



Faculty of Resource Science and Technology

**Characterisation and Pathogenicity of *Pyricularia oryzae* from
Different Regions of Sarawak**

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Characterisation and Pathogenicity of *Pyricularia oryzae* from Different
Regions of Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.


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ABSTRACT

Rice blast disease (causal agent: *Pyricularia oryzae*) is an important rice disease in Sarawak. However, crucial information on *P. oryzae* of Sarawak such as genetic diversity, variation of avirulence (*Avr*) genes, resistance of Sarawak rice towards *P. oryzae* and potential alternative host for *P. oryzae* of Sarawak are still lacking. This study aims to characterise *P. oryzae* collected from rice and weed host from rice field in Kuching, Serian, Sri Aman, Betong and Sibu divisions of Sarawak, to assess the sequence variation of three *Avr* genes; *AvrPiz-t*, *Avr-Pik* and *AvrPi-54* in *P. oryzae* isolates collected from Sarawak rice field, and screen six Sarawak rice landraces (Kanowit, Selasih, Merah, Merawi, Pandan Wangi and Bario) for resistance towards the newly isolated *P. oryzae* isolates. A total of 25 *P. oryzae* isolates were successfully isolated using spore-drop method and identified using conidia morphology and internal transcribed spacer (ITS) sequence. These 25 *P. oryzae* isolates were characterised for their morphological characteristics, growth rate and sporulation rate on two different media, oatmeal agar (OMA) and potato dextrose agar (PDA). The colony surface of all 25 *P. oryzae* isolates varied on different growing media whereas the pigmentation of upper colony surface varied from black, grey to white. There were no morphological traits that was unique to the division of the isolates originating from same location. The growth rate and sporulation rate for 24 *P. oryzae* isolates varies depending on the type of media used and differs among isolates. A total of 11 *P. oryzae* isolates from this study together with 15 Sarawak *P. oryzae* isolates from stock cultures of previous study and FRST UNIMAS Collection were used for *AvrPiz-t*, *AvrPik* and *AvrPi-54* gene isolation. A total of 26 sequences of *AvrPiz-t*, 11 *AvrPik* and 17 *AvrPi-54* genes from Sarawak isolates were successfully amplified and verified. The open reading frame (ORF) of the *Avr* genes were aligned with *AvrPiz-t*, *AvrPik* and *AvrPi-54* ORF (respectively) from other countries that are

available in National Centre for Biotechnology Information database. Unique sequences were identified and translated into amino sequence. The amino acid sequences were aligned with haplotype sequences from previous studies. In total, there were 19 *AvrPiz-t* haplotypes, with eight newly identified haplotypes, 12 *AvrPik* haplotypes with one newly identified haplotype and seven newly identified *AvrPi-54* haplotypes. Neutrality tests were performed and suggested that *AvrPiz-t* and *AvrPi-54* gene were under negative selection while *AvrPik* were under positive selection. Pathogenicity assay was performed on six selected Sarawak rice (Kanowit, Selasih, Merah, Merawi, Pandan Wangi and Bario) against five *P. oryzae* isolates from Sarawak (POS22, POS24, POSA20, POSA23 and POSI7) using detached-leaf inoculation. All rice landraces showed different levels of susceptibility towards all five *P. oryzae* isolates. Isolate POS22 was the most virulent isolate. Kanowit landrace was the most susceptible landrace and Merawi landrace was the least susceptible landrace. From alternative host, seven *P. oryzae* isolates from *Eleusine indica* were successfully obtained using the spore-drop method and confirmed through conidia morphology identification and ITS sequence. Morphological characterization revealed variations in upper colony surface on different growing media, with pigmentation ranging from black, grey, light grey to white. The growth and sporulation rates differed among the isolates and were influenced by the type of media used. The data collected is crucial for disease management in Malaysia, particularly in Sarawak, and serves as a reference for future rice breeding programs in the region.

Keywords: Rice blast fungus, growing media, avirulence gene, Sarawak rice resistance, alternative host

Pencirian dan Kepatogenan Pyricularia oryzae dari pelbagai kawasan di Sarawak

ABSTRAK

Penyakit karah padi (agen penyebab: Pyricularia oryzae) merupakan penyakit padi yang penting di Sarawak. Tetapi maklumat penting mengenai P. oryzae seperti kepelbagaian genetik, variasi gen avirulen (Avr), kerintangan padi Sarawak dan juga potensi hos alternatif bagi P. oryzae di Sarawak amatlah terhad. Kajian ini bertujuan untuk 1) menciri pencilan P. oryzae dari hos padi dan rumpai yang diperoleh dari jelapang padi di bahagian Kuching, Serian, Sri Aman, Betong dan Sibu, mentaksir variasi jujukan tiga gen Avr; AvrPiz-t, AvrPik dan AvrPi-54 di dalam pencilan P. oryzae diperoleh dari jelapang padi di Sarawak, dan menyaring kerintangan enam padi Sarawak (Kanowit, Selasih, Merah, Merawi, Pandan Wangi dan Bario) terhadap pencilan P. oryzae diperoleh dari kajian ini. Terdapat 25 pencilan P. oryzae berjaya dipencilkan dan dikenal pasti melalui morfologi konidia dan penjujukan 'internal transcribed spacer' (ITS). Kesemua 25 pencilan P. oryzae dicirikan dari segi morfologi, kadar pertumbuhan dan kadar pensporulan menggunakan dua jenis medium pengkulturan, agar mil oat (OMA) dan agar dekstroza kentang (PDA). Permukaan koloni bagi kesemua 25 pencilan P. oryzae berbeza mengikut medium pengkulturan digunakan, dan pempigmenan (dari permukaan atas koloni) bervariasi dari hitam, kelabu kepada putih. Tiada ciri morfologi yang unik kepada kawasan atau bahagian dimana pencilan tersebut diperolehi. Kadar pertumbuhan dan pensporulan berbeza bagi setiap pencilan dan dipengaruhi oleh jenis medium pengkulturan digunakan. Sejumlah 11 pencilan P. oryzae dari kajian ini dan 15 pencilan P. oryzae dari stok kultur dari kajian lepas dan koleksi FRST UNIMAS digunakan untuk pemencilan gen AvrPiz-t, AvrPik dan AvrPi-54. Terdapat 26 jujukan gen AvrPiz-t, 11 jujukan gen AvrPik dan 17 jujukan gen AvrPi-54 diperoleh dari pencilan P. oryzae Malaysia berjaya diamplifikasi dan disahkan. Rangka

bacaan terbuka bagi gen *Avr* telah diujikan dengan rangka bacaan terbuka gen *AvrPiz-t*, *AvrPik* dan *AvrPi-54* dari negara lain yang tersedia ada di pangkalan data 'National Centre for Biotechnology Information'. Jujukan yang unik dikenal pasti dan diterjemahkan kepada jujukan asid amino dan diujikan dengan jujukan haplotip dari kajian lepas. Terdapat 19 haplotip *AvrPiz-t*, lapan darinya adalah haplotip baru dikesan; 12 haplotip *AvrPik* dimana terdapat satu haplotip baru; dan tujuh haplotip *AvrPi-54* baru dikenal pasti. Ujian keneutralan menunjukkan gen *AvrPiz-t* dan *AvrPi-54* di bawah pemilihan negatif, manakala gen *AvrPik* di bawah pemilihan positif. Asai kepatogenan enam padi Sarawak (Kanowit, Selasih, merah, Merawi, Pandan Wangi dan Bario) melalui kaedah inokulasi daun terpotong menunjukkan tahap kerentanan yang berbeza terhadap lima pencilan *P. oryzae* Sarawak (POS22, POS24, POSA20, POSA23 dan POSI7). Pencilan POS22 merupakan pencilan yang paling virulen, manakala padi Kanowit merupakan padi paling rentan dan padi Merawi adalah paling kurang rentan. Terdapat tujuh pencilan *P. oryzae* dari rumpai *Eleusine indica* telah berjaya dipencilkan dan dikenal pasti melalui morfologi konidia dan jujukan ITS. Pencirian morfologi menunjukkan perbezaan dari segi permukaan atas koloni pada medium pengkulturan berbeza, dan pempigmenan bervariasi dari hitam, kelabu, kelabu cerah dan putih. Kadar pertumbuhan dan pensporulan adalah berbeza bagi setiap pencilan dan dipengaruhi oleh jenis medium pengkulturan yang digunakan. Data yang diperoleh dari kajian ini amatlah penting untuk pengurusan penyakit karah di Malaysia, terutama sekali di Sarawak dan boleh dijadikan rujukan untuk program pembiakbakaan padi di Sarawak pada masa yang akan datang.

