow nutsedge, Texas panicum, Bermuda grass, broadleaf signal grass, sickle pod and spiny amaranth (Gigaitis *et al*.,

2002). These species were responsible for the center rot of onion and presumed to have narrow host range. The onion will possess white lesions along the leaf margin with water-soaked symptoms and eventually cause it to darken, dry and wilt. In time, the interior and center of the onion bulb turns discoloured and rotten. In another report, *Pantoea allii* was isolated from onion seeds and plants that exhibited similar symptoms of center rot (Brady *et al*., 2011).

2.4.5 Media used and Factors affecting the growth of Pantoea spp.

In general, bacteria need certain micronutrient such as iron for growth, replication and metabolism. Pathogenic bacteria develop various mechanism in order to obtain iron from host. This includes production of certain bioactive compounds such as siderophores. Siderophores known as powerful ferric iron-chelating molecules that is usually produced by microorganism which act as effective iron carrier from host environment (Page, 2019). One of the types of siderophores is hydroxamate. Hydroxamate is an organic compound bearing the functional group N-hydroxy amide and has ability to sequester iron from the host which is essential for bacterial metal nutrient.

Temperature and pH value also plays an important role for bacterial growth. *Pantoea* species have wide ecological niches. Thus, these bacteria are able to grow and adapt in various environment. *Pantoea agglomerans* is stated as mesophilic bacterium with –6° C minimum temperature (Costa *et al*., 2002). Isolated *Pantoea* sp from sub-alpine soil was found to be cold-tolerant bacterium which able to grow in temperature ranging from 4 to 42°C (Selvakumar *et al*., 2007). In contrast, *Pantoea* sp. was found to grow in arid climate soil which relatively high temperature (Abbas *et al*., 2017). In Colorado onion-growing field, leaf blight pathogen, *Pantoea ananatis* can grow in average high temperature, ranged about 28 to 35°C (Schwartz *et al*., 2003). *Pantoea*-belonging bacteria has also been reported to be acid-tolerant. *Pantoea agglomerans* was observed grew in acidified media with pH value of 5.5 to 4.0 (Cañamás *et al*., 2007). The acidic environment induces high acid tolerance response (ATR) of bacteria that

make them survive in malic and citric acid. However, the acid-tolerance of bacteria depends on the level of acidity and type of organic acid used. High pH of acetic acid is found to be toxic to *Pantoea* spp. growth.

Most of the bacterial media for Enterobacteriacea can be used to culture *Pantoea*-belonging species, such as blood agar, nutrient agar, tryptic soy agar or MacConkey and Hektoen media (Grimont & Grimont, 2005). General media such as nutrient agar and Trypticase Soy Agar (TSA) are also commonly used in plant pathogen isolation. In order to obtain target bacteria, selective media are required. *Pantoea ananatis* from infected rice crops can be isolated selectively by using NSCV-In media which are developed by Hasegawa *et al*., (2003). Other than that, PA 20 semi-selective media for *P. ananatis* that are isolated from infected onion seeds also have been designed by Goszczynska *et al*., (2006). The media was developed based on the utilisation of D (+) arabitol by the bacteria and not for the other microbial community associated with the infected seed. However, it requires long incubation period for the bacteria to form colonies. Rapid isolation media was developed for onion-pathogenic bacteria including *P. ananatis* and *P. agglomerans* and both bacteria grew well and appeared yellow and pale yellow respectively on selective Onion Extract Media (OEM) at 28°C in 36 hours incubation (Zaid *et al*., 2012). The selectivity of OEM might be due to the addition of crystal violet, cycloheximide and anti-microbial properties of onion extract itself that can inhibit many fungal and bacterial species. This semi-selective media is made for rapid identification of the pathogen associated with onion.

2.5 Recent management of bacterial leaf blight

Various approaches to control rice leaf blight caused by *X. oryzae* have been applied since the first outbreak of the disease. Proper strategy and planning are required in order to manage the disease comprehensively, including pre- and post-treatment. Rice field soil are commonly treated by using calcium carbonate ($CaCO_3$) powder 25 days before planting in order to increase soil pH and to control certain bacteria. According to Guo and others in 2019, application of $CaCO_3$ potentially inhibit the bacterial growth from Xanthobacteraceae family. Certain Xanthobacteriaceae bacteria such as *Xanthobacter xylophilus* are found to be moderately acidophilic which live in soil pH ranged about 4.8-6.8 (Zaichikova *et al*., 2010). Thus, by increasing soil pH, some acidophilic bacterial community including pathogenic species could be declined. $CaCO_3$ powder also act as a pre-planting fertilizer which supply minerals such as calcium and magnesium to the soil. Two types of $CaCO_3$ that is typically used for soil treatment of rice field are dolomite and calcite.

The most common and affordable practices is by chemical spraying. Chemical substance that possesses antimicrobial properties is required to control bacterial leaf blight effectively. Chitosan solution is proven to have strong antibacterial activity against plant pathogen including rice leaf blight pathogen. Chitosan involves directly in the cell lysis, destruction of the bacterial biofilm and increase bioactive production in rice such as phenylalanine ammonia lyase, peroxidase and polyphenol oxidase (Li *et al*., 2012). Chitosan can be applied as seed coating solution, foliar treatment and soil amendment. Pre-treatment of rice seed could be done by soaking seed with 80 ppm polymeric chitosan that could improve disease resistant ability (Boonlertnirun *et al*., 2017). According to Bhaskara Reddy *et al*., (1999), chitosan application as seed coating agents induces resistant of wheat seed against *Fusarium graminearun* and improves germination rate.

Chitosan provides broad spectrum antibacterial properties against pathogenic bacteria including *Xanthomonas campestris, Erwinia carotovora* and *Pseudomonas fluorescens* (Entsar *et al*., 2003). However, the effectiveness of chitosan is heavily dependent on the degree of acetylation, the concentration used and types of pathogens encountered.

Chemical approaches to overcome rice diseases began as early as 1950s with the application of Bordeaux mixture and toxic compounds such as mercury and copper. Thus, the application of those compounds eventually would affect rice grains during harvesting. Spraying of probenazole-based agrochemical can be used as pre- and post-planting to inhibit bacteria growth. It is commonly used as seedling box treatment and granular treatment in rice agrosystem (Oostendorp *et al*., 2001). Probenazole is known as plant activator that are capable to invoke plant systemic acquired resistance (SAR) and have been used by Japanese rice farmers since 1975 against rice blast (Iwata *et al*., 2001). Probenazole able to activate plant defense system through formation of resistance-type lesion, activation of defense-related phenylpropanoid pathway, amplification of superoxide production and induction of defense-related genes (Iwata *et al*., 2004). According to Mahmood *et al*., (2009), pathogenesis-related protein 5 (PR5) in probenazole-treated rice seedlings was significantly increased, indicating systemic resistance successfully activated against *X. oryzae.* In plant defense system, production of reactive oxygen including superoxide (O_2) is important against pathogenic microbial infection.

Another discovered plant activator that is commonly used against pathogenic *Xanthomonas* spp. is acibenzolar-S-methyl (ASM). ASM is a synthetic molecule that analogous to salicylic acid. The plant activator enhances the production of pathogenesis-related proteins (PR-proteins) and concentration of secondary metabolites in plant's intercellular spaces and lead to systemic resistance against pathogen attack (Nascimento

et al., 2015). Babu *et al*., (2003) reported that *X. oryzae* unable to colonise ASM-treated rice after 3 days of being inoculated. Furthermore, secondary metabolites related to plant defense mechanism such as chitinase and β-1,3-glucanase were observed more prior to ASM treatment. According to Talreja *et al*., (2017), synthetic plant activator acibenzolar-S-methyl was able to reduce multiple virulence determinants of Xoo.

Recently, biological approaches have caught farmers' and researchers' attentions as there are very sustainable and biological-based approaches that enlighten alternative ways on how to control crop diseases. Discovery of antagonistic microbes that able to suppress BLB causal agents such as *Bacillus* spp. (Vasedevan *et al*., 2002), *Pseudomonas aeruginosa* (Yasmin *et al*., 2017), *Trichoderma* spp. (Verma *et al*., 2007) might accelerate more studies on this control. Isolated chemoorganotrophic bacteria, *Serratia nematodiphila* from rice field soil was found to have antagonistic effect to BLB-causal agent which can be observed through a dual-cultural test (Khoa *et al*., 2016). It is also capable to reduce pathogenesis under both greenhouse and field conditions. As other living things, microbial communities also compete for essential elements for living. In this case, iron is the most important macronutrient for bacteria. Under iron-depleted condition, bacteria that is capable to significantly produce siderophore that functions as iron chelating compound, will be aggressively outcompete other pathogenic bacteria. A study conducted by Khoa *et al*., (2016) revealed the bacterial strain, *S. nematodiphila* has great siderophore production compared to others which can be used to suppress pathogenic bacteria populations. According to Compant *et al*., (2005), *Serratia* spp. biocontrol activities were mediated by siderophore and lytic enzyme production. Furthermore, the application of *S. nematodiphila* can be considered as safe as this genus are quite common in the environment and no evidence found yet that it can cause any disease to human or animal.

The application of beneficial fungal also has been reported to give better outcome in combating crop diseases. Up until now, genus *Trichoderma* has been registered for more than 60% as biocontrol for various crop worldwide (Singh *et al*., 2018). The application of *Trichoderma*-based formulation can be considered as environmental-farmer-friendly as it gives no harm for both human and plants. For the case of BLB, the integrated application between *Trichoderma harzianum* and *Pseudomonas fluorescens* resulting in effective suppression effect against causation agent (Jambhulkar *et al*., 2018). Leaf blight was successfully reduced after application of *T. harzianum* as foliar spray on rice after the first appearance of disease symptoms (Gangwar *et al*., 2012). Harman (2006) revealed that *Trichoderma* spp. have the capability to release wide range of extracellular enzymes that can function as plant-pathogen inhibitor. It was believed that the induction of plant defenses is due to the production of microbe-associated molecular patterns (MAMPs) by *Trichoderma* spp. (Hermosa *et al*., 2012). The ability of the fungal to release MAMPs is associated with the molecules signaling that lead to resistance induction within plant such as salicylic acid, ethytlene and jasmonic acid. Study conducted by Yoshioka *et al*., (2011) revealed that salicylic acid, jasmonic acid and ethylene signaling pathway is important in induction of systemic resistance in *Arabidopsis thaliana* when treated with *Trichoderma asperellum*. The anti-microbial properties of the fungal species have been utilized as biocontrol agents, plant growth promoter and biological fertilizer. *Trichoderma*-belonging fungi are known to have various functions including promoting systemic and localized resistance plant defenses against pathogenic microbes and adaptation towards abiotic constraints (Bigirimara *et al*., 1997; Doni *et al*., 2018).

CHAPTER 3: METHODOLOGY

3.1 Disease monitoring

The aerial and monitoring observation of the leaf blight disease at IADA Barat Laut Selangor rice fields were carried out from October until December 2018. Disease surveillance using UAV had identified five plots infected with leaf blight disease at three locations named Sekinchan (3°33'27.4'N 101°06'01.7'E); Pasir Panjang (3°34'16.1'N 101°05'29.4'E); and Sungai Besar (3°66'2937'N 101° 04'78.59'E). Observation was carried out to detect the infestation and distribution of the disease in nature. Disease images were taken by using Unmanned Aerial Vehicle (UAV), DJI Phantom 4 Pro with an imbedded camera (Figure 3.1)**.** The setting was adjusted for 24 mm focal length, 100 ISO and 1/320 of shutter speed. Image focus was set using the control panel and followed by saving the focused image.

All the data images captured were visualized via DJI Go 4 software. Images were taken during mid-day at 10m, 20m, 30m and 100m high from the sea level. These heights were designated to ensure the disease pattern and distribution can be clearly observed from aerial view. The symptoms appeared in field were visually detected, observed and recorded (Figure 3.2). Infected field images recorded at height of 30m were selected for infestation coverage. The disease can be clearly identified by brownish coloration of the infected plants at this height. Images were processed and cropped using photo mobile application Lightroom. The grid was manually drawn on the images using mobile application Grid#. The percentage of infestation was determined by counting the grid (as pixel) having areas with infected plants. The images were processed by inserting grids squares in the image to calculate the percentage of rice fields infected with the leaf blight disease (Figure 3.3). The percentage infestation was calculated based on the infected area within the square grid.

Figure 3.1: Location of three observation sites of leaf blight disease in Selangor

Figure 3.2: DJI Phantom Pro 4 (FOV 84° 8.8 mm/24 mm, f/2.8 - f/11 auto focus at 1 m - ∞, 100 - 3200 (Auto)) with iPad Apple for disease aerial monitoring.

Figure 3.3: Detected rice leaf blight disease-infected plot (a) and comparison between healthy plot (b) in IADA Barat Laut Selangor.