Kata kunci: Kulat karah padi, medium penkulturan, gen avirulen, kerintangan padi Sarawak, hos alternatif

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LIST OF ABBREVIATIONS

μL	Microliter
ANOVA	Analysis of variance
APIP	<i>AvrPiz-t</i> interacting protein
<i>Avr</i> gene	Avirulence gene
BLASTn	Nucleotide basic local alignment search tool
ca.	circa
CIA	Chloroform: iso-amyl alcohol
cm	Centimetre
CTAB	Cetrimonium bromide
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DOA	Department of Agriculture
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector-triggered immunity
IPM	Integrated Pest Management
ITS	Internal transcribed spacer
MADA	Muda Agricultural Development Authority
MAFI	Ministry of Agriculture and Food Industries
MARDI	Malaysian Agriculture Research and Development Institute
MEGA	Molecular Evolutionary Genetic Analysis
MgCl ₂	Magnesium chloride
NB-LRR	Nucleotide-binding leucine-rich repeat

NCBI	National Centre for Biotechnology Information
OMA	Oatmeal agar
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PRR	Pattern recognition receptor
Psi	Pounds per square inch
PTI	PAMP-triggered immunity
QTL	Quantitative trait loci
R gene	Resistance gene
SSL	Self-sufficiency level
TAE	Tris-acetate-ethylenediaminetetraacetic acid
WA	Water agar

CHAPTER 1

INTRODUCTION

1.1 Study Background

Rice feeds 50% of the world population (Zhou, 2016). It is one of the major industries in Malaysia and a staple food for Malaysians. In 2019, approximately 1.8 million metric tonnes of rice were produced, and 2.7 million metric tonnes of rice were consumed in Malaysia (Khazanah Research Institute, 2019). To overcome the differences in rice production and consumption, Malaysian government aims to increase the production up to 100% self-sufficiency level (SSL) in the Eleventh Malaysia Plan (Firdaus *et al.*, 2020). As on 2021, Malaysia rice SSL is at 65% (MAFI, 2021). Rice production can be challenged by diseases and pest infestations, which may lead towards economic losses and threat to food security.

Rice blast is one of the most important rice diseases, caused by ascomycetes fungus *Pyricularia oryzae* (teleomorph: *Magnaporthe oryzae*). This disease has caused 10 - 30% global rice yield loss annually (Skamnioti & Gurr, 2009; Zhou, 2016) and up to 70% yield loss in Malaysia (Gianessi, 2014; Latiffah & Norsuha, 2018). A survey conducted by Malaysian Agricultural Research and Development Institute (MARDI) in 2015 showed 43% of rice field in Peninsular Malaysia were infected by rice blast (Harun, 2015). In 2017, rice blast disease had infected 10% of rice field area under Muda Agricultural Development Authority (MADA) and approximately 10,000 tons of rice grains were lost, causing severe economic loss up to 50% of production cost (Suzalina, 2017). In Sarawak, 58% of the surveyed rice fields (128 farms in total) between the year 2009 to 2012 were infected by *P.*

oryzae with disease severity ranging from moderate (6 - 25% affected leaf area) to high (> 50% affected leaf area) (Lai, 2016; Lai & Eng, 2011, 2013).

Management on the disease by using chemicals such as fungicide can be expensive and may cause the pathogen to buildup resistance towards the active ingredients in the fungicides (D'Avila *et al.*, 2022). Breeding for resistant rice cultivars by incorporating resistance (*R*) gene into susceptible rice cultivars is a way to manage rice blast disease. Following gene-for-gene interaction proposed by Flor (1971), the resistance (*R*) gene in plant recognises the avirulent (*Avr*) gene in the pathogen which result in resistance against the pathogen. On the other hand, to overcome the *R*-gene mediated resistance of the host, *P. oryzae* population will evolve rapidly through sexual recombination and genetic mutation. This co-arms evolution results in new variation of *Avr* gene that cause resistance breakdown (Huang *et al.*, 2014; Yeo, 2014).

Sarawak is blessed with a wide variety of rice landraces which can be the potential genetic materials in building disease-resistant rice variety. There were up to 1000 Sarawak rice accessions reported (Khazanah Research Institute, 2023). Assessing the pathogenicity and resistance of Sarawak rice landraces would be beneficial to provide early insights on Sarawak rice resistance especially towards rice blast. Previous study by Zhen (2015) reported that Biris landrace was found to be resistant towards *P. oryzae* isolate from Bario. While another study by Hussin (2021) had reported that 15 tested Sarawak rice landraces were resistant towards four Sarawak *P. oryzae* isolates (POK3, POK6, POS2 and POMI1). While nine Sarawak rice landraces were moderately resistant towards one isolate (POSA2) from Sri Aman division. Results from both fore mentioned studies suggested that rice landraces that showed resistance towards *P. oryzae* can be a potential resistance donor for resistant rice breeding in Sarawak.

Pyricularia oryzae is recognized for its capacity to adapt and broaden its host range (Chung *et al.*, 2020). It was previously reported to be able to infect wide range of host in different countries (Mackill & Bonman, 2002). Additionally, it has been documented to have the capability to infect as many as 50 species of *Poaceae* (Ou, 1985). This includes domesticated crops like wheat (*Triticum* spp.), barley (*Hordeum vulgare*), corn (*Zea mays*) and wild grasses such as crabgrass (*Digitaria sanguinalis*) and goosegrass (*Eleusine indica*) (Zhong *et al.*, 2016; Chung *et al.*, 2020; Pak *et al.*, 2021). Host jump and host expansion are one of the common evolutionary mechanisms in plant pathogens which enables them to shift from one host to another (Zhong *et al.*, 2016).

1.2 Problem Statement

Pyricularia oryzae is a rapidly evolving species with high genetic diversity in nature (Huang *et al.*, 2014) due to the sexual recombination and genetic mutation (Sirisathaworn *et al.*, 2017). This nature of *P. oryzae* allow them to be able to break the resistance of a rice cultivar over time (Huang *et al.*, 2014) and able to shift and expand host range (Chung *et al.*, 2020), which makes it hard to curb rice blast disease.

For curbing *P. oryzae* in rice fields, knowledge on the variations of *Avr* genes of Sarawak *P. oryzae* would be beneficial, as well as assessing the resistance of Sarawak rice landraces to provide insights on the co-arms evolution status between Sarawak rice *R*-genes and *P. oryzae* *Avr*-genes. This will allow strategic stacking or pyramiding of *R* genes (Ning *et al.*, 2020) in building resistant varieties which allow recognition of the different variations of *Avr* genes in *P. oryzae* population. In Malaysia, only one study has been done on *Avr*-genes of *P. oryzae* (Jee *et al.*, 2017). The author detected *AvrPik*, *AvrPiz-t*, *AvrPi-54* and *AvrPital* in *P. oryzae* isolate 7' which was isolated from Kuching division in Sarawak,

Malaysia. However, the variations and selection of these genes are still unknown in Sarawak as well as in Malaysia.

The management of weeds, particularly in rice cultivation areas in Malaysia, appears to be suboptimal, potentially attributed to the laborious and costly nature of weed control, particularly when resorting to herbicides. The labour-intensive and financially burdensome aspects of weed management have led many rice farmers to limit their efforts and focusing more on insect and pest management rather than weed management (Dilipkumar *et al.*, 2017). Notably, certain weed species, particularly those belonging to the *Poaceae* family, may act as inoculum reservoirs for *P. oryzae*, potentially prompting a shift towards their primary host, *Oryza sativa* (Zhong *et al.*, 2016; Chung *et al.*, 2020; Pak *et al.*, 2021).

In Sarawak, there is no information regarding potential alternative hosts for *P. oryzae*. Investigating the host range of *P. oryzae* in Sarawak could be instrumental in identifying potential inoculum reservoirs for the pathogen during non-planting seasons. Simultaneously, such research has the potential to enhance weed management, particularly within Sarawak's rice field areas dominated by *Poaceae* family.

Addressing this challenge requires a more proactive approach. A targeted investigation into the host range of *P. oryzae* in Sarawak could provide valuable insights, enabling farmers to identify and eliminate potential reservoirs. By understanding and targeting these reservoirs, farmers can significantly reduce the risk of exposure to *P. oryzae* infections, all while optimizing cost, time, and labour investments. This not only promotes a more sustainable and efficient weed management strategy but also underscores the importance of informed decision-making in safeguarding rice crops against potential threats.

1.3 Objectives

This study aims:

- i. To characterise *P. oryzae* collected from rice field in Kuching, Serian, Sri Aman, Betong and Sibu divisions of Sarawak.
- ii. To assess the sequence variation of three avirulence (*Avr*) genes; *AvrPiz-t*, *Avr-Pik* and *AvrPi-54* in *P. oryzae* isolates collected from Sarawak rice field.
- iii. To screen Sarawak rice landraces for resistance towards the newly isolated *P. oryzae* isolates.
- iv. To characterise *P. oryzae* isolated from weed host in rice field of Kuching, Serian, Sri Aman and Sibu divisions of Sarawak.

CHAPTER 2

LITERATURE REVIEW

2.1 Rice Status in Malaysia

Rice holds a significant position within Malaysia's economic landscape, standing as the third-largest industry in the country, trailing behind the dominant palm oil and rubber sectors (Dorairaj & Govender, 2023). As of the year 2021, rice has solidified its status as a staple food among Malaysians, with an average per capita consumption of 79 kg, according to the Ministry of Agriculture and Food Industries (MAFI, 2021). The nation's commitment to rice production is evident in the recorded figures for 2021, which indicate a substantial production of 1.68 million metric tons. In the latest statistics, Malaysia has achieved 65% self-sufficiency level in rice (MAFI, 2021; Department of Agriculture, 2022).

The state of Sarawak is known as the second-largest hub for rice farmers after Kedah (Khazanah Research Institute, 2023). As of 2022, Sarawak contributed 88,419 metric tonnes to the national production, as reported by the Department of Agriculture (DOA, 2023). Despite its significant role, Sarawak faced a decline in rice production after 2018, leading to a reduction in its self-sufficiency level, which stood at 51% in 2019 with a per capita consumption of 92 kilograms (DOA, 2022). Recognizing the importance of revitalizing its rice sector, Sarawak has set an ambitious goal to become the new 'rice bowl of Malaysia' by 2030. To achieve this objective, Sarawak has undertaken proactive measures, including the designation of over 10,000 hectares of land for rice cultivation (Sheblee, 2023). This strategic move aims to increase the overall rice production in the region and contribute significantly to the national self-sufficiency level. The concerted efforts in expanding

cultivation areas underscore Sarawak's commitment to revitalizing its role in the Malaysian rice industry.

2.2 Rice Blast Disease

Rice blast disease is caused by the fungus *Pyricularia oryzae*. It is one of the world-wide diseases of rice plant that is disastrous. Saccardo (1880) established two genus names for the fungus that cause rice blast, *Pyricularia* and *Dactylaria*. Then Asuyama (1965) concluded that the blast fungus is reasonably included in the genus *Pyricularia*. Caavra (1891) first described a *Pyricularia* species on rice in Italy and named it *P. oryzae* (Ou, 1972). It was concluded by Asuyama (1965). *P. oryzae* is the name of the blast fungus at anamorph stage while *Magnaporthe oryzae* is the name during teleomorph stage. *M. oryzae* is rarely found in the field as mating occurs with opposite compatible mating type is required while *P. oryzae* is commonly found in the field as they can reproduce asexually (TeBeest *et al.*, 2007).

Rice blast fungus affects all parts of the plants such as leaves, leaf collars, panicles and seeds. The symptoms shown on the plant differ with the age, resistance of rice cultivars and the environmental condition (TeBeest *et al.*, 2007). In general, rice blast symptoms can be seen by looking at the spots or lesion development on the leaves, nodes and different parts of panicles and the grains. The leaf spots are typically elliptical with the centre of the spots are whitish or grey and the margin of the spot is usually brown or reddish brown (Ou, 1972). Resistant cultivars tend to have smaller lesions compared to susceptible cultivars and the old leaves of susceptible cultivar have light tan lesion with necrotic border (TeBeest *et al.*, 2007). When the infection occurs on the seed, the seed will have spots on the surface of the seed. Infection also might occur at the pedicels of the rice plant causing them not able to produce rice grain. This condition is called blanking (TeBeest *et al.*, 2007).

The first report of rice blast incidence in Peninsular Malaysia was in the year 1945. Jaya rice variety was planted at that time, which was susceptible to rice blast and had 20 - 70% yield loss (Habibuddin, 2012; Nurul Nahar *et al.*, 2020).

2.3 Life Cycle of Rice Blast

Rice blast has a polycyclic life cycle, and a complete disease cycle may take up to one week under favourable condition (Figure 2.1). It was reported that each lesion can generate more conidia up to 20 days (Sopialena & Palupi, 2017). Infection begins when the conidia of *P. oryzae* are deposited on the leaves (TeBeest *et al.*, 2007). These conidia adhere on the leaves and germinate when there is enough moisture on the plant surfaces. A germ tube will emerge from the narrow part of the conidia and extend until a flat and round structure known as appressorium develops at the tip of the germ tube. The formation of appressorium requires a hard surface (Talbot, 2003; Agrios, 2005; Wilson & Talbot, 2009).

Next, the appressorium penetrates the leaf epidermal cell using a turgor pressure of up to 8.0 MPa. The infection then spreads to the neighbouring cells through plasmodesmata (Kankanala *et al.*, 2007). The entire process from germination to penetration spans a duration of 7 to 8 hours. Following successful penetration, the fungus develops specialized biotrophic invasive hyphae, which adeptly colonize the cells without causing the plant to show visible symptoms for the initial four days (Valent & Khang, 2010). This stage is known as biotrophic stage of *P. oryzae*. After an additional three to four days of infection, the infected cells become necrotic causing the emergence of symptoms on the surface of the affected leaves (Wilson & Talbot, 2009). This stage is known as necrotrophic stage of *P. oryzae*. The subsequent invasion of hyphae marks a transition to both biotrophic and necrotrophic phases, categorizing *Pyricularia* as a hemi biotrophic pathogen.

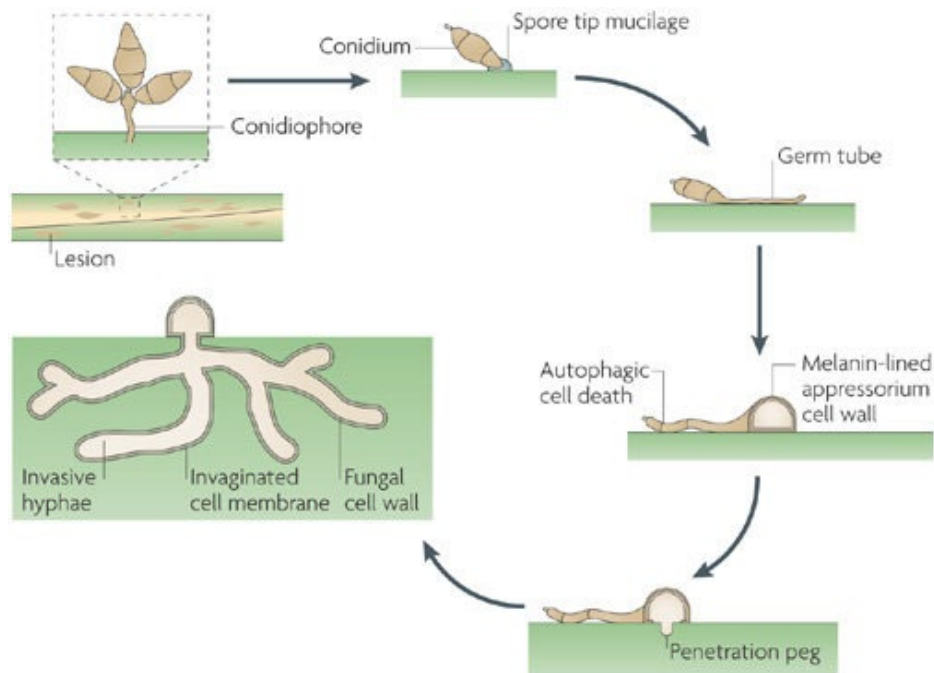


Figure 2.1: Life cycle of *Pyricularia oryzae* (adapted from Wilson & Talbot, 2009).

2.4 Epidemiology of Rice Blast

The infection predominantly occurs at temperatures conducive to the mycelial growth, sporulation, and conidial germination of *P. oryzae*. The relationship between temperature and host susceptibility varies based on factors such as the duration of heat treatment, air and soil conditions, water temperatures, leaf age, and cultivar type (Ou, 1972). Neupane and Bhusal (2020) found that the ideal conditions for a blast outbreak include overcast weather, elevated relative humidity (ranging from 93% to 99%), lower nighttime temperatures (15 - 20 °C), and an extended period of dew formation.

Moreover, light and darkness play a role in influencing rice blast fungus infection (Ou, 1972). Slight shading during the early stages of lesion development encourages longer lesion growth (Imura, 1940). Lesion development is further stimulated by increasing sunlight intensity, while shading inhibits this process (Ou, 1972). Additionally, Kikawa (1900)

supported by Sakamoto (1940) indicating that wind enhances the pathogenicity of the pathogen. In tropical regions, air-borne conidia of *P. oryzae* persist throughout the year, and the fungus can exist in infected rice plants and alternative hosts at any time (Ou, 1972). Occurrence of rice blast in rice plant is usually at its peak during one month after planting and also during grain filling stage (Shahriar *et al.*, 2020). It was reported that the disease resistance of rice plant towards *P. oryzae* is growth stage dependent where the genes for resistance or defence may be effective at early growth stage but not the later stage (Challagulla *et al.*, 2015; Yeo, 2014)

2.5 Management of Rice Blast

In 1985, Integrated Pest Management (IPM) was implemented in Malaysia (Majid *et al.*, 1985) but the system faced challenges due to lack of resource to train farmers and plant pathologist to achieve an effective IPM (Mohammad, 2014). A proper cultural practice needs to be implemented among farmers. This includes crop rotation, weeding maintenance, seed treatment and good farm hygiene practice.

Fungicides are used traditionally and widely among farmers to control rice blast disease. Some of the commonly used fungicides includes benomyl, azoxystrobin, carbendazim, mancozeb, and difenoconazole + propiconazole (Kato, 2001; Kongcharoen *et al.*, 2020). However, *P. oryzae* may develop resistance against the fungicides in a prolonged time. This situation can be observed by the emergence *P. oryzae* population with reduced sensitivity towards fungicide in Brazil (D'Avila *et al.*, 2021). These chemicals also are hazardous to the environment, livestock, and humans (van der Wurff *et al.*, 2016). Fungicides residues may be present in crop after harvests and consumption of the crop becomes the source of toxic exposure to humans (Fantke *et al.*, 2016).

Biological approaches in managing rice blast were also widely researched. This includes application of aerobic rhizobacteria such as *Bacillus subtilis*, *Pseudomonas* sp. and *Trichoderma viridae* which have shown positive result against rice blast pathogen (Filippi *et al.*, 2011; Ghimire *et al.*, 2017; Wasimfiroz *et al.*, 2018). Apart from that, *Bipolaris sorokiana* and *Saccharomyces cerevisiae* were found effective to control rice blast pathogen (Malik *et al.*, 2022). However, the efficacy of bio-agent in controlling *P. oryzae* was reported to be lower than fungicides (Ghimire *et al.*, 2017).