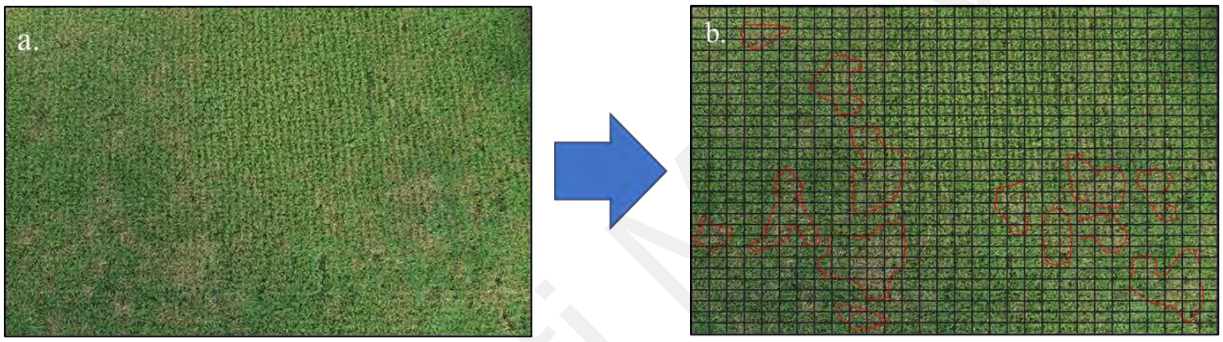

Figure 3.4: Images of detected leaf blight infected plots were converted into grid to estimate infestation area (indicated by red line) (a) Before converting into grid-formed image (b) After converting into grid-formed images.

3.2 Samples Collection

A total of 16 bacterial isolates were collected from IADA Barat Laut Selangor in December 2018. The bacterial isolates were collected at three different fields by extracting the ooze from the infected leaf. The symptomatic leaf was visually observed and characterized based on the lesion colour, appearance and length. The infected leaf was cut approximately 5 cm and partially soak in peptone water for 5 minutes. Bacterial ooze was carefully observed to ensure it mix well with peptone water (Figure 3.3). The collected samples were then stored at 4 °C for long term storage.

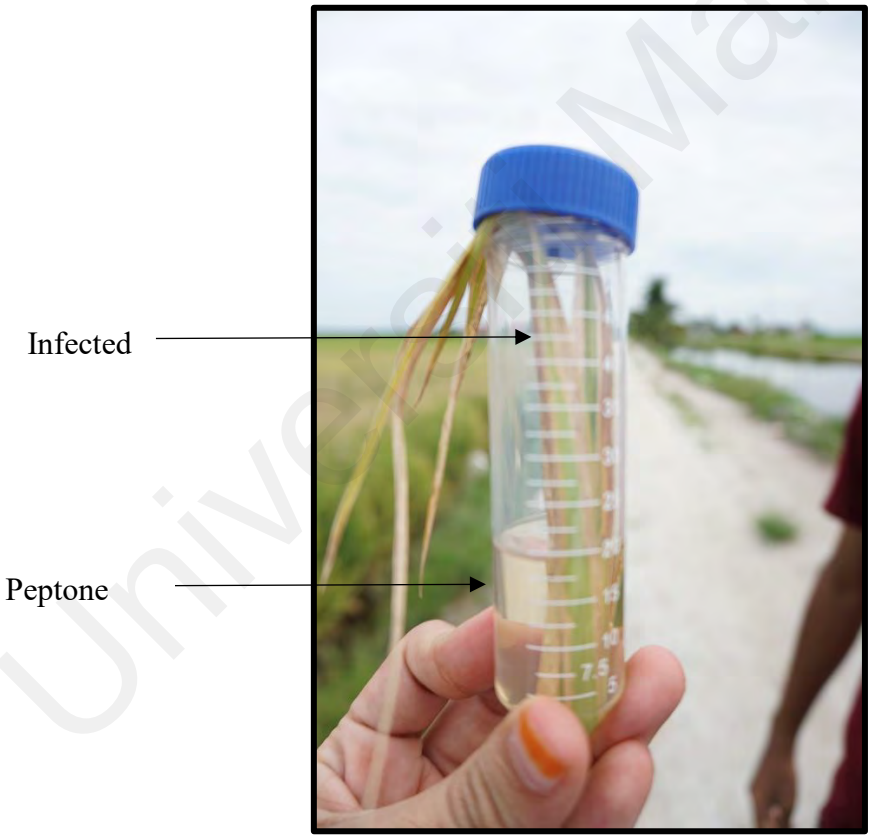

Figure 3.5: Infected leaf was dipped into peptone water for 5 minutes.

3.3 Pathogen Isolation

Isolation of the bacteria was carried out from the fresh infected leaf. The infected leaf showed typical brownish lesion on the leaf, starting from the tip. This water-soaked lesion then spread along the margin to the lower part of the leaf. The infected leaf was cut approximately 5 cm, including the healthy tissues. The leaf pieces were then surface-sterilized by using 95% ethanol for 3 minutes, followed by washing with sterilized distilled water. The leaf pieces were then partially soaked into peptone water for 5 minutes or until bacterial ooze appear. The bacterial suspension was prepared for serial dilution. Each diluted suspension was then spread-plated on Tryptic Soy Agar (TSA) media. The inoculated plates were incubated at 25 °C for 24 hours. After the growth of bacterial colony was observed, the plates were stored at 4 °C for long term storage.

3.4 Evaluation of morphological characteristics

The plates of pure culture were subjected to evaluation of their morphology. A total of sixteen isolates were evaluated based on their colour, colony form, margin, and elevation through visual screening under the light microscope.

3.5 Biochemical test

3.5.1 Gram Staining

The bacterial isolates collected from the fields were tested with gram staining in order to differentiate Gram-negative and positive bacteria. Smeared bacterial culture was stained with crystal violet for 1 minute. Iodine was added to bind with crystal violet. Acetone was added for 15 minutes in order to decolorize bacterial stains. Safranin was added for 30 seconds for counter-stain purposes. The glass slide with bacterial stain was observed under the microscope for their colour and form (Jonit *et al.,* 2016)

3.5.2 Catalase Test

Catalase test was conducted to differentiate aerobic and facultative bacteria. A drop of Hydrogen peroxide (H_2O_2) was added to the glass slide. Small amount or a colony of bacterial was taken from well isolated media plate and mix with 3% of H_2O_2 on the glass slide. The formation of bubble was recorded. Bubble formation indicates the presence of catalase which is positive reaction (Jonit *et al.,* 2016).

3.5.3 Oxidase Test

Oxidase test was used to identify cytochrome-C-oxidase-producing bacteria. Positive reaction will be indicated by the presence of purple colour. A drop of oxidase was added onto Whatman filter paper. A colony of bacteria was picked using platinum loop and

smeared on the filter paper. The immediate colour changes were observed (Jonit *et al.,* 2016)

3.5.4 KOH Test

Potassium hydroxide (KOH) test was conducted to identify gram negative bacteria. 3% of KOH was applied on the glass slide. A colony of bacteria was collected and mix with 3% KOH on glass slide. The mixture was stirred carefully. The viscous solution will form mucoid string within 30 seconds. Sticky and viscous solution will indicate the gram-negative bacteria (Jonit *et al.,* 2016)

3.5.5 Anaerobic/Oxidation-Fermentation Test

Isolates were inoculated into the media tubes (consisted of 2 g peptone; 5 g NaCl; 0.3 g KH_2PO_4; 3 g bromothymol blue; 1000 ml distilled water) by stabbing method approximately ¼ inch from the bottom. Media tubes were incubated at 35 ^{o}C for 14 days. Colour changes were observed daily after inoculation.

3.5.6 Starch Hydrolysis

2 g of nutrient agar (NA) was dissolved in 80 ml distilled water. The solution was heated and stirred successively. 2 g of starch was added into the 10 ml distilled water and added to molten agar. The plate then was inoculated with bacteria colony. The plate was incubated at 25 °C for 7 days. Lugol iodine was added into the plate. Appearance of clear zone around colony was observed and recorded (Jonit *et al.,* 2016)

3.6 Pathogenicity test against MR220

3.6.1 Plant Materials and Experimental Design

The experiment of the BLB-resistant assessment of rice was carried out in glasshouses at the Faculty of Science Glasshouse Complex, Universiti Malaya.

3.6.2 Bacterial Isolates and Xanthomonas oryzae Suspension Preparation

Bacterial suspension was prepared in Tryptic Soy Broth (TSB) and incubated at 25 ^{o}C for 24 hours (Figure 3.5). To standardize the concentration, the bacterial suspension was adjusted to 10^{8}/ml spectrophotometrically. Bacterial suspension was stored overnight before inoculation day.

Figure 3.6: Example of the bacterial suspension prepared in TSB. The presence of bacterial colony was indicated by cloudy color of the suspension.

3.6.3 Inoculation of selected isolates to cultivated rice

Leaf clipping method (Kauffman 1974) was performed with selected isolates against local rice cultivar MR220 **(**Figure 3.5). Isolates were grown on TSA at 25 °C for 24 hours. Bacterial suspensions were made in TSB to obtain approximately 10^7 colony forming units (CFU)/ml. For this preliminary pathogenicity test, cultivated variety MR220 was selected. The seven day-olds MR220 rice seedlings were grown in a tray with soil mixture. The seedlings were transplanted into pots filled with autoclaved soil mixture with one plant per pot. Plants were grown up to 30 days-old in a greenhouse at Rimba Ilmu, Institute of Biological Sciences, University of Malaya (3°13'N, 101°66'E) at 29 °C and relative humidity of 95%. The test was conducted with five replicates per isolate, and two leaves were inoculated for each isolate (Figure 3.6). Leaves were cut 2 cm from the tip with a scissor that was dipped with bacterial suspension for 60 s. To ensure maximum transmission, the wounded site of the leaf was dipped into bacterial suspension. Negative control plants were inoculated with peptone water and positive control with Xoo suspension. Plants were maintained in a greenhouse with 29 °C day/night temperatures and observed daily for four weeks for the development of symptoms. Lesion length was measuredon day 30^{th} and recorded.

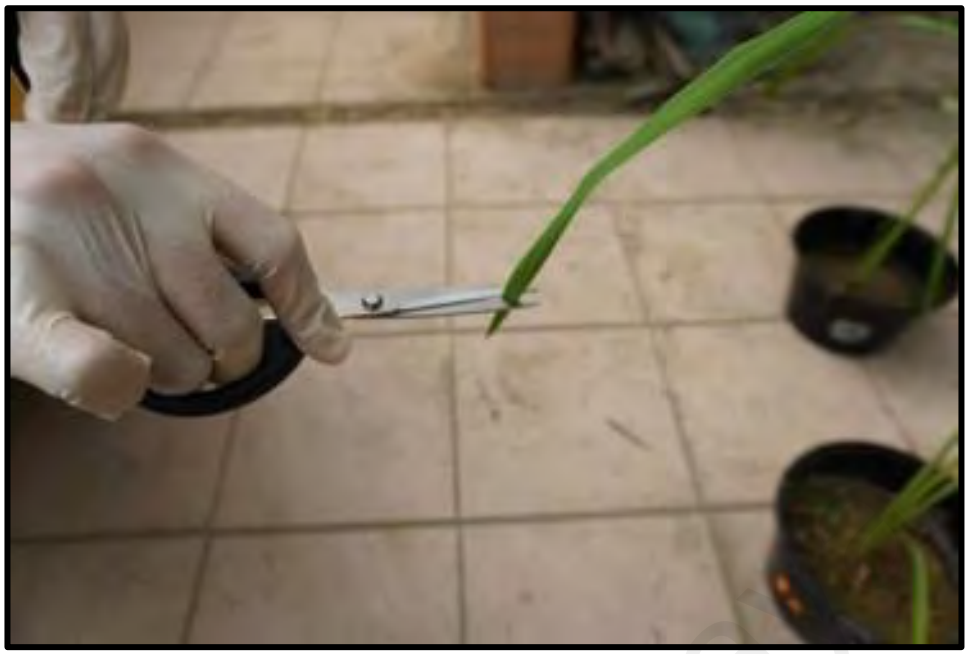

Figure 3.7: Leaf clipping method used to inoculate bacterial suspension into the host plant.

Figure 3.8: First pathogenicity test was designed withfive replicates per treatment.