Application of nanotechnology in rice blast disease management is also widely studied. Some nanoparticles such as zinc oxide, silver, chitosan and copper have been reported to possess antifungal properties (He *et al.*, 2011; Kim *et al.*, 2012; Kanhed *et al.*, 2014; Rosa-Garcia *et al.*, 2018; Oh *et al.*, 2019). In Sarawak, study on antifungal effect of zinc oxide nanoparticles against *P. oryzae* isolates of Sarawak was reported by Tang *et al.*, (2023). The authors reported that zinc oxide nanoparticles at certain concentrations inhibited the colony growth of *P. oryzae*. The effect is isolate/genotype dependent.

The utilization of resistant rice cultivars stands out as the most effective method for managing rice blast, offering both economic and environmentally friendly advantages. Currently, the rice genome hosts more than 100 mapped rice blast resistance (*R*) genes, with some providing race-specific resistance and others conferring broad-spectrum resistance (Skamniotti & Gurr, 2009). Extensive research has identified and cloned 25 *R*-genes, and over 500 quantitative trait loci (QTLs) associated with rice blast resistance were identified (Ashkani *et al.*, 2015; Li *et al.*, 2019). Despite these advancements, the resilience of rice cultivars based on *R* genes against *P. oryzae* may diminish over time due to the pathogen's rapid evolution (Huang *et al.*, 2014). For instance, the MARDI rice cultivar MR219, introduced as a resistant cultivar in 2001, faced severe infections by *P. oryzae* during the

2008/2009 planting seasons, leading to its withdrawal due to resistance breakdown (Zainal Abidin, 2015).

To address the challenge of resistance breakdown, gene pyramiding or stacking is employed, involving the stacking of multiple *R*-genes into a single rice variety. This strategy, supported by studies of Castilla *et al.* (2003), Skamniotti and Gurr (2009), and Ning *et al.* (2020), has proven effective in enhancing rice resistance and has successfully managed resistance breakdown in the Philippines and India (Hittalmani *et al.*, 2000). Another promising approach involves breeding for quantitative resistance based on QTLs, as demonstrated by Ballini *et al.* (2008). This method provides an additional layer of defence against rice blast and contributes to a more comprehensive and sustainable strategy for disease management.

2.6 Plant Defence Mechanism

Plants are exposed continuously against microbes that may be pathogenic towards the plant. Pathogenic microbes need to gain access into the plant tissues through penetration of the cell wall through wounds or natural openings such as the stomata for a successful penetration and colonization. However, plants possess defence mechanisms that protect the plant against infection by pathogens. There are two lines of plant defence mechanism; the first line involves physical and chemical barrier of the plant. While the second line of defence involves innate immunity against pathogen which consists of primary and secondary layers. On the primary layer of defence, an extracellular receptor known as PRR (pattern-recognition receptor) is triggered by pathogens' PAMP (pathogen-associated molecular pattern) for direct interaction or DAMP (damage-associated molecular pattern) for indirect interaction. These interactions will induce PTI (PAMP-triggered immunity) (Chisholm *et al.*, 2006).

Pathogens need to avoid from being recognized by PRR to successfully infect the plant. Hence, effectors are secreted by the pathogens into the apoplast or cytoplasm of the plant to induce susceptibility of the plant which usually would interfere with plant signalling pathway after PRR recognition. However, plants can detect the pathogen effectors through intracellular receptors which is known as nucleotide-binding leucine-rich repeat (NB-LRR) protein that is encoded by R gene (Wang *et al.*, 2017). Recognition of effectors through plant NB-LRR protein would activate effector-triggered immunity (ETI) which is a stronger response that leads to programmed cell death known as hypersensitivity response (HR). This is known as the secondary layer of defence. New variants of effector will be produced by the pathogens to avoid induction of ETI. Meanwhile, plants will also increase their R genes frequency to match with the new effector variants to activate ETI. The arm race between the effector and the R genes goes on (Jones & Dangl, 2006 ; Yeo, 2014).

Population of *P. oryzae* can be genetically diverse and can vary according to time and place which would lead to evolution and adaptation towards the dynamics of environmental condition (McDonald & Linde, 2002; Silva *et al.*, 2009). The genetic diversity may lead to emergence of more virulent pathotype of *P. oryzae* which may overcome or evade the resistance based on R genes.

2.7 Avirulence Gene of *Pyricularia oryzae*

Effectors of *P. oryzae* are known as the avirulence (*Avr*) gene. *Avr* genes of *P. oryzae* are unstable and diverse which could act as a pathogenicity tool (Huang *et al.*, 2014). To date, there are 40 *Avr* genes identified (Ning *et al.*, 2020) and 12 of them have been cloned and characterised; *Avr-Pita* (Orbach *et al.*, 2000) , *Avr-CO39* (Farman & Leong, 1998), *PWL1* (Kang *et al.*, 1995), *PWL2* (Sweigard *et al.*, 1995), *ACE1* (Fudal *et al.*, 2005), *AvrPiz-t* (Li *et al.*, 2009), *AvrPia*, *AvrPii*, *AvrPik/km/kp* (Yoshida *et al.*, 2009), *AvrPib* (Zhang *et*

al., 2015), *AvrPi9* (Wu *et al.*, 2015) and *AvrPi-54* (Ray *et al.*, 2016). It was reported that some *Avr* genes of *P. oryzae* has higher level of variations compared to other regions of genome (Chen *et al.*, 2014). For example, *AvrPital* gene showed polymorphism among 30 Thai isolates and were under positive selection (Kasetsomboon *et al.*, 2013). A study on *AvrPital* isolates from USA were also found to be under positive selection and deletion mutation at the coding region which resulted in frameshift and amino acid alteration have showed to be responsible in defeating the *Pi-ta* genes (Dai *et al.*, 2010; Zhou *et al.*, 2007). Apart from that, a study on the *AvrPiz-t* gene sequence variation showed that there is low diversity in *AvrPiz-t* coding region and is under positive selection. The study also suggested that single nucleotide polymorphism or frameshift leads to altered protein sequences causes the protein to lose its function (Chen *et al.*, 2014).

2.7.1 *AvrPiz-t* gene

AvrPiz-t was cloned from *P. oryzae* isolate 81278ZB15 and the size is 327 bp. It is predicted as secreted protein with no homology to other known protein (Li *et al.*, 2009). *AvrPiz-t* interact indirectly with *Piz-t* and encodes 108 amino acid polypeptides of predicted secreted protein into BIC (biotrophic interface complex) before translocated into plant cell (Li *et al.*, 2009; Park *et al.*, 2012; Wang *et al.*, 2017). The product of *AvrPiz-t* does not show any similarities to any known protein in *P. oryzae* as well as in other organism (Zhang *et al.*, 2013). *AvrPiz-t* has six-strand β sandwich fold and in the solution structure of *AvrPiz-t*, Cys 62 forms a disulfide bond with Cys75 and the presence of disulfide bond is believed to be important for avirulence function of *AvrPiz-t* (Li *et al.*, 2012; Jee *et al.*, 2017). There are 12 APIPS (*AvrPiz-t* interacting protein) interacts with *AvrPiz-t* and four of them APIP2, APIP6, APIP8 and APIP10 are involved in ubiquitin proteasome system (Park *et al.*, 2012; Zhang *et al.*, 2013). APIP6 and APIP10 regulate PTI in response to *P. oryzae* and both interacts

with *AvrPiz-t* which cause ubiquitination of their E3 ligase activity thus promoting *AvrPiz-t* degradation (Park *et al.*, 2012). The interaction of *AvrPiz-t* with APIP6 and APIP 10 posed challenges for plants in developing an *R* protein capable of directly identifying *AvrPiz-t*. This interaction is essential for maintaining a low protein level within the plant cell (Wang *et al.*, 2017).

Several studies on *AvrPiz-t* sequence variations have been conducted. Chen *et al.* (2014) revealed eight *AvrPiz-t* haplotypes out of 243 *P. oryzae* isolated from 38 countries. Low nucleotide diversity of *AvrPiz-t* was observed in the open reading frame. *AvrPiz-t* from the *P. oryzae* population was found to be under positive selection (Chen *et al.*, 2014). In Thailand, four *AvrPiz-t* haplotypes were detected out of 42 *P. oryzae* isolates. The *AvrPiz-t* gene of the 42 *P. oryzae* isolates were low in diversity and not under selective pressure (Sirisathaworn *et al.*, 2017). While in Yunnan, China, 100 *P. oryzae* isolates revealed 11 *AvrPiz-t* haplotypes with no selection pressure (Wang *et al.*, 2020).

2.7.2 *AvrPik* gene

The *AvrPik/km/kh* was cloned from *P. oryzae* isolate Ina68 and encodes for putative secreted protein of 113 amino acids with signal peptide at the N-terminus (Yoshida *et al.*, 2009; Jee *et al.*, 2017; Li *et al.*, 2019). The *AvrPik-D* structure has six stranded β -sandwich structure with di-sulphide bond between Cys54 and Cys70 (Maqbool *et al.*, 2015; Wang *et al.*, 2017)

AvrPik requires two *R*-gene homologue *Pik1* and *Pik2* to act together for a direct interaction to occur (Zhai *et al.*, 2014). Up to date, there are 11 *AvrPik* alleles have been reported; *AvrPik-A*, *AvrPik-B*, *AvrPik-C*, *AvrPik-D*, *AvrPik-E*, *AvrPik-F*, *AvrPik-G*, *AvrPik-H*, *AvrPik-I*, *AvrPik-J* and *AvrPik-K* (Yoshida *et al.*, 2009; Longya *et al.*, 2019; Zhang *et al.*, 2020). Different *Avr-Pik* alleles are detected by different *Pik* alleles (Kanzaki *et al.*, 2012).