3.7 Molecular Identification

3.7.1 DNA extraction

Bacterial samples were cultured in Tryptic Soy Broth (TSB) for DNA extraction. DNA was extracted using Presto Mini gDNA Bacteria kit (Geneaid), according to manufacturer's protocol. 2 ml suspension was transferred into 2.0 ml microcentrifuge tube. The tube then was centrifuged at 13,000 rpm for 2 minutes. Supernatant was discarded from the tube and 180 ul of GT Buffer was added. The cell pellet was re-suspended by a vortex. 20 ul of proteinase K was added into the solution. The suspension was incubated at 60 °C for 10 minutes. 200 ul of GB Buffer was added into the suspension and mixed by vortex for 10 seconds. The suspension then was incubated again at 70 °C for 10 minutes or until the sample lysate clear. 200 ul of absolute ethanol was added into the sample lysate and immediately mixed by shaking vigorously. The sample lysate then was transferred into GD column and collection tube and was centrifuged at 13,000 rpm for 3 minutes. The collection tube containing flow-through was discarded and replaced by new collection tube. 400ul of WI Buffer was added into the GD column and centrifuged at 13,000 rpm for 60 seconds. The collection tube containing flow-through was discarded and replaced by new collection tube. 600ul of wash buffer was added into the GD column and centrifuged at 13,000 rpm for 30 seconds. The collection tube containing flow-through was discarded and replaced by new collection tube. The sample was then centrifuged again at 13,000 rpm for 3 minutes in order to dry the matrix column. Collection tube was discarded and replaced by 2.0 ml microcentrifuge tube. 60ul pre-heated elution buffer was added to the center of matric column and centrifuged at 13,000 rpm for 45 seconds to elute the purified DNA.

3.7.2 Gel electrophoresis

Samples were analyzed on 1% of agarose gel. For 40 ml of 1% agarose gel, 0.4 gram of agarose powder was added to 40 ml of Tris-borate EDTA (TBE) buffer of 1X solution. Then, the agarose solution was heated for about 1 minute and cooled down. 3µl of marker was added into the first well, and 1 µl of sample mixed with 1 µl of loading dye were added into the subsequent wells. Gel electrophoresis was set up at 100 volts (V) for 30 minutes and viewed under UV light.

3.7.3 Polymerase Chain Reaction

Eluted DNA was stored at -20 °C for further downstream technique. 16SrRNA gene amplification was carried out by using Exten 2x PCR Master Mix (1st BASE). DNA was amplified by polymerase chain reaction (PCR) in a 25-µl total volume mixture containing 12.5 µl of Master Mix 2X (1st BASE), 1.5 µl of template DNA, 0.5 µM each primer, and sterile ddH2O to the final volume. Amplifications were carried out in Veriti 96 Well thermal cycler (Applied Biosystem) (Figure 3.8) and optimized to the following conditions: denaturation step at 94 °C (1 min), annealing at 60 °C (15 s), and extension at 68 °C (1 min 30 s) for 30 cycles. Amplicons were further purified and analysed by electrophoresis on l.5% (w/v) agarose gel and visualized under UV light (300 nm) after EtB"Out" (Yeastern Biotech Corporation, Taiwan) staining. Purified amplicons were submitted to a commercial company for sequencing and the results were analysed using the National Centre for Biotechnology Information (NCBI) BLAST System (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Figure 3.9: Veriti 96 Well thermal cycler (Applied Biosystem) used for polymerase chain reaction of the isolates.

3.8 Inoculation of identified isolates and positive control, *Xanthomonas oryzae* to cultivated rice

Identified bacterial isolates were chosen for the second pathogenicity test, based on their symptoms in the first pathogenicity test. For this second test, another cultivated rice variety, MR220CL2 was chosen. This is because the infected samples identified in the field belong to this variety.Seeds of MR220CL2 were germinated in 30 °C incubator for 7 days and the seedlings were grown in a tray filled with fined soil mixture to initiate better root growth (Figure 3.8). The rice seedlings were transplanted into pots, with one plant per pot. The pots were filled with autoclaved soil mixture of garden soil, burnt husk and sand and placed in a greenhouse at Rimba Ilmu, Institute of Biological Sciences, University of Malaya (3°13'N, 101°66'E). Each of plant was fertilized with Nitrogen, Phosphorus and Potassium fertilizer (NPK) once per month. For this second pathogenicity test, the experiment was carried out with 10 replications per treatment (Figure 3.9.). Artificial inoculation was done following the same procedure as the first pathogenicity test. After the inoculation, plants were allowed to grow under continuous light at 30 ^{O}C and 75% relative humidity (RH). Plants were scored every 3 days after inoculation. The disease symptoms were observed and recorded.

Figure 3.10: Tray of fined soil mixture for rice seedlings.

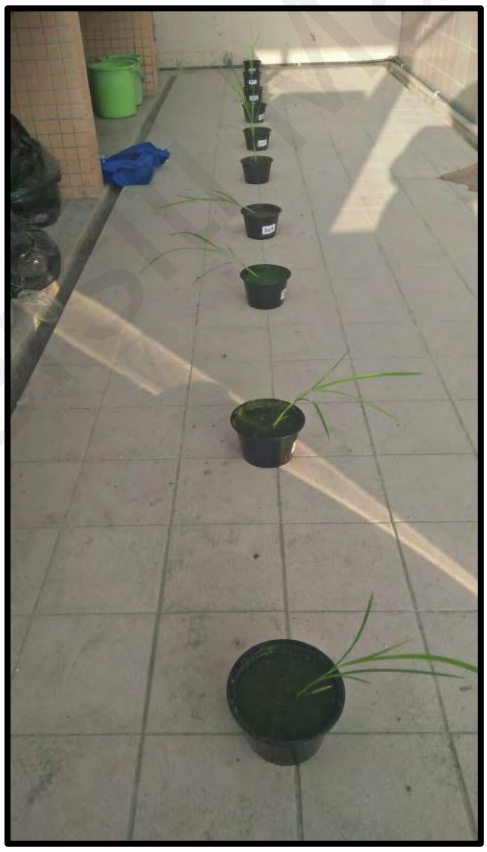

Figure 3.11: Second pathogenicity test was designed with ten replicates per treatment.

3.9 Disease Assessment and statistical analysis

Disease assessment of the plants was evaluated based on the lesion percentage of infected plants. Lesion percentage was calculated based on the lesion length (cm) per leaf length (cm). Disease length was measured from the site of inoculation (tip of the leaf) to the end-point of dried/brown lesion observed (Figure 3.12). Two leaves per pot were selected for disease severity assessment. The disease scoring was compared and analysed using Student t-test with the level of significance at $p<0.05$.

Figure 3.12: Disease was measured by recording the length of the lesion which start from the inoculation site until the last symptomatic area on the leaf.

CHAPTER 4: RESULTS

4.1 Disease monitoring using Unmanned Aerial Vehicles (UAV)

From aerial view, the symptoms of leaf blight that can be seen were dried patterns/spots. The infestation not only occurred on an individual plant but also spread to neighbouring plants which led to a wide range of infestations. All the images were taken after 60 days of transplanting/direct seeding. Thirty meter height was chosen based on the clarity of the images, the wide-ranged area covered and less interception with other disturbances including wind from drone's propellers. Figure 4.1 showed four different heights taken to give an early estimation of the area covered. In comparison, 10 m and 20 m height provide less area covered in an image. Thus, 30 m was chosen as the most suitable height in deploying UAV to estimate the leaf blight infestation per 5000 m^2.

In total, 15 separate datasets were collected, processed and available for further analysis. The field data and the infected rice crop's images were recorded and shown (Figure 4.2, 4.3 and 4.4), approximately at the reproduction stage which is 60 to 80 days old. All remotely sensed data was taken at different timeframes due to unfavourable weather conditions and different plantation schedules. After December 2020, the crops were harvested and the study was concluded as no further data could be collected.

The percentage of infestation was determined by counting the infested area using grid scoring on the captured images. For Pasir Panjang, Plot 1.1 had the highest infestation percentage per 5000 m^2 with 32% while the lowest is Plot 1.4 with 6.6%. In Sekinchan, Plot 2.3 recorded the highest infestation percentage with 48.2% while Plot 2.1 had the lowest with only 7%. Plot 3.4 had the highest percentage with 32.2% while Plot 3.1 recorded the lowest infestation percentage with only 7.6%. Relatively, the five plots in Sekinchan have recorded the highest average infestation percentage with 20.6% as

compared to Pasir Panjang and Sungai Besar with 19.9% and 18.7%, respectively (Table 4.1).

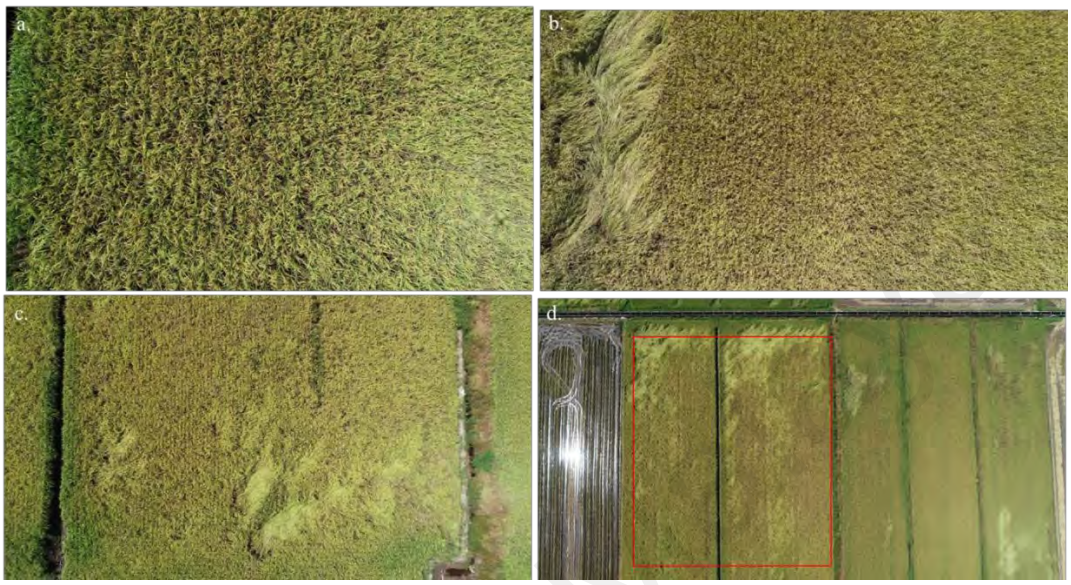

Figure 4.1: Aerial view images from four different heights. (a) 10 m ;(b) 20 m ;(c) 30 m ;(d) 100 m. The red box indicates leaf blight infestation.

Table 4.1: Bacterial leaf blight (BLB) infestation percentage at three (3) areas in IADA Barat Laut Selangor rice fields.

Area	Infestation (%)*
Pasir Panjang	19.9 (±11.38)
Sekinchan	20.6 (±16.09)
Sungai Besar	18.7 (±11.37)

* The percentage of infestation scored per 5000m^2 area. The value in parenthesis is the standard deviation of the mean percentage of the infestation.

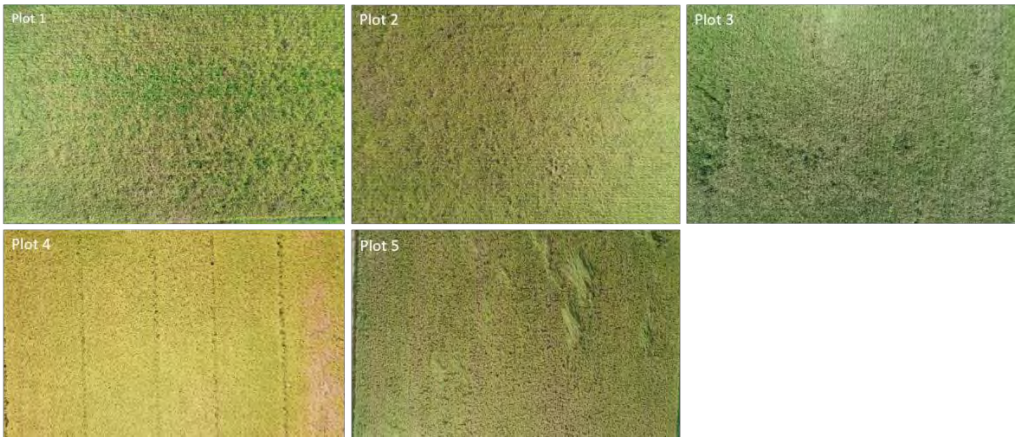

Figure 4.2: Infected plots observed at Pasir Panjang at a height of 30 m.

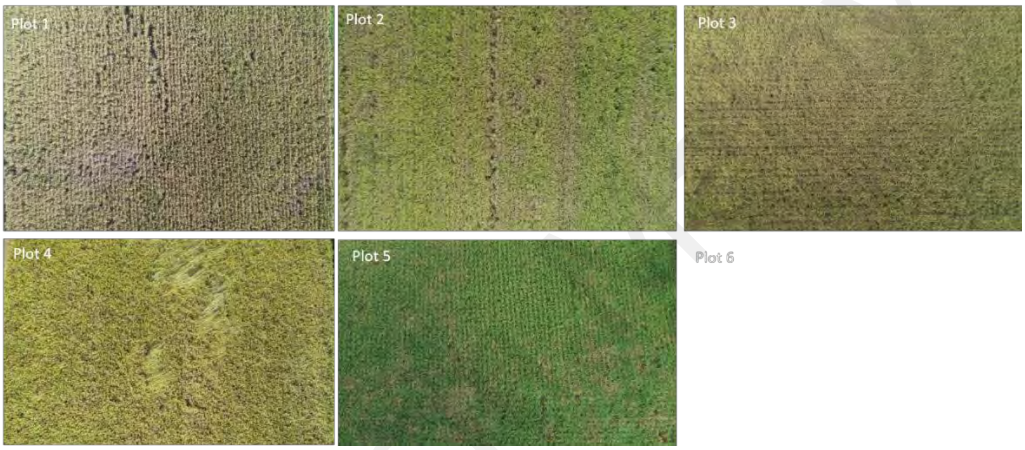

Figure 4.3: Infected plots observed at Sekinchan at a height of 30 m.

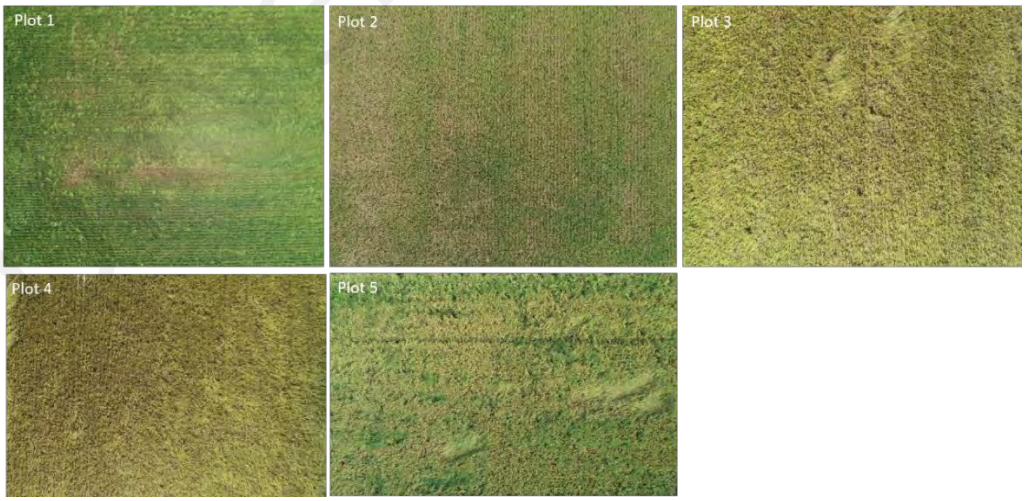

Figure 4.4: Infected plots observed at Sungai Besar at a height of 30 m.

4.2 Morphological characterisation of bacterial isolates

The infected leaf samples with leaf blight were collected from rice granaries at Sekinchan, Peninsular Malaysia. The isolation technique was conducted by collecting samples of bacterial ooze from leaves with dried and brown lesions and not from the seed or rotten leaf tissues that are usually overpopulated with various microorganisms. The ooze from the fresh lesions is a better sampling material as compared to the whole infected leaf because of minimal potential contamination. Furthermore, the isolation of bacteria from symptomatic leaves is easy compared to seeds, due to the presence of other microbes that commonly overpopulated in seeds. Trypticase Soy Agar (TSA) was used as isolation media and screening of the isolates. The serially diluted bacterial ooze was plated on the TSA at 25±2 °C for 24-48 hours. After a few series of re-streaking technique, 16 purified bacterial colonies were successfully isolated (Figure 4.5).

The 16 isolates showed variation in their morphological characteristics. For colony form, 69% (11/16) of the colonies are circular while others showed irregular form. In terms of colony colour, 50% (8/16) of the isolates manifest yellow pigmentation while others were observed white or milky in colour. For the colony margin, various types were observed, and it can be simplified into undulate, entire, and lobate. 50% (8/16) of the isolates exhibit undulate-typed while 38% (6/16) exhibit entire-typed, and 1% (2/16) exhibit lobate-typed. Colony elevation was observed as raised for all the collected isolates. Based on the microscopic observation, most isolates displayed rod-shaped cells except SK07, SK10, and SK11. These bacterial colonies showed cocci, strepto-rod and strepto-bacilli (SK07, SK10 and SK11, respectively). The yellow colour and mucoid colonies are the typical characteristics of the common pathogen of rice leaf blight, *Xanthomonas oryzae* due to the production of extracellular polysaccharides (EPS) in a media containing sugar.

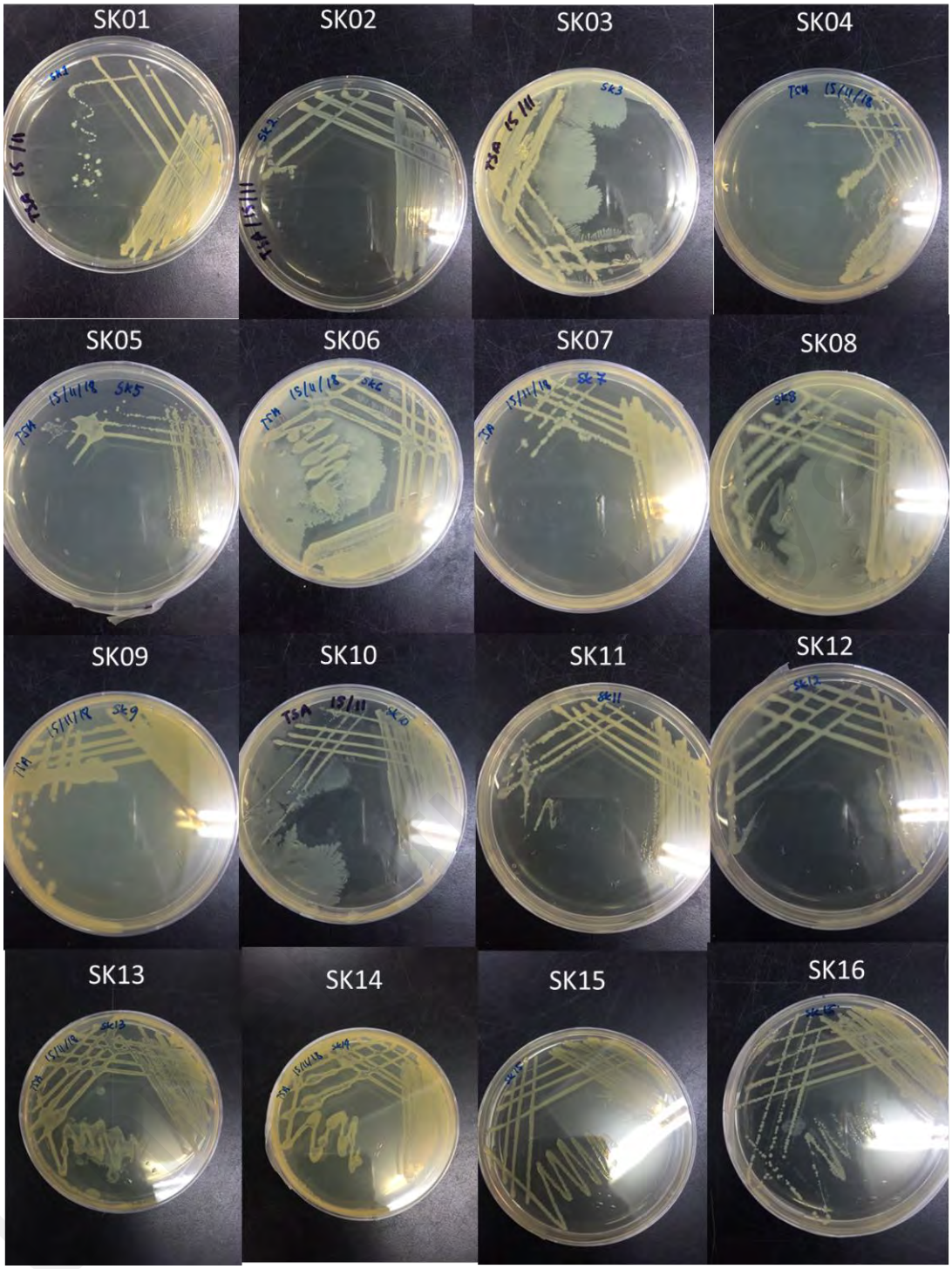

Figure 4.5: 16 purified bacterial colonies isolated from the symptomatic leaf blight-infected rice leaves. The isolates were labelled as SK01, SK02, SK03, SK04, SK05, SK06, SK07, SK08, SK09, SK10, SK11, SK12, SK13, SK14, SK15, and SK16.

Table 4.2: Morphological characteristics of the 16 isolates.

Isolate ID	Location	Colony Form	Colour	Margin	Elevation	Form
SK01	Pasir Panjang	Irregular	Milky	Undulate	Raised	Rod
SK02	Pasir Panjang	Circular	Milky	Undulate	Raised	Rod
SK03	Pasir Panjang	Circular	Milky	Entire	Raised	Rod
SK04	Pasir Panjang	Circular	Milky	Entire	Raised	cocci
SK05	Pasir Panjang	Circular	Yellow	Entire	Raised	Rod
SK06	Pasir Panjang	Irregular	Yellow	lobate	Raised	Rod
SK07	Pasir Panjang	Irregular	Milky	Entire	Raised	Strepto rod
SK08	Pasir Panjang	Irregular	Milky	Entire	Raised	Rod
SK09	Sekinchan	Irregular	Yellow	Undulate	Raised	Rod
SK10	Sekinchan	Circular	Yellow	Lobate	Raised	Strepto-bacilli
SK11	Sekinchan	Circular	Yellow	Undulate	Raised	Coccus
SK12	Sekinchan	Circular	Yellow	Undulate	Raised	Rod
SK13	Sekinchan	Circular	Yellow	Undulate	Raised	Rod
SK14	Pasir Panjang	Circular	Milky	Undulate	Raised	Rod
SK15	Sekinchan	Circular	Yellow	Entire	Raised	Rod
SK16	Sekinchan	Circular	Yellow	Undulate	Raised	Rod
X. oryzae	-	Circular	Yellow	Convex	Smooth	Rod

4.3 Biochemical Profiling of Bacterial Isolates

In general, there are no exact similarities in biochemical characteristics of the bacterial isolates in this study as compared to the known pathogen causing bacterial leaf blight, Xoo. The biochemical profiles of the 16 isolates from the bacterial ooze of infected rice leaf were distinct from Xoo. The biochemical profiles displayed an inconclusive determination of putative causative agents for leaf blight disease (Table 4.3).

All purified isolates were Gram-negative (Figure 4.6). Positive reaction for Catalase test indicated by the formation of bubble formation after H_2O_2 being added to the bacterial slide. The result confirmed that the isolates possess catalase enzyme and most probably the isolates are not belonging to the anaerobic bacterial group due to the lack of this enzyme. There were 11 isolates which showed negative reactions against oxidase (SK01, SK03, SK05, SK08, SK09, SK10, SK11, SK12, SK13, SK14 and SK16, indicating that most of the isolates are not capable of producing enzyme cytochrome oxidase or indophenol oxidase. Only 31% of the isolates possess the enzyme and are subsequently categorised as aerobic bacteria. Positive oxidase is indicated by the presence of deep purple or blue colour while negative oxidase is indicated by no colour changes observed (Figure 4.7). For KOH test, only SK06 isolate displayed negative result which indicated by no viscous string present during the assay. For example, isolate SK01 showed viscous string after being added with potassium hydroxide (Figure 4.8).

Other than catalase, starch hydrolysis is also commonly used for Xanthomonads identification by having the property of amylase production such as a-amylase and oligo-1,6-glucosidase. The amylase production is essential to hydrolyse starch into maltose (Samanta *et al*., 2014). Positive reaction is indicated by the presence of a clear zone around bacterial growth while dark blue colour that indicating negative reaction for starch hydrolysis (Figure 4.9). Both positive and negative reactions were recorded for starch

hydrolysis by these isolates. About 62% of the isolates showed negative reactions while 38% were capable to hydrolyse starch including SK01, SK03, SK06, SK08, SK10 and SK11. Based on the study conducted by Swings *et al*., 1990, the Xanthomonads were able to break down starch after seven days of incubation. On the other hand, different studies conducted in Venezuela did not find this profile in their bacterial isolates (Guvera *et al*., 1999). Both reactions of starch hydrolysis were recorded in this study. The colour changes of inoculated media on the plates from purple to yellow after 24 hours of incubation, indicated positive reaction. For anaerobic test, all isolates were observed negative and unable to grow in anaerobic conditions. In anaerobic growth study, all sixteen putative isolates showed negative reaction for anaerobic activity, a distinct difference compared to *X. oryzae* which gave a positive result for anaerobic test.