AvrPik-D can be recognised by seven *Pik* alleles (*Pik*, *Pikm*, *Pikp*, *Pikh*, *Piks*, *Pi7* and *Pil*) (Longya *et al.*, 2019; Hu *et al.*, 2022). *AvrPik-A* and *AvrPik-K* can be recognised by two *Pik* alleles; *Pikm* and *Pikh* (Yoshida *et al.*, 2009; Li *et al.*, 2019). While *AvrPik-E* can be recognised by three *Pik* alleles; *Pik*, *Pikm* and *Pikh* (Li *et al.*, 2019). Both *AvrPik-H* and *AvrPik-I* can be recognized by four *Pik* alleles; *Pik*, *Pikm*, *Pikp* and *Pikh* (Li *et al.*, 2019) whilst *AvrPik-C* can only be recognised by one *Pik* allele; *Pi7* (Longya *et al.*, 2019). *AvrPik-B* and *AvrPik-F* were not recognised by any *Pik* alleles (Longya *et al.*, 2019).

In a study by Li *et al.* (2009) revealed five *Avr-Pik* haplotypes (H6-H10) from *P. oryzae* isolates of Yunnan. The study indicated that the *Avr-Pik* loci of Yunnan may undergo population expansion or purifying selection. While a study by Suttiaphai *et al.* (2021) had revealed that *AvrPik* gene of *P. oryzae* isolates from Thailand undergo decrease in population size or balancing selection.

2.7.3 *AvrPi-54* gene

The *AvrPi-54* gene directly interacts with the *Pi-54* gene, as reported by Wang *et al.* (2019). This gene encodes a predicted secreted protein consisting of 462 amino acids, featuring a signal peptide at the N-terminal region. The 3D structure analysis of *AvrPi-54* reveals the presence of six β -sheets, while the mature SP truncated protein lacks α -helices, as demonstrated by Ray *et al.* (2016). Co-expression of *AvrPi-54* and *Pi-54* genes induces a robust hypersensitivity response (HR) in *Nicotiana benthamiana*, as observed in the study by Ray *et al.* (2016).

In a study by Saklani *et al.* (2022), the direct interaction between *AvrPi-54* and *Pi-54* protein on the surface of the host plasma membrane was demonstrated using in vivo method. The authors revealed that *AvrPi-54* protein might function as transcription factor or as a chromatin remodeler in the nucleus of the cell which is very important in

immunocompromising the host. However, the presence of *Pi-54* protein restricts the *AvrPi-54* protein movement towards the nucleus by interacting directly with *AvrPi-54* protein at the plasma membrane and triggering defence response.

2.8 Alternative Hosts of *Pyricularia oryzae*

Rice is a domesticated member of *Poaceae* family consists of up to 10,000 species which might allow pathogens to shift from the main host to alternative host of the same family (Kelog, 2001; Gaut, 2002; Zhong *et al.*, 2016; Chung *et al.*, 2020). It has been reported that *P. oryzae* is able to switch hosts and expand their range, allowing them to transition from one host to another and establish themselves in new hosts to ensure their survival (Zhong *et al.*, 2016). This can be demonstrated by the emergence of wheat blast caused by *Magnaporthe oryzae* in Brazil in the year 1985 which imposed challenges for farmers and researchers (Maciel *et al.*, 2014). *P. oryzae* can infect more than 50 *Poaceae* species which includes crops such as barley, corn, and millets and grasses such as *Digitaria sanguinalis*, *Setaria viridis* and *Eleusine indica* (Ou, 1985). The strains of *P. oryzae* are divided into a several pathotype; *Oryza* pathotype (isolated from *O. sativa*), *Panicum* pathotype (isolated from *Panicum miliaceum*), *Eleusine* pathotype (isolated from *Eleusine coracana*), *Triticum* pathotype (isolated from *Triticum aestivum*), *Avena* pathotype (isolated from *Avena sativa*), and *Lolium* pathotype (isolated from *Lolium perenne*) (Castroagudin *et al.*, 2016). To date, there are no reports of *P. oryzae* of different pathotypes infecting different hosts in natural condition. Artificial cross-infectivity of *P. oryzae* isolates between rice host and weed host was reported by Mackill and Bonman (1986). The cross-pathogenicity result showed that *Brachiaria distachya*, *chinochloa colona*, *Leersia hexandra*, *Leptochloa chinensis* and *Rottboellia exaltata* were susceptible towards *P. oryzae* isolated from rice host. While *P. oryzae* isolates from *B. distachya*, *E. colona*, *L. hexandra*,

and *R. exaltata* were able to infect rice. It was also reported that *P. oryzae* isolated from *Eleusine* hosts were highly fertile with *P. oryzae* from different hosts (Asuke *et al.*, 2020).

CHAPTER 3

METHODOLOGY

3.1 Preparation of Media and Buffers

Table 3.1 shows the media and buffers used in this study. The pH level for all media were adjusted to 6.5 ± 0.5 . All media and buffers except for Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffers were autoclaved at 120 psi for 15mins.

Table 3.1: List of ingredients for media and buffers preparation which were used in this study.

Ingredients	Measurement	Reference
<u>Water Agar (WA)</u>		
• Agar straws	10 g	
• Distilled water	500 mL	
<u>Oatmeal Agar (OMA)</u>		
• Instant oatmeal	15 g	Kumar <i>et al.</i> , 2005
• Agar stick	7.5 g	
• Distilled water	500 mL	
<u>Potato Dextrose Agar (PDA)</u>		
• HiMedia™ Potato Dextrose Agar	19.5 g	
• Distilled water	500 mL	
<u>CTAB Buffer</u>		
• Tris HCl (1M, pH 8.0)	100 mL	Yang, 2017
• NaCl (5M)	280 mL	
• EDTA (0.5M)	40 mL	
• CTAB	20 g	
• Distilled water	Bring to 1000 mL	
<u>50x TAE Buffer</u>		
• Tris	242 g	Behle & Pawlowski, 2018
• EDTA (0.5M)	100 mL	
• Glacial acetic acid	57.1 mL	
• Distilled water	Bring to 1000 mL	

3.2 Rice Blast Sample Collection

Rice blast sampling was conducted during rice planting season from October 2020 to December 2020 covering Kuching, Serian, Sri Aman, Betong and Sibu divisions of Sarawak. All rice fields visited in all five divisions were owned by smallholder farmers. Rice blast samples were collected from five different points per hectare of rice field. If different landraces were planted at the same field, the samples were separated following each landrace. Table 3.2 summarized all visited rice fields during the sampling period.

Table 3.2: List of rice blast sampling locations in this study.

Division	District	Location	Coordinate
Kuching	Padawan	Kpg Garung, Padawan	N1°17'47.4", E110°16'54.7"
	Tebedu	Kpg Tema Penggal	N1°5'41.51", E110°23'35.88"
	Tebakang	Kpg Riih Mawang	N1°8'5.81", E110°31'26.84"
Serian	Serian	Kpg Slabi Plaman	N1°8'23.00", E110°33'53.00"
		Kpg Slabi Plaman	N1°8'26.00", E110°33'59.00"
		Kpg Semukoi A	N1°3'38.40", E110°39'27.60"
		Kpg Semukoi A	N1°3'38.40", E110°39'27.60"
		Kpg Semukoi A	N1°3'38.40", E110°39'27.60"
		Kpg Semukoi A	N1°3'38.40", E110°39'27.60"
		Kpg Semukoi A	N1°3'38.40", E110°39'27.60"
Sri Aman	Pantu	Kpg Dadak B	N1°4'58.26", E111°2'28.20"
		Kpg Dadak B	N1°4'58.26", E111°2'28.20"
	Simanggang	Kpg Skra	N1°15'50.04", E111°25'50.16"
		Kpg Sg. Pasir, Tanjung Bijat	N1°19'23.04", E111°23'54.66"
		Kpg Pangkat Melayu, Stumbin	N1°18'06.8", E111°22'46.6"
		Kpg Sunga	N1°19'45.18", E111°17'54.12"
	Lingga	Kpg Seduku	N1°23'22.48", E111°15'8.99"
		Kpg Seduku	N1°23'22.48", E111°15'8.99"
		Kpg Seduku	N1°23'9.61", E111°13'54.23"
	Lubok Antu	Engkilili	N1°8'2.28", E111°38'50.64"
		Lubok Antu	N1°13'53.89", E111°42'30.38"
Betong	Betong	Issu/Bangat	N1°19'49.68", E111°32'32.40"
Sibu	Sibu	Jln Kanowit-Durin	N2°8'16.80", E112°0'40.92"
		Near Igan Bridge	N2°20'54.42", E111°48'30.54"
		Jln Sibu-Durin	N2°10'47.16", E112°1'5.46"
	Kanowit	Jln Kanowit	N2°10'40", E112°3'48.18"

3.3 Isolation and Identification of *Pyricularia oryzae*

3.3.1 Single Spore Isolation and Pre-identification

Spore-drop isolation method was conducted according to Hussin *et al.* (2020). Each rice blast lesion on an infected leaf was cut in half with each half of the lesion having a section of healthy part at one end. The leaf segments were surface sterilised with 1% sodium hypochlorite for 1 minute and rinsed three times with sterilized distilled water (each lesion was treated separately). Each leaf segment from a lesion was then attached onto the lid of a Petri dish containing water agar (WA), with adaxial surface facing towards the medium. Subsequently, the plates were incubated in a humidity box at room temperature and observed daily for single conidium colony of *P. oryzae* developed on WA, under a light microscope (ECLIPSE E100LED MV R).