Table 4.3: Biochemical characteristics of the isolates.

Isolate ID	Gram Staining	Catalase Test	Oxidase Test	KOH Test	Starch hydrolysis	Anaerobic Test
SK01	-	+	-	+	+	-
SK02	-	+	+	+	-	-
SK03	-	+	-	+	+	-
SK04	-	+	+	+	-	-
SK05	-	+	-	+	-	-
SK06	-	+	+	+	+	-
SK07	-	+	+	+	-	-
SK08	-	+	-	+	+	-
SK09	-	+	-	+	-	-
SK10	-	+	-	+	+	-
SK11	-	+	-	+	+	-
SK12	-	+	-	+	-	-
SK13	-	+	-	+	-	-
SK14	-	+	-	+	-	-
SK15	-	+	+	+	-	-
SK16	-	+	-	+	-	-
X. oryzae	-	+	+	+	+	+

*Positive reaction indicated by (+) and negative reaction indicated by (-).

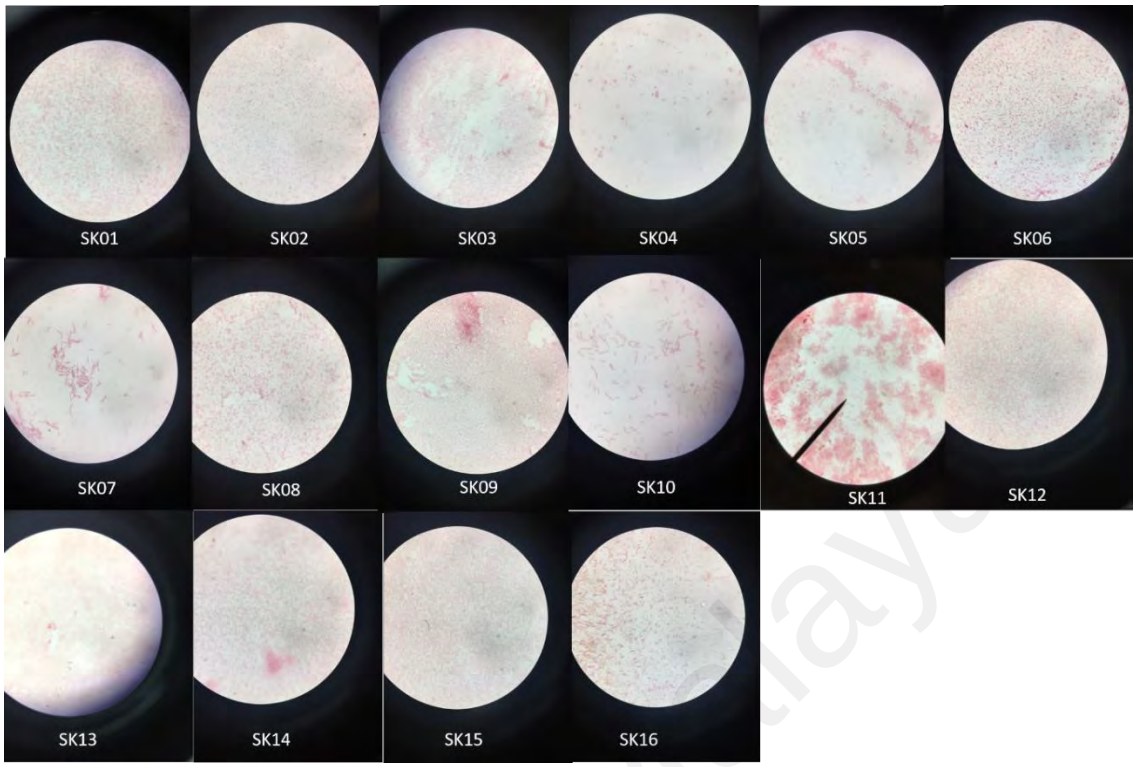

Figure 4.6: Gram-staining result of the 16 isolates after observing under microscope.

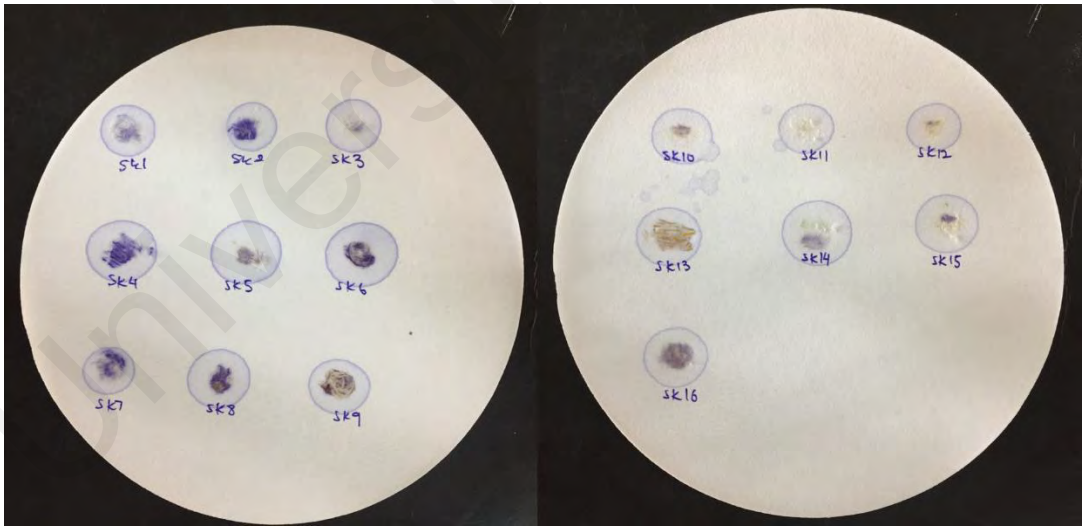

Figure 4.7: Oxidase test result of 16 isolates; purple indicating positive while colourless indicating negative result.

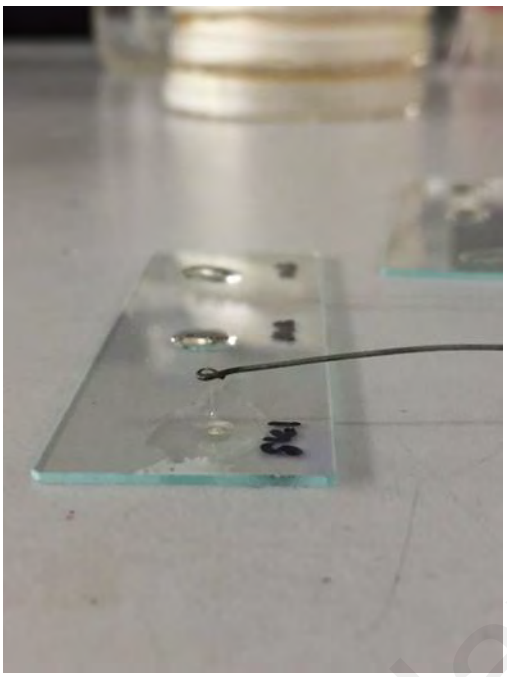

Figure 4.8: Viscous String was observed as an indicator for a positive result in KOH test.

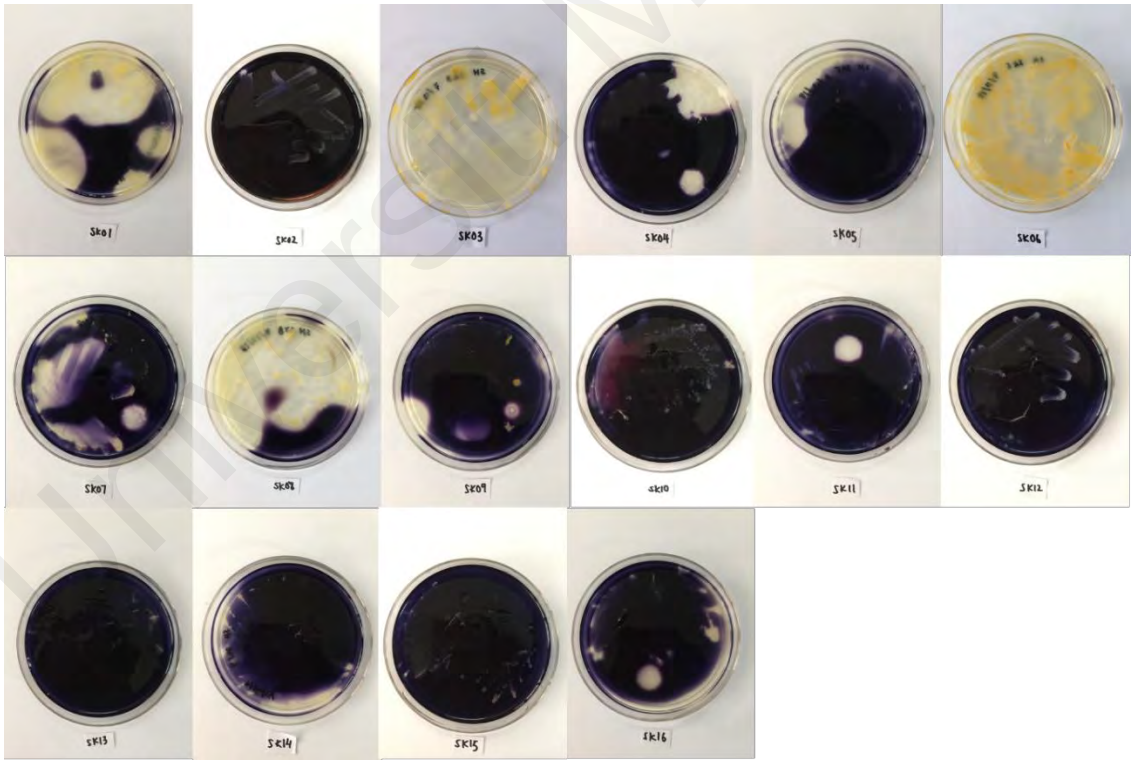

Figure 4.9: Starch hydrolysis result of the 16 isolates; plate with clear zone indicating positive while purple-brown or no clear zone indicating negative result.

4.4 Preliminary pathogenicity test on bacterial isolates.

Twelve isolates were selected based on their close similarities with the known pathogen, *X. oryzae*. These isolates were further tested for pathogenesis screening on local cultivars MR 220 with Xoo as positive control and distilled water as the negative control. The result in Figure 4.10 revealed that leaf blight symptoms appeared clearly at 30 days after inoculation. The symptoms were indicated by scorching and dried lesions along the leaf margin. The severity of the blight increased with the age of the plants which consequently lead to total necrosis of the leaf cell.

The highest disease lesion was recorded by SK09 with 28.01% and the lowest lesion percentage was recorded by isolate SK12 with only 3.19%. Seven isolates were observed to cause low disease severity percentage (<10%) to the rice cultivar MR 220 including SK02, SK03, SK05, SK08, SK12, SK13 and SK14. The infected leaves were exhibiting minor injury from the infection which indicated by short and dried disease lesions. Isolate SK04, SK06 and SK15 were observed to have moderate symptoms against the rice cultivar with lesion percentage of 17.3%, 15.1% and 14.84%, respectively.

SK02, SK04, SK06, SK09, SK13 and SK15 were further subjected to molecular identification. These isolates were selected due to the similarity of the symptoms observed with the leaf blight lesions observed in the infected field.

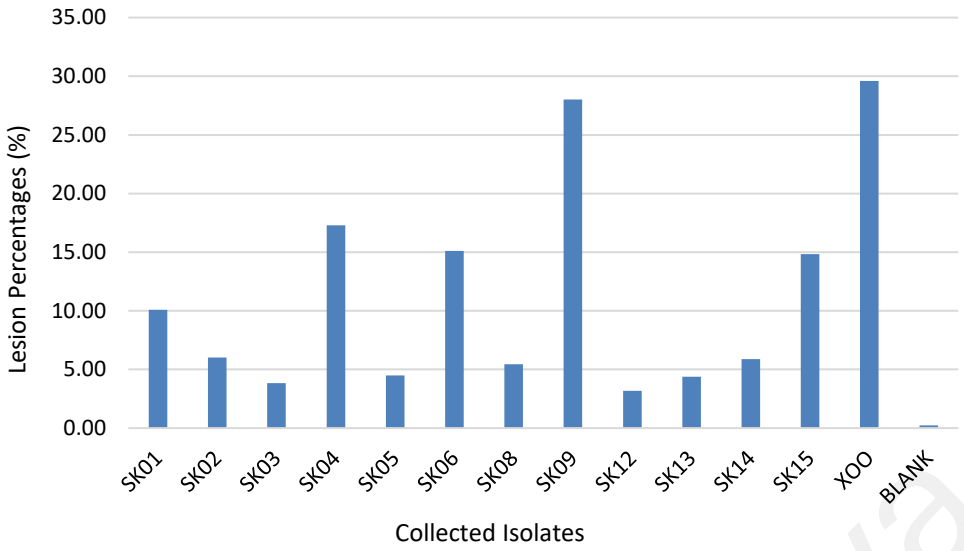

Figure 4.10: Percentage of lesions caused by the isolates against rice cultivar MR 220.

4.5 Molecular Identification based on 16SrRNA gene amplification and sequencing

PCR amplification was performed by using 16S rRNA universal primers, 27f and 1492r targeting the 16SrRNA (~1500 bp) gene of the 5/6 selected isolates based on the pathogenicity test. The sequences were deposited into the GenBank database. Accession numbers are shown in Table 4.4. A BLAST search of the EMBL/GenBank database conducted with the sequences revealed a high degree of sequence identity (> 98%) with previously determined sequences of bacteria belonging to the genus *Pantoea*. Figure 4.1 shows the phylogenetic relationship derived from a neighbour-joining analysis of the pairwise comparison among the 16S rRNA sequences of five isolates from this study with nine sequences of six described species of the genus *Pantoea*. Phylogenetic tree was constructed by Kimura algorithms (Figure 4.11).

For the subsequent molecular identification, PCR amplification was performed by using 16S rRNA universal primer, 27f and 1492r. BLAST analysis of the 16S rRNA sequences of two isolates designated as SK09 (Genbank Accession No. MN209959) and

SK15 (Genbank Accession No. MN209957) were identified to have 98.84% similarities as *Pantoea wallisii* LMG 26277 (Genbank Accession No. MLFS01000124) and 99.3% similarities as *Pantoea agglomerans* DSM 3493(T) (Genbank Accession No. AJ233423), respectively (Table 4.4).

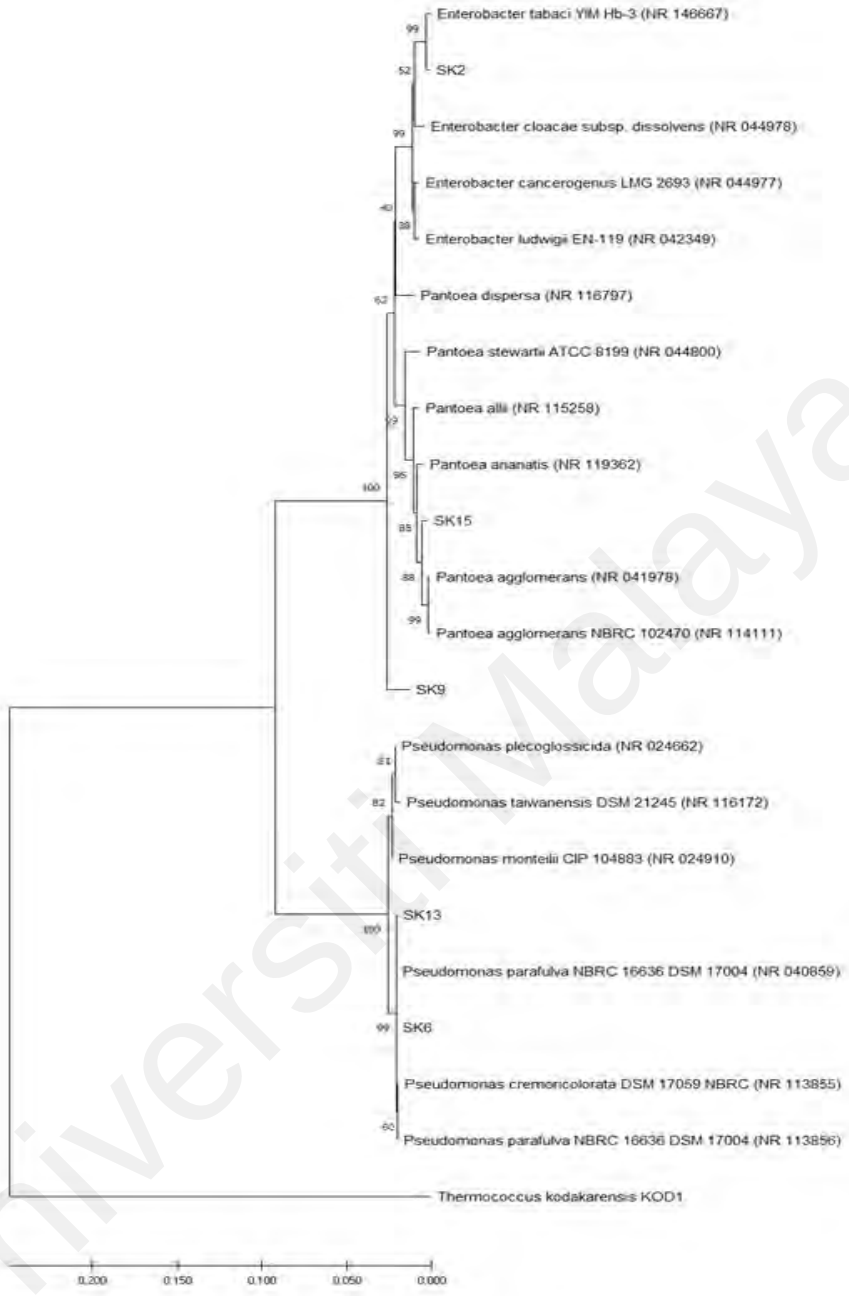

Figure 4.11: Phylogenetic tree based on the 16SrRNA gene sequences of the five selected isolates from leaf blight-infected leaves

Table 4.4: Isolates with identified bacterial species

Isolate ID	Species Identified	Similarities (%)	GeneBank Accession	Location
SK15	*Pantoea agglomerans*	99.30	AJ233423	Sekinchan
SK09	*Pantoea wallisii*	98.84	MLFS01000124	Sekinchan
SK13	*Pseudomonas parafulva*	99.93	-	Sekinchan
SK06	*Pseudomonas parafulva*	100.00	-	Pasir Panjang
SK02	*Enterobacter tabacii*	99.52	-	Pasir Panjang
SK04	Unidentified	-	-	Pasir Panjang

4.6 Confirmation of putative agent of leaf blight disease

The susceptibility of local variety towards leaf blight varies with the isolates tested and therefore, a second pathogenicity test was conducted in a greenhouse by using only two identified potential pathogenic agents of rice leaf blight. The results (Figure 4.12) and (Figure 4.13) revealed that the local variety MR 220 CL2 is susceptible to infection at the vegetative stage but the clear symptoms only can be observed 45 days after the artificial inoculation (Figure 4.14). At the 1st and 2nd week after inoculation (WAI), there were no obvious symptoms identified among the treatments with SK09 (*Pantoea wallisii*), SK15 (*Pantoea agglomerans*), and controls. Clear symptoms were observed at the 3rd and 4th WAI. *P. agglomerans* caused the longest lesion length about 14.24cm at the 4th week of observation which covered 49.19% of the total surface area of the leaf. *P. wallisii* caused moderate infection/symptoms by causing 8.88 cm lesion length and covered 27.95% leaf surface area. After the 4th WAI, the leaf of the infected plant wilted, the brown lesion covered the whole leaf area and the symptomatic plant started to die due to the severe infection. Thus, the ideal duration for observation of the treatments is within 30 days after inoculation.

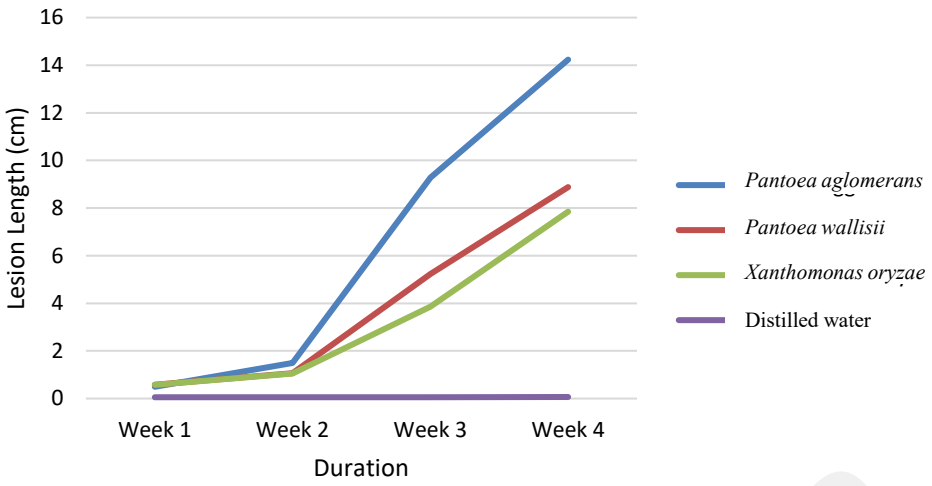

Figure 4.12: Average Lesion Length of infected leaves by isolate for 4 weeks.

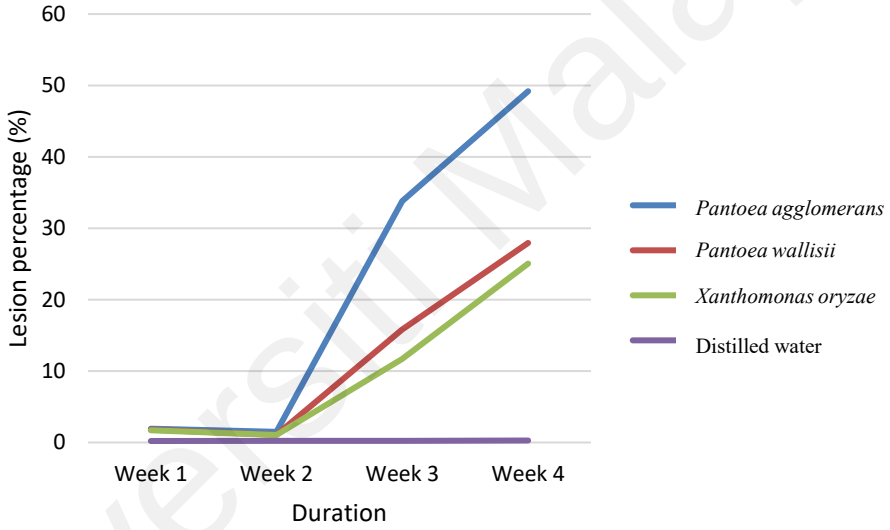

Figure 4.13: Average Lesion Percentage of infected leaves by isolate for 4 weeks.

Figure 4.14: Different disease intensity among the treatments against 30-day-old rice variety MR 220 CL2.

CHAPTER 5: DISCUSSION

5.1 Utilization of UAV for disease monitoring

The application of the UAV demonstrated the efficiency of rice monitoring for leaf blight disease. A total of 18 ha of rice field was surveyed within three months of rice monitoring. UAV application is far more easy-to-operate and energy-efficient compared to conventional monitoring. Based on the report by Rafia *et al*., (2017), conventional wheat monitoring conducted in Punjabi cost 1,418 workers to finish the task in 136 days for one growing season. In the same report, it is stated that modern technique by using precision agricultural tools only required 26 employees to finish the same task within 40 days. Surveys from this study indicated that Sekinchan was badly infested with leaf blight disease as compared to Pasir Panjang and Sungai Besar.

Sekinchan's rice fields have been reported to have leaf blight infestation since 2016-2019 (Jonit *et al.,* 2016; Toh *et al.,* 2019). This rice growing region is one of the most extensive agricultural areas with a total yield reaching 10 t/ha (Tan 2016). The record was due to the heavy application of chemical fertilizer. Excessive use of chemical fertilizer might be one of the factors contributing to the continuous disease infestation. However, there were no significant differences in the rate of infestation for the three studied areas, although the disease infestation had affected a large number of rice plots. Furthermore, based on the one whole season's observation, no infestation occurred before 60 days of plant age. This may indicate the slow rate of disease development. Rice commonly undergoes tillering stage at the age of 60 days and produces grain at the age of 70-90 days. Based on the pattern observed, many factors, directly or indirectly, might contribute to the leaf blight infestation. This factor includes the movement of rice workers from infested fields to healthy fields. The straight lines of disease pattern from one end to

another end of the rice field indicate the pathogens were spread along the working line by farmers (Figure 5.1).

Figure 5.1: Example of leaf blight infestation pattern following the farmer's working line as indicated between the dashed red lines. (Photo credit: This photo was taken by Dr. Muhamad Shakirin Mispan in December 2017).

From another perspective, leaf blight surveillance is efficiently detected by the advancement of monitoring technology. Currently, research in monitoring technology has been focusing on agricultural activities, especially in crop disease detection and precision agriculture-based programs. As shown in the result, the disease patterns can be differentiated by comparing the different color gradients. Infected leaves appeared dried with brown lesions while the healthy leaves appeared green (depending on the stages). In our study, the identification of color differences between infected and healthy crops was done manually after the images were captured and saved. This can be improved by deploying a multispectral drone equipped with an advanced camera (Ipate *et al*., 2015). The application of UAV is contributing to the fast detection and wide-range image captured. The images of straight lines and scattered leaf blight patterns in three locations were taken using UAV with specific grid tools application. This method is capable and

effective in making early detection and estimation of infestation areas and this can lead to the enforcement of efficient control measures.