Single conidium colony was then picked and transferred onto oatmeal agar (OMA). The plates were incubated under dark condition for 5 days and under fluorescent light condition for the subsequent days, at room temperature maintained at ca. 28 °C. Alternatively, if spores were observed on leaf segment but no single conidium colony on WA, the spores were dislodged with 250 µl of sterilized distilled water and spread on a new plate of WA. The plate was then incubated under light condition at room temperature. Single conidium colony of *P. oryzae* was picked and cultured as described above. Conidia from pure culture were used for morphological pre-identification of *P. oryzae*. A wet slide mount was prepared by scraping a small piece of mycelia from the candidate *P. oryzae* culture and mixed with a drop of lactophenol blue solution on microscope slide. Then the sample was covered using coverslip and viewed under light microscope. The species was pre-identified as *P. oryzae* when the morphology of the conidia matched the description by Hussin *et al.*

(2020). Only pre-identified isolates of *P. oryzae* were further identified using internal transcribed spacer (ITS) sequence (Hussin *et al.*, 2020).

3.3.2 Molecular Identification of *Pyricularia oryzae*

Genomic DNA of preidentified fungal isolates was extracted using ionic detergent cetyl trimethylammonium bromide (CTAB) method (Lee *et al.*, 1988) with modification. Fungal mycelia were scraped off from the OMA plate (seven to ten days-old) and ground into powder in liquid nitrogen using pestle and mortar. One millilitre of pre-heated CTAB was added to the sample and mixed well. A total of 500 μ L homogenized sample was pipetted into microcentrifuge tube and incubated in water bath at 65 °C for one hour. Then, 300 μ L of chloroform: iso-amyl alcohol (CIA; 24:1) was added into the tube and centrifuged for 15 minutes at 13,500 rpm. The supernatant was transferred into a new microcentrifuge tube containing pre-chilled isopropanol for overnight precipitation at 4 °C. After precipitation, DNA was pelleted by centrifuging at 13,500 rpm for 15 minutes and the supernatant was discarded. The DNA pellet was washed by using 1mL of 70% ethanol and centrifuged again for 15 minutes at 13,500 rpm. The supernatant was discarded, and the DNA pellet was air dried to remove excess ethanol. Lastly, the DNA pellet was dissolved by adding 50 μ L of ultrapure water.

ITS region was amplified using primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') for each preidentified isolate. Polymerase chain reaction (PCR) was performed using 1X *Taq* Buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 μ M of each primer and 1.25 U of *Taq* DNA Polymerase (1st BASE, Axil Scientific Pte. Ltd.). PCR was performed using T100™ Thermal Cycler (Bio-Rad Laboratories, USA). The thermal cycling profile follows Tan *et al.* (2021);

pre-denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1 min; and final extension at 72 °C for 10 min.

PCR amplicons were purified using QIAquick Gel Extraction Kit™ and then sent to Apical Scientific Sdn. Bhd for sequencing. The consensus sequences obtained were searched using nucleotide basic local alignment search tool (BLASTn) from National Centre for Biotechnology Information (NCBI) Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The species verification was determined through percentage of identity and expectation value (E-value).

3.4 Characterization of *Pyricularia oryzae* Isolates on Different Media

3.4.1 Morphological Description of *Pyricularia oryzae* isolates on Different Media

Morphological characterisation method described by Mohammadpourlima *et al.* (2017) was adapted. There were two types of media used for morphological characterization, OMA and potato dextrose agar (PDA). A total of 25 isolates were cultured on OMA and PDA.

Ten days old cultures were used as the inoculum source. An inoculum plug (5 mm Ø) was cut from the edge of actively growing mycelia and transferred (mycelium-side down) onto the centre of a Petri dish containing 20 mL medium. For each medium, there were ten replicates per isolate. The Petri dishes containing fungal plugs were then incubated for 5 days in dark condition and subsequent days under light condition, at room temperature maintained at ca. 28 °C. Morphological characteristics of the colonies (form, elevation, margin, colour, and surface) were described on the 10th day after inoculation.

3.4.2 Growth Performance of *Pyricularia oryzae* on Different Media

The colony radial growth was measured along the perpendicular lines drawn on Petri dish (Figure 3.1). Measurement was done daily until the 10th day. Average radial growth was calculated by using Equation (1) followed by growth rate using Equation (2). Then, conidia were harvested on the 20th day. Approximately 1 mL of 0.05% Tween 20 solution was added onto the surface of fungal mycelia. A sterilised spatula was used to scrap the mycelia off the agar, and the conidia suspension was filtered by using sterilised muslin cloth. A volume of 10 μ L of conidia suspension was added into the loading chamber of haemocytometer. Sporulation rate was estimated based on conidia count on the haemocytometer.

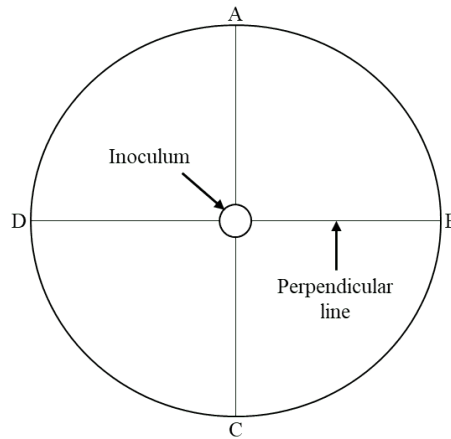


Figure 3.1: Illustration of *P. oryzae* inoculum position and perpendicular lines drew on Petri dish used in experiment.

$$\text{Average radial growth } (r) = \frac{a + b + c + d}{4} \quad \text{Equation 3.1}$$

$$\text{Growth rate} = \frac{(r_2 - r_1) + (r_3 - r_2) + \dots}{N - 1} \quad \text{Equation 3.2}$$

a, b, c, d –Reading on axis A, B, C and D (respectively) of Petri dish.

r1, r2, r3 – average radial growth on day 1, 2, 3...

N - Total days of incubation period.

Statistical analysis was carried out using SPSS software (Version 27). Two - way analysis of variance (ANOVA) was conducted to see any significant interaction between media and isolates. Student's T-test was used to compare the mean growth rate isolates between media at 0.05 significance level except for isolate POS23. POS24, POSA14, POSA19 and POSA20 due to total number of sample lesser than 30 ($n < 30$) and instead were analysed using Mann-Whitney test at 0.05 significance level. One – way analysis of variance (ANOVA) was used to compare the mean growth between isolates in each media at 0.05 significance level while for isolates POS23, POS24, POSA14, POSA19 and POSA20, Kruskal-Wallis was used due to total number of sample lesser than 30 ($n < 30$). To compare which media gives better sporulation rate in general, Mann-Whitney test was used where isolates were used as number of samples (n).

3.5 Isolation of Avirulence Genes from *P. oryzae* Isolates

3.5.1 Amplification of Avirulence Genes and DNA Sequencing

A total of 26 *P. oryzae* isolates were used in this study (11 isolates from this study, 15 isolates from stock cultures of Hussin *et al.* (2020), Rafael *et al.* (2023) and UNIMAS Collection). For the amplification of *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes in *P. oryzae* isolates, three pairs of primer designed by Jee *et al.* (2017) were used (Table 3.3). PCR was performed using 1x *Taq* Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μ M of each primer and 1.25 U of *Taq* DNA Polymerase (1st BASE, Axil Scientific Pte. Ltd.). PCR was performed

using T100™ Thermal Cycler (Bio-Rad Laboratories, USA) following Jee *et al.* (2017). The PCR products were electrophoresed on 2% agarose gel and viewed under UV transilluminator. PCR products were purified by using QIAquick Gel Extraction Kit™ and sent to Apical Scientific Sdn. Bhd. (Malaysia) for DNA sequencing

Table 3.3: List of *Avr* gene primer sequences used in this study.

Target <i>Avr</i> gene	Primer	Primer Sequence (5' – 3')	Optimised T _a (°C)	Amplicon size (bp)
<i>AvrPiz-t</i>	MOPIZTF1	ATGCAGTTCTCAACCATCATCA	60.7	327
	MOPIZTR1	CTATTGGCGCTGAGCCTGA		
<i>AvrPik</i>	MOPIKF1	ATGCGTGTTACCACTTTTAACA	58.6	342
	MOPIKR1	TTAAAAGCCGGGCCTTTT		
<i>AvrPi-54</i>	MOPI54F1	ATGCAGTTCACCGCCACCAT	59.5	462
	MOPI54R1	CTAGCAGCCATAGGTGAGGA		

3.5.2 Alignment of *AvrPiz-t* gene ORF and Haplotype Analysis

The *AvrPiz-t* ORF sequences of 27 *P. oryzae* isolates from Malaysia (Sarawak) [including one isolate from Jee *et al.* (2017)] were analysed together with the *AvrPiz-t* ORF sequences of 123 isolates retrieved from NCBI Genbank database [Thailand (42 isolates), India (80 isolates), China (one isolate)] (Appendix D) and 100 *AvrPiz-t* ORF sequences from Wang *et al.* (2020) (not available in NCBI). MEGAX (Kumar *et al.*, 2018) was used to align the sequences. Unique sequences were selected and translated into amino acid sequence from the alignment of 250 *AvrPiz-t* ORF sequences.

The unique amino acid sequences were then aligned with the eight *AvrPiz-t* haplotype amino acid sequences from Chen *et al.* (2014) using CLC Sequence Viewer (version 8.0). Distinct amino acid sequences were declared as different haplotypes. Haplotype numbering was continuous from Chen *et al.* (2014). A Venn diagram was constructed to show the presence of *AvrPiz-t* haplotypes in different countries based on haplotypes found from Chen *et al.* (2014), Wang *et al.* (2020) and this study.

3.5.3 Alignment of *AvrPik* gene ORF and Haplotype Analysis

The *AvrPik* ORF sequences of 12 *P. oryzae* isolates from Malaysia (Sarawak) [including one isolate from Jee *et al.* (2017)] were analysed together with the *AvrPik* ORF sequences of 169 isolates retrieved from NCBI GenBank database [Thailand (144 isolates), Japan (6 isolates), China (8 isolates) and Korea (11 isolates)] (Appendix E). MEGAX (Kumar *et al.*, 2018) was used to align the sequences. From this alignment of 181 *AvrPik* ORF sequences, unique sequences were selected and translated into amino acid sequence.

The unique amino acid sequences were then aligned with the five *AvrPik* haplotype amino acid sequences from Zhang *et al.* (2020). The amino acid sequences were then aligned using CLC Sequence Viewer (version 8.0). Distinct amino acid sequences were declared as different haplotypes. Haplotype numbering was continuous from Zhang *et al.* (2020). A Venn diagram was constructed to show the presence of *AvrPik* haplotypes in different countries based on haplotypes found from Zhang *et al.* (2020) and this study.

3.5.4 Alignment of *AvrPi-54* gene ORF and Haplotype Analysis

The *AvrPi-54* ORF sequences of 18 *P. oryzae* isolates from Malaysia (Sarawak) [including one isolate from Jee *et al.* (2017)] were analysed together with the *AvrPiz-t* ORF sequences of 77 isolates from India retrieved from NCBI GenBank database (Appendix F). MEGAX (Kumar *et al.*, 2018) was used to align the sequences. From this alignment of 95 *AvrPiz-t* ORF sequences, unique sequences were selected and translated into amino acid sequence. The amino acid sequences were then aligned using CLC Sequence Viewer (version 8.0). Distinct amino acid sequences were declared as different haplotypes. Haplotype numbering was numbered from H1 to subsequent number. A Venn diagram was constructed to show the presence of *AvrPi-54* haplotypes in different countries based on haplotypes found from this study.

3.5.5 Genetic Diversity Analysis of Avirulence Genes

The *AvrPiz-t* ORF sequences of 150 *P. oryzae* isolates from Malaysia (Sarawak), Thailand, India and China (one isolate), together with the 100 *AvrPiz-t* ORF sequences from Wang *et al.* (2020) were used for population genetic analysis. For *AvrPik* gene, 181 *AvrPik* ORF sequences coming from Malaysia (Sarawak), Thailand, Japan, Korea and China, and 95 *AvrPi-54* ORF sequences from Malaysia (Sarawak) and India, were used.

DnaSP5.0 software (Librado & Rozas, 2009) was used to estimate the number of DNA haplotypes, polymorphic sites, nucleotide diversity (π), and haplotype diversity (Hd). Additionally, sliding window analysis, Tajima D's test (Tajima, 1989), Fu and Li's F test, Fu and Li's D test (Fu & Li, 1993) were also performed.

3.6 Pathogenicity assay of *Pyricularia oryzae* isolates on Selected Sarawak Rice Landraces

3.6.1 Plant materials preparation

A total of six Sarawak rice landraces were selected; Kanowit, Selasih, Merah, Merawi, Pandan Wangi and Bario. Rice seeds were soaked in distilled water and kept in a dark environment at room temperature for a week. The germinated seeds were then transplanted onto a planting tray filled with potting mix (3:2:1 ratio of topsoil: peat moss: sand). The rice seedlings were grown under direct sunlight in a net house until they reached four-leaf stage (approx. six weeks old).

3.6.2 Preparation of *Pyricularia oryzae* Conidial Suspension

A total of five Sarawak rice blast isolates, POS22, POS24, POSA20, POSA23 and POSI7 were selected. All five isolates were grown at c a. 25°C, five days in dark condition and on subsequent days exposed under fluorescent light. On the tenth day, the conidia were harvested as described in Section 3.5.1.

3.6.3 Detached Leaf Inoculation Assay using Filter paper Method

Detached-leaf inoculation using filter-paper method were adapted and modified from Takahashi *et al.* (2009). The fourth leaf of rice seedling (approx. six weeks old) was cut into 8cm length and surface sterilised using 1% sodium hypochlorite for one minute and rinsed thrice with sterilised distilled water. The leaf was then placed on sterilised moist tissue paper inside a sterile Petri dish, with adaxial surface facing upwards. A small (approximately 4cm x 2 cm), folded moist tissue paper was placed at both cut ends of the leaf to avoid the leaf from shrivel and to maintain position. Next, a small piece of sterile filter paper (0.2 cm x 3 cm) was placed at the centre of the leaf. The leaf was inoculated by pipetting 10µL of *P. oryzae* conidial suspension (5×10^5 of conidia /mL) onto the filter paper. The filter paper was

then flipped, facing the surface of the leaf. The inoculation steps were repeated for every replicates. For negative control, the leaves were not inoculated. The plates were sealed with parafilm to maintain humidity and incubated in an inoculation box in full darkness for 24 hours at room temperature. After 24 hours, the plates were taken out from the inoculation box and the filter paper were removed from the leaves. Any excess suspension droplets on the leaves were blotted with sterile tissue paper. The plates were placed on laboratory bench with 8hrs light exposure and 16hrs in darkness at 25 °C. The tissue paper was kept moist by adding sterile distilled water onto the tissue paper whenever necessary.

Rice blast symptom development was observed every day and scored on the 7th day post-inoculation by using disease rating scale adapted and modified from Challagulla *et al.* (2015) and Takahashi *et al.* (2009, 2014) (Table 3.4). The assay was performed with 25 replicates per rice landrace and repeated once. The average score of the replicates was used to determine the susceptibility of each rice landrace. Re-isolation of *P. oryzae* from inoculated rice leaves following Koch's postulate was also performed to further proving the pathogenicity of the isolates. A 100µL sample taken from the third rinse water during the surface sterilisation process was spread on PDA for each replication to confirm that the identified pathogen originated specifically from the diseased leaf.

Table 3.4: Disease rating scale used in pathogenicity test

Symptom description	Score	Susceptibility
No visible symptom	0	Resistant
Dark brown, non-sporulating lesions	1	
Expanding, dark brown, non-sporulating lesions	2	Moderately resistant
Small circular or diamond-shaped lesions	3	
Large expanding lesions with sporulating areas	4	Susceptible

The disease rating scale was adapted and modified from Challagulla *et al.* (2015) and Takahashi *et al.* (2009, 2014).

Disease severity index among six Sarawak rice landrace were calculated following Equation (3) which was adapted from McKinney (1923). Statistical analysis was performed by using SPSS software (Version 27). One-way ANOVA was used to compare (1) the mean disease score among six Sarawak rice landraces at significance level of 0.05 and (2) to compare the average earliest day of detection of observable rice blast symptom on leaves among six Sarawak rice landraces at significance level of 0.05.

Disease Severity Index (DSI)%

$$= \frac{\text{sum of scales of all observatios}}{\text{number of observations} \times \text{maximum scale value}} \times 100 \quad \text{Equation 3.3}$$

3.7 Isolation of *P. oryzae* from Alternative Hosts

3.7.1 Sampling Location

Weed having rice blast symptom was sampled simultaneously with rice blast sampling during rice planting season from October 2020 to December 2020. Rice fields with weeds occurrence within 5 m perimeter were scanned for rice blast-like symptoms on the leaf of weed (Figure 3.2). Weeds with rice blast symptom were collected (leaf and whole plant) for herbarium preparation and fungal isolation. Table 3.5 shows sampling location with weed blast occurrence in the rice field.

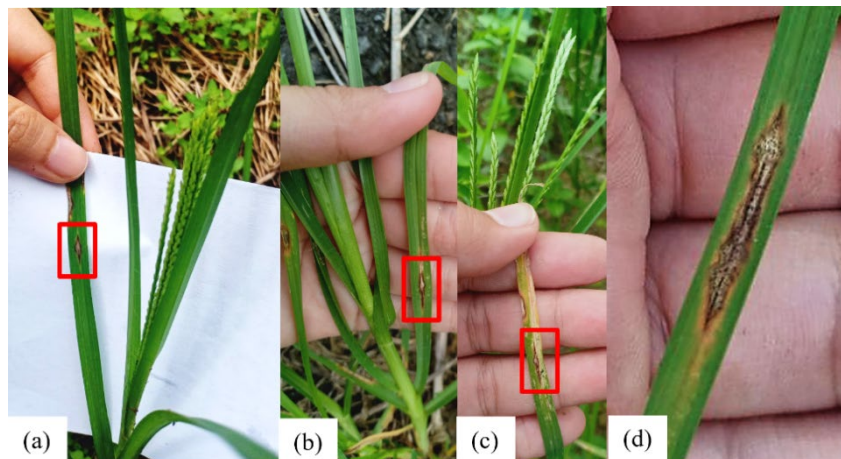


Figure 3.2: Field observation of blast symptom found on weed host. (a) – (d) Symptoms of rice blast found on *Eleusine indica*.