High technological tools have been developed to promote precision agriculture in these modern days. Hyperspectral or multispectral imaging is one of the common ways in crop monitoring due to the valuable information that can be extracted from the captured image data. More information can be interpreted from hyperspectral images because hyperspectral imaging (HSI) provides a wide range of wavelengths from the spectral reflectance of each pixel (Sankaran *et al.,* 2010). The wavelengths may include the visible and infrared regions of the electromagnetic spectra. Specific spectral bands are required to obtain preferred and precise data images. Thus, hyperspectral imaging is suitable for plant disease detection methods using UAVs. Hyperspectral imaging significantly improves the efficiency of disease detection in terms of spatial resolution and hyperspectral images across the crop growing phases. By deploying these functions, crops can be monitored efficiently over time and more data can be collected regarding growth rate or disease infestation for research purposes.

Furthermore, these applications have been extensively used in food quality control and crop monitoring. Fungal disease such as yellow rust of wheat was efficiently detected by using high resolution hyperspectral images at the late of growing stages (Zhang *et al.,* 2019). Other than that, hyperspectral and multispectral imaging were also used in assessing fruit and food quality and safety (Aleixos *et al.,* 2002; Gowen *et al.,* 2007).

Hyperspectral or multispectral images also can be used to analyse plant physiology-associated data. Plant physiological data usually can be done traditionally by using destructive methods. In order to measure chlorophyll content in plants, laborious and destructive methods are required, which eventually will lead to high variability outcomes (Brito *et al.,* 2011). Thus, simple and instant techniques need to be developed to study the

plant physiology. Multispectral imaging can provide insight into physiological-related inputs such as plant photo pigmentation and water content. This can be achieved by analysing the reflectance at a visible wavelength which provides the information on the photo pigmentations while, reflectance at infrared wavelength gives the physiological data of the plant (Huang *et al.,* 2007). Each spectral areas delivers and interprets useful data about the plant.

Air-borne hyperspectral imaging has been used to identify basal stem rot caused by *Ganoderma* spp. in oil palm (Shafri & Hamdan, 2009). On top of that, the result was quite precise which up to 84% successful rate and this finding indicate that the aerial monitoring using hyperspectral imaging can be used in mass disease screening in oil palm plantation. The application of aerial hyperspectral imaging was also tested to detect citrus greening disease is caused by bacterial species *Candidatus liberibacter* (Lee *et al.,* 2008). The study was conducted to identify the citrus disease by screening through the canopy level. In another study conducted by Qin *et al.,* (2009), the common red grapefruit disease, citrus canker was identified by the hyperspectral imaging with 96% precision. Based on all these reports, it can be concluded that hyperspectral imaging is reliable, and it can improve crop disease detection especially in large scale screening.

Precision agriculture has been implemented globally and influenced the way of agriculture on another level, especially for commodity crops. For instance, in the field of crop improvement and phenotyping. Plant phenotyping is essential in collecting data of ideal agronomic traits such as plant architecture, days to heading and disease resistance. By deploying UAV, beneficial agronomic traits from thousands of lines can be detected efficiently in a short of time and subsequently improve crop breeding program in the future. The application of UAV has been used in the rice agroecosystem to monitor panicle's position which is an important phenotypic that affects biomass and grain yield

(Ogawa *et al.,* 2019). The method developed has high sensitivity for the detection of plant phenotypic and it is time-effective, compared to the traditional method.

In this study, one of the major challenges in aerial imaging-based plant disease detection is the selection of specific timing for monitoring which very much depends on the field conditions. Monitoring time would affect the images captured. This is because excessive light from the sun would disrupt the colour gradient and it would be difficult to differentiate between infected and healthy plants. Suitable plant age also needs to be standardised to avoid confusion between infected and matured crops. Despite the challenges, available tools such as hyperspectral and multispectral sensors would improve this aerial monitoring method. However, the application of high-tech sensors required a specific spectral band and selection of statistical classification algorithm which depends on the targeted crop disease and particular field conditions. For instance, 1000nm to 1340nm were a good spectral band for bruise detection on apples (Lu, 2003). Whilst, spectral bands in a range of 558nm to 960nm were the best selection to distinguish bruises on apples from healthy apples based on different coloration (Xing *et al.,* 2005; ElMasry *et al.,* 2008). Therefore, some of the tools required stable internet connectivity for an excellent outcome. In particular, it is hard to conduct aerial monitoring with unstable or poor internet connection because it is vital for the UAV to detect target field precisely; as well as the location through Global Positioning Satellite (GPS).

This study confirmed that the proposed deep learning architecture and hyperspectral imaging have potential for crop disease detection. The future work will be to validate the proposed model on more UAVs hyperspectral image datasets with various crop species and different types of crop diseases. Moreover, new dimensionality algorithms on large hyperspectral images will also be further developed for efficient data analysis, image processing methods, as well as a well-trained pilot.

5.2 New emerging pathogen of rice leaf blight, *Pantoea* species in Selangor, Malaysia

A total of 16 putative isolates were successfully retrieved from the collected leaf blight-infected rice leaves and cultured on a TSA medium. Isolation of the pathogenic agent of blight disease is easier to be retrieved from the leaf compared to the seeds due to diverse microorganisms in the seeds (Jonit *et al.,* 2016). Based on the result, all isolates showed variation in their morphological and biochemical characteristics. Variability of the observation might be due to the different collected bacterial communities in the phyllosphere. Endophytic bacteria can be found higher in the phyllosphere due to the lack of competition among microbial communities (Pedraza *et al.,* 2009). Different locations and ages also influence the morphological variability of the bacteria (Naqvi *et al.,* 2013). This could be the potential factors of the various morphological characteristics observed. Biochemical characterization is essential to determine and profile the isolated bacteria. However, biochemical tests for the isolates of this study displayed inconclusive determination of putative causal pathogen for rice leaf blight disease.

A total of 12 putative isolates were selected based on morphology and biochemical test for the preliminary pathogenicity test against local rice cultivar MR220. Based on the result, four isolates caused leaf blight symptoms on the tested rice variety (Figure 4.10). The symptoms were indicated by the formation of brown and dried lesions that started from the tip of the leaf and spread along the leaf margin. From the molecular identification through PCR amplification using 16S rRNA universal primer, five isolates designated as SK02, SK06, SK09, SK13 and SK15 were identified as *Enterobacter tabacii, Pseudomonas parafulva, Pantoea wallisii, Pseudomonas parafulva* and *Pantoea agglomerans* respectively. *Pantoea* spp. comprises two of the identified isolates have been reported to cause the same disease in various rice growing regions around the world

(Aksoy & Boluk, 2019; Doni *et al.,* 2019). Thus, this study focused on these two isolates. Few findings have reported that *P. ananatis*, *P. dispersa,* and *P. stewartii* have infected MR269 and MR284 rice varieties with leaf blight disease in Malaysia (Toh *et al.,* 2019; Azizi *et al.,* 2019).

Results from this study had identified the two isolates as *P. agglomerans* and *P. wallisii*. These isolates have shown differences in terms of disease lesions as compared to other collected isolates. *P. agglomerans* and other *Pantoea* strains have been reported to have caused leaf blight disease in various rice growing areas around the world (Aksoy and Boluk 2019a, b; Doni *et al*., 2019). In Malaysia, recent findings reported *P. ananatis*, *P. dispersa,* and *P. stewartii* have infected MR269 and MR284 rice varieties with leaf blight disease in Malaysia (Toh *et al*., 2019; Azizi *et al*., 2019a,b). *P. agglomerans* and *P. wallisii* shared three common phenotypic characteristics which are yellow and raised elevation type of colonies and rod-shaped cells. Both isolates also have the same colour and colony form as *X. oryzae*. Furthermore, the recorded characteristics of these two isolates are similar to the *Pantoea* spp. as reported worldwide. For instance, *Pantoea* spp. isolated from maize white spot (MWS) infected crop showed yellowish colonies when observed on the TSA media (Sauer *et al.,* 2015). *Pantoea* spp. from ornithogenic soil appeared as short straight rods with circular-shaped and yellow coloured at 5 to 40 °C and were able in producing biosurfactants when growing on hydrocarbon media (Vasileva-Tonkova & Gesheva, 2007). The production of yellow pigment by *Pantoea*-belonging bacteria was associated with the ability of the bacteria to survive in a wide range of environments. Production of carotenoid associated with pigment that is able to absorb light in the 400- to 550-nm range which will manifest yellow-orange coloration. Carotenoid biogenesis of *Pantoea* species enable them to survive in oxidative stress condition which commonly occurred in plant host (Mohammadi *et al.,* 2012). Thus, the bacteria are to thrive against various types of reactive oxygen species (ROS) such as

hydrogen peroxide and superoxide anions. However, there was a report stating that *Pantoea*-belonging bacteria able to form blue pigments on G agar plate and have water solubility (Fujikawa & Akimoto, 2011).

Generally, TSA is a general media for the cultivation of microorganisms and was used in this study. The ingredients of soybean meal and casein make it suitable for a wide range of microbes including plant pathogenic bacteria. The application of TSA with 5% sheep blood has been used to cultivate collected *Pantoea* sp. from a patient who suffered from dyspnea and bilateral ankle edema (De Baere *et al.,* 2004). In another study, *Pantoea* sp. isolated from diseased eucalyptus was also grown on TSA media and incubated at 28ºC for 24 hours (Brady *et al.,* 2012). However, new semi-selective media for *Pantoea* spp. has been developed based on their halophilic properties which is named *Pantoea* Genus-specific agar (PGSA) (Kini *et al.,* 2019). Prior to halophilic properties, PGSA was developed specifically for the genus *Pantoea* by using the combination of crystal violet, sodium thiosulphate, peptone, sucrose and 65% of sodium chloride and adjusted to pH 7.1. With a high salt concentration in PGSA, the media efficiently inhibit the growth of unwanted bacteria and fungi that did not have halophilic properties as *Pantoea* species. Alternatively, the development of PGSA can be used as an efficient tool for *Pantoea* screening media.

Based on the previous culturing method, *Pantoea*-belonging species can be categorized as halophilic microbes as they are able to grow in high salt concentrations, compared to other bacteria. Costa and others in 2002 stated that the growth of bacteria was activated by the presence of sodium chloride under optimum temperature. The isolated rhizobacteria *P. agglomerans* was found to have a higher growth rate when 100 to 300 mM of NaCl was used in culturing media (Silini-Cherif *et al*., 2012). Furthermore, bacteria's performances such as siderophores production and biogenesis of Indole Acetic

Acid (IAA) are significantly better in the presence of salt. Isolated bacteria from chickpea's rhizosphere, *Pantoea ananatis* was stated to be salt-tolerant species (Panwar *et al*., 2016).

Biochemical profiling for the isolates in this study is inconclusive due to distinctive results. Gram staining assay was adopted to differentiate gram negative from gram positive bacteria. *Pantoea* spp. is gram-negative bacteria with a thick layer of peptidoglycan. Based on the current studies, all sixteen isolates were identified as gram-negative bacteria (Shyntum *et al.,* 2014; Vinodhini *et al.,* 2017). However, compared to the common pathogen of leaf blight, *X oryzae* which is a gram-negative bacterium and positive for catalase, oxidase, KOH, starch hydrolysis and anaerobic tests, there is no isolate matched with *X. oryzae* biochemical characteristics. This indicates that the collected isolates could be different genus from the common leaf blight pathogenic agent. In this study, biochemical characterization showed that *P. agglomerans* and *P. wallisii* from leaf-blight infected leaves had similar characteristics as *X. oryzae* for gram staining, catalase, and KOH test but different for oxidase, starch hydrolysis and anaerobic test. Thus, these isolates cannot be concluded as *X. oryzae* at the early screening. Advance biochemical profiling systems such as API20E would be worthy for biochemical characterization before the isolates are subjected to further taxonomic investigations.

For decades, the bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo) has been widely known as the only bacterial pathogen for vascular disease leaf blight in rice with more than 30 races of Xoo identified worldwide (Banarjee *et al.,* 2018; Chien *et al.,* 2019). Despite that, *Pantoea*-belonging bacterial species have emerged as a new causative pathogen for leaf blight disease in rice-growing regions globally.

In this study, two different bacterial species belonging to the genus *Pantoea* were reported to cause leaf blight symptoms on rice. *P. wallisii* and *P. agglomerans* were

isolated from infected leaves and confirmed to induce identical symptoms on rice hosts in pathogenicity tests. The symptoms are similar to those caused by *X. oryzae* (Yang & Bogdanove, 2013; Yasmin *et al.,* 2016). Recently, *Pantoea* spp. has been discovered as a new emergence pathogen of rice leaf blight as reported in Korea (Lee *et al.,* 2010), India (Mondal *et al.,* 2011), Venezuela (González *et al.,* 2015), and for local cases, it has been stated by Toh *et al.,* (2019) and Azizi *et al.,* (2019) indicated that *P. ananatis*, *P. dispersa* and *P. stewartii* were recorded to be the pathogens for leaf blight outbreaks in Malaysia's rice granaries in Kedah and Selangor.

On the other hand, isolate SK06 and SK04 were found to be able to cause mild disease lesions on the rice cultivar which were subsequently identified as *P. parafulva* and one unidentified species, respectively. That one unidentified isolate was due to the unusual lesion which is not similar to the common leaf blight lesion. Until now, no report stated that *P. parafulva* a phytopathogen, especially for rice crops.

Rice leaf blight symptoms caused by *Pantoea* spp. undistinguished with the symptoms caused by *X. oryzae.* Furthermore, symptoms observed from *Pantoea*-related blight disease are very similar to Xoo-caused leaf blight. This is due to the similar coloration shown by *Pantoea* spp infection showed brown and dried lesion on leaf margin as commonly observed. In Russian rice fields, *P. ananatis* was reported to cause typical leaf blight which was observed by the symptom of water-soaked lesions which subsequently turned to the brown colour on plants' lemma (Egorova *et al.,* 2015). In 2016, *P. agglomerans* and *P. ananatis* were reported as a pathogen of blight disease in Turkey rice-growing regions and indicated to cause brown-red circular lesions on the tip of the leaf blade (Aksoy & Boluk, 2018). In Benin, *Pantoea*-related blight was reported and observed in local rice granaries which was indicated by orange-brown lines along the leaf margin on symptomatic leaf and caused by *P. ananatis* and *P. stewartii* (Kini *et al.,* 2017).

Pantoea-related leaf blight cases in Venezuela were caused by *P. agglomerans* also showed almost identical symptoms as reported in other infected rice-growing regions from various nations (Gonzalez *et al.,* 2015). In Malaysia, reported leaf blight incidences which were found to be caused by *P. stewartii* and *P. ananatis* also showed similar disease expressions of leaf blight, showing water-soaked lesions at the upper part of the leaves, and turned into brown-dried lesions along the leaf blade (Toh *et al.,* 2019).

Based on the typical leaf blight disease, bacterial pathogen commonly infects the host plant via hydathode or wound that is caused by physical injuries (Shaheen *et al*., 2019). Then the pathogen dwelled in the vascular tissues, reproduced and overpopulated the vessel. Due to the infection, xylem passage will be blocked by the excessive bacterial colonies and lead to the water-soaked lesions. For example, Stewart's wilt in onion is induced by *P. stewartii* subsp. *stewartii* was initially triggered by the microbial colonization in apoplastic space which will create water-soaked appearance. Due to the infection, the pathogen colonizes the xylem, moves systematically through the plant and blocks water flow, leading to wilting symptoms (Burbank *et al.,* 2015). Interestingly, *Pantoea*-caused rice leaf blight also can be observed by water-soaked lesions on the upper part of the leaf which eventually turned yellow-brown coloured (Azizi *et al.,* 2019). This will lead to less water intake in the leaf tissues, reduces photosynthetic surface area and lead to the reduction of grain production and quality.

A series of disease monitoring conducted in this study which focused on leaf blight infestation in Selangor, had shown similar patterns. The collected and tested isolates which were eventually identified as *P. agglomerans* and *P. wallisii* were also caused identical symptoms in pathogenicity test. These symptoms are the result of the pathogen spreading through the vascular system to adjacent leaf tissue forming parallel dried lesions along the leaf blades, causing brownish or scorching lines. Leaf blight symptoms

caused by *Pantoea* spp. are similar to the primary agent of rice leaf blight, *X. oryzae* (Doni *et al.,* 2019). The symptoms caused by *P. wallisii* and *P. agglomerans* in this study appeared as parallel water-soaked lesions on the leaf vein, causing yellowing and dried-look scorching patterns. However, differences in virulence among tested isolates in the first and second pathogenicity tests and two identified isolates, provide a possibility for the selection or instability of the pathogenic bacteria compared to *X. oryzae*. Interestingly, *P. wallisii* caused leaf blight symptoms more significantly than *P. agglomerans* which only caused mild infection to the inoculated rice in pathogenicity tests done in this study, suggesting that some *Pantoea* spp. could be opportunistic pathogen. *Pantoea* is a comparatively earlier phytopathogen which has lost and subsequently developed different plant-associated Type III Secretion Systems (T3SSs) (Kirzinger *et al.,* 2015). T3SS is a membrane-embedded nanomachine that is commonly found in Gram-negative and essential virulence factors in many pathogenic bacteria (Puhar & Sansonetti, 2014). Different types of virulence factors in pathogenic bacteria could affect the symptoms shown on the host plants. This could be the factor of virulence instability for certain *Pantoea* species. When compared with other reported *Pantoea* spp., the isolate *P. agglomerans* is commonly associated with rice leaf blight as reported in Turkey and Venezuela while *P. wallisii* has not been reported yet until now.

Leaf blight is a problematic disease in rice-growing countries, causing huge economic losses in rice production worldwide (Chukwu *et al.,* 2019). The adverse effects of rice leaf blight on agricultural activities can be seen in the increasing the input such as bactericides and yield reduction which can be up as high as 70% in various countries (IRRI 2019). Fluctuation of local temperature could be an external factor for the shifting pathogenic bacteria of leaf blight from Xoo to *Pantoea*-related species. Temperature oscillation is a key factor of microbial infestation in plant hosts (Cheng *et al.,* 2013). Moreover, local rice production is predicted to lose about 5.5% due to global warming

and the new invasion of plant pathogens (Siwar *et al.,* 2014). Prolonged loss in rice production in Malaysia will lead to reduction of self-sufficient level (SSL) and cause instability of food security. If the problems remain unsolved, the current 689,810 ha of Malaysian rice granaries would not be enough to provide sufficient rice for the growing population and will adversely affect the local socio-economic stability in the coming years.

In other climatic regions, *Pantoea* spp is widely known to cause diseases in other crops. A common disease of maize (*Zea mays*), Stewart's wilt caused by *Pantoea stewartii* subsp. *stewartii.* The disease symptoms are indicated by water-soaked lesions on the leaves of maize seedlings which lead to the plant's stunted growth. Similar species of *Pantoea* was found to be the causative agent for fruit bronzing of jackfruits (*Artocarpus heterophyllus*) in Philippines and Mexico (Gapasin *et al.,* 2014; Hernandez-Morales *et al.,* 2017). In other cases, certain *Pantoea*-belonging bacteria are found to have transitioned from saprophyte to host-specific pathogen. *P. agglomerans* was known as common epiphyte and endophyte in various plants. However, Barash and Manullis-Sasson (2007) stated that two pathovars *P. agglomerans* pv. *gyspophilae* and *P. agglomerans* pv. *betae* were found to be tumorigenic bacteria that induced gall formation for both flowering plant: gypsophila and beet crop. The transition to host-specific plant pathogen of *P. agglomerans* is influenced by the acquisition of plasmid-borne pathogenicity island (PAI) (Barash and Manulis-Sasson, 2009). The bacteria prevent the root development, cause hypersensitive reaction (HR) and limit the propagation of the plant. In addition, rice grain discoloration has been reported to be caused by *Pantoea* spp. in China and Russia (Yan *et al.,* 2010; Egovora *et al.,* 2015). Immature, lighter and discoloured grains were observed at infected rice fields in both regions upon harvesting.

5.3 Significant impacts of newly emerged *Pantoea*-related leaf blight towards Malaysian rice production

In the early 1980's, leaf blight infestation was first detected in Malaysian rice granaries, brought about an estimated loss of MYR 50 million but the spread was limited to a certain region (Saad &Habibuddin 2010; Jonit *et al.,* 2016). Since then, control measures were implemented to control the disease including the introduction of new rice cultivars MR 219 and MR 220 which have less susceptibility to leaf blight disease. The utilization of these varieties was able to reduce leaf blight disease infestation significantly (Rafidah *et al.,* 2018). However, the re-emergence of leaf blight disease in the major Malaysian granaries within this decade has put on red alert in our rice industry. The shifting of the pathogenic agent of leaf blight from Xoo to *Pantoea* spp. has altered the rice agrosystem landscape in Malaysia. The current control might not be successful because of the different species of pathogen that emerged. Furthermore, the current development of rice breeding for leaf blight disease resistant cultivars is chiefly focusing on Xoo as the pathogen (Awaludin *et al.,* 2020; Rafidah *et al.,* 2018; Razak *et al.,* 2020). Therefore, the focus on rice breeding program needs to be altered and shift to this newly identified pathogen of leaf blight.

5.4 Potential management of *Pantoea*-causing leaf blight disease in rice

Various approaches have been implemented to control the leaf blight of rice, including the application of bactericides and certified seeds. However, the exact strategy to manage leaf blight infestation caused by *Pantoea* spp. have yet to be determined. For instance, nitrogen-containing heterocyclic compound 1,2,4-Triazole is common derivative in chemical substances that is used in agriculture against phytopathogen (Yang and Bao, 2017). Leaf blight infestation that was caused by Xoo also can be controlled by certain bactericides (Khan *et al.,* 2012). Over the last decade, there is a shifting trend from

chemical to biological approaches in disease and pest management in agriculture. This is due to the rapid development of resistance pathogen to chemical derivatives used (Chukwu *et al.,* 2019). Moreover, frequent application of agrochemical inputs in rice farming may cause adverse impact to ecosystem and human health.

Alternatively, biological controls are recommended and used in disease and pest management. For example, certain species of bacteria has been used in prevention of leaf blight disease such as *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp. (Elshakh *et al.,* 2016; Yasmin *et al.,* 2017; Jambhulkar *et al.,* 2018). The application of bacteriophages also can be used as biological agent in controlling *P. ananatis* infestation in rice (Azegami, 2013). By applying biological approach alone may not solve the problem, especially in mass crop production. Integrated disease management is suggested in handling crop diseases for a better, more efficient and farmer-friendly style. Thus, by combining biological controls and proper agricultural practices, leaf blight disease in rice can be overcome. System of Rice Intensification (SRI) provides comprehensive and better way in managing rice diseases while able to increase rice production. This method is an agroecological way of rice management that provides optimal conditions for healthy crop development. SRI methods can be applied by creating better interspace between individual plant, sustain suitable soil condition with organic wastes, provide efficient irrigation and better selection of young seedlings for transplanting (Doni *et al.,* 2019). SRI methods have been proven in boosting-up plants' fitness against crop pathogen by increasing plant immunity system, maximising healthy soil conditions and creating ideal spaces in-between individual crop (Thakur *et al.,* 2016; Doni *et al.,* 2019). For better early prevention of leaf blight, certified disease-resistant rice varieties must be used. Thus, for future control of *Pantoea* spp., breeding for disease-resistant towards this species is a main priority.

CHAPTER 6: CONCLUSION

Pantoea agglomerans and *Pantoea wallisii* have been identified as pathogenic bacterial species for rice leaf blight in Malaysia rice granaries, with *P. wallisii* being newly described as crop pathogen. Both species of *Pantoea* in this study has been characterised for their biochemical properties and morphological characteristics. The pathogenicity of both *P. agglomerans* and *P. wallisii* was showed positive result with both caused typical dried and brown lesions on local rice cultivars MR220 CL2. However, the result varied among the species and isolates tested. This might be due to the external factors that has been stated in the discussion. This study can be extended to prevail comprehensive biochemical profiling which can be done by advance biochemical profiling system such as API20E. Precise *Pantoea* isolates also can be cultured using selective media such as PGSA.

On the other hand, UAV was proved to be reliable monitoring tools, especially for large scale disease screening. In this study, UAV was utilized and deployed for aerial monitoring with 30 m as suitable height for better data imaging of rice leaf blight. Nevertheless, UAV equipped with advanced sensors such as hyperspectral imaging sensor is highly recommended due to more data can be extracted. Since this study only focuses on the identification of the pathogens and their pathogenicity relations with the plant host without investigating the virulence factors of the bacteria itself, evaluating virulence mechanism and its function in pathogenesis in host-pathogen relationship should not be abandoned. Many recent findings reported that different virulence factors would affect their association with the plant host, which is either opportunistic or obligatory pathogen. By understanding the mechanism of pathogenesis of the bacteria and relationship with rice host, control measures could be made and reduce the *Pantoea*-blight infestation. For that reason, future research needs to be done by identifying

virulence factors of the pathogenic *Pantoea* species, relating the factors with the rice crop relationship and the adaptability towards native environment.

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