Table 3.5: Location with weed blast occurrence in rice field in different Sarawak division.

Division	District	Location	Coordinate
Kuching	Sematan	Kpg Siru Melayu	N1°49'51.9", E109°43'38.7"
	Padawan	Kpg Karu	N1°15'59.46", E110°17'1.86"
Serian	Serian	Kpg Slabi Plaman	N1°8'26", E110°33'59"
Sri Aman	Pantu	Kpg Dadak B	N1°4'58.26", E111°2'28.20"
	Engkelili	Along Roadside	N1°8'2.28", E111°38'50.64"
Sibu	Sibu	Near Igan Bridge	N2°20'54.42", E111°48'30.54"
	Kanowit	Along Roadside	N2°10'40.00", E112°3'48.18"

3.7.2 Alternative Host Identification and Herbarium Preparation

The whole plant with rice-blast symptoms were used for plant identification and herbarium preparation. Preparation of herbarium specimen was modified from method by Anderson (1999). Collected samples were placed within a newspaper and carefully folded in 'V' or 'W' shape. Then, the samples were soaked in 70% ethanol inside a plastic bag for a week. Next, the samples were taken out and placed inside a new sheet of newspaper and pressed using herbarium press. Then, the press was placed inside a drying oven for two weeks. After drying, the samples were mounted on an acid-free herbarium paper (41cm x 29cm) using Missouri Botanical Garden type glue and let dried.

3.7.3 Isolation and Identification and Characterisation of *Pyricularia oryzae* from Alternative Host

The method for isolation and identification of *P. oryzae* from alternative host were the same as mentioned in Section 3.4. The method for characterisation of *P. oryzae* from alternative host on different media follows the description in Section 3.5.

CHAPTER 4

RESULTS

4.1 Isolation and Identification of *Pyricularia oryzae* from Rice Host

All infected rice leaves collected from the field had typical rice blast symptom, where the lesions were diamond-elliptical shaped with a greyish centre and brown margin (Figure 4.1). A total of 25 *P. oryzae* isolates were obtained from five divisions of Sarawak (Table 4.1). The distribution of Sarawak's *P. oryzae* isolates collected in this study was mapped as shown in Figure 4.2. All 25 isolates were pre-identified as *P. oryzae* based on the morphology of the conidia which was teardrop-shaped, three celled with two septa and hyaline pigmentation (Figure 4.3). The conidium morphology matched with the description from previous studies (Ou, 1987; TeBeest *et al.*, 2007; Hussin *et al.*, 2020). ITS amplification was performed on 13 out of the 25 isolates and the length of the ITS sequence ranging from 322 bp – 543 bp (Table 4.3). All 13 isolates were identified as *P. oryzae* with a 98-100% percentage of identity. The other 12 isolates had problem with DNA extraction due to problems with CTAB reagents which upon investigation, have expired. Therefore, their identification was solely based on conidium morphology.



Figure 4.1: Rice blast symptom on rice leaf. Scale bar = 1 cm.

Table 4.1: List of *Pyricularia oryzae* isolates collected from rice host from different locations in this study.

Isolate	Location	District	Division	Coordinate	Rice Landrace*
POB1	Issu/Bangat	Betong	Betong	N1°19'49.68", E111°32'32.40"	Unknown
POK 9	Kpg Garung, Padawan	Padawan	Kuching	N1°17'47.4", E110°16'54.7"	Unknown
POS 13	Kpg Tema Penggal	Tebedu	Serian	N1°5'41.51", E110°23'35.88"	Unknown
POS 20	Kpg Riih Mawang	Tebakang	Serian	N1°8'5.81", E110°31'26.84"	Unknown
POS 21	Kpg Slabi Plaman	Serian	Serian	N1°8'23.00", E110°33'53.00"	Padi Belacan
POS 22	Kpg Slabi Plaman	Serian	Serian	N1°8'26.00", E110°33'59.00"	Unknown
POS 23	Kpg Semukoi A	Serian	Serian	N1°3'38.40", E110°39'27.60"	Padi Nibong
POS 24	Kpg Semukoi A	Serian	Serian	N1°3'38.40", E110°39'27.60"	Padi Nibong
POS 25	Kpg Semukoi A	Serian	Serian	N1°3'38.40", E110°39'27.60"	Padi Nibong
POS 26	Kpg Semukoi A	Serian	Serian	N1°3'38.40", E110°39'27.60"	Unknown
POSA 13	Kpg Dadak B	Pantu	Sri Aman	N1°4'58.26", E111°2'28.20"	Unknown
POSA 14	Kpg Dadak B	Pantu	Sri Aman	N1°4'58.26", E111°2'28.20"	Unknown
POSA 15	Kpg Skra	Simanggang	Sri Aman	N1°15'50.04", E111°25'50.16"	Padi Hitam
POSA 16	Kpg Sg. Pasir, Tanjung Bijat	Simanggang	Sri Aman	N1°19'23.04", E111°23'54.66"	Unknown
POSA 17	Kpg Pangkat Melayu, Stumbin	Simanggang	Sri Aman	N1°18'06.8", E111°22'46.6"	Padi Hitam
POSA 18	Kpg Sunga	Lingga	Sri Aman	N1°19'45.18", E111°17'54.12"	Padi Bario
POSA 19	Kpg Seduku	Lingga	Sri Aman	N1°23'22.48", E111°15'8.99"	Padi Hitam
POSA 20	Kpg Seduku	Lingga	Sri Aman	N1°23'22.48", E111°15'8.99"	Padi Hitam
POSA 21	Kpg Seduku	Lingga	Sri Aman	N1°23'9.61", E111°13'54.23"	Unknown
POSA 22	Engkilili	Lubok Antu	Sri Aman	N1°8'2.28", E111°38'50.64"	Padi Kanowit

Table 4.1: continued

POSA 23	Lubok Antu	Lubok Antu	Sri Aman	N1°13'53.89", E111°42'30.38"	Unknown
POSI 3	Jln Kanowit-Durin	Sibu	Sibu	N2°8'16.80", E112°0'40.92"	Padi Balik
POSI 5	Near Igan Bridge	Sibu	Sibu	N2°20'54.42", E111°48'30.54"	Unknown
POSI 6	Jln Sibu-Durin	Sibu	Sibu	N2°10'47.16", E112°1'5.46"	Unknown
POSI 7	Jln Kanowit	Kanowit	Sibu	N2°10'40", E112°3'48.18"	Unknown

* Rice landrace names were given according to farmers.

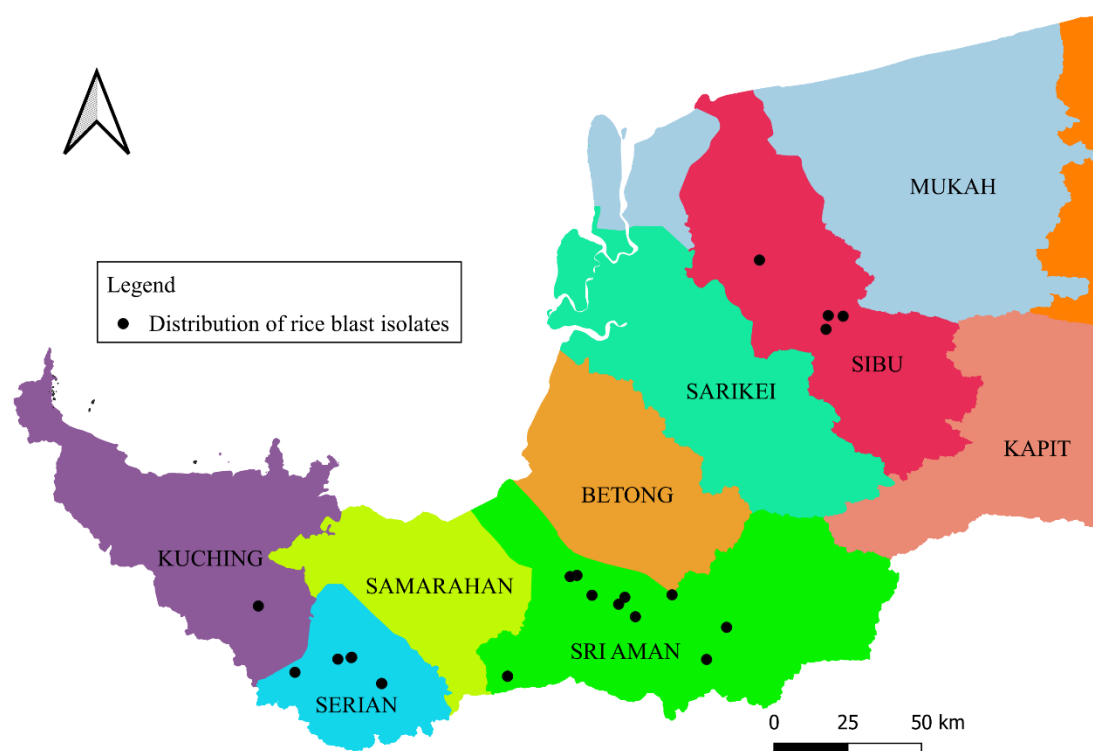


Figure 4.2: Distribution of the 25 *Pyricularia oryzae* isolates (black dots) collected in this study across the Sarawak's division.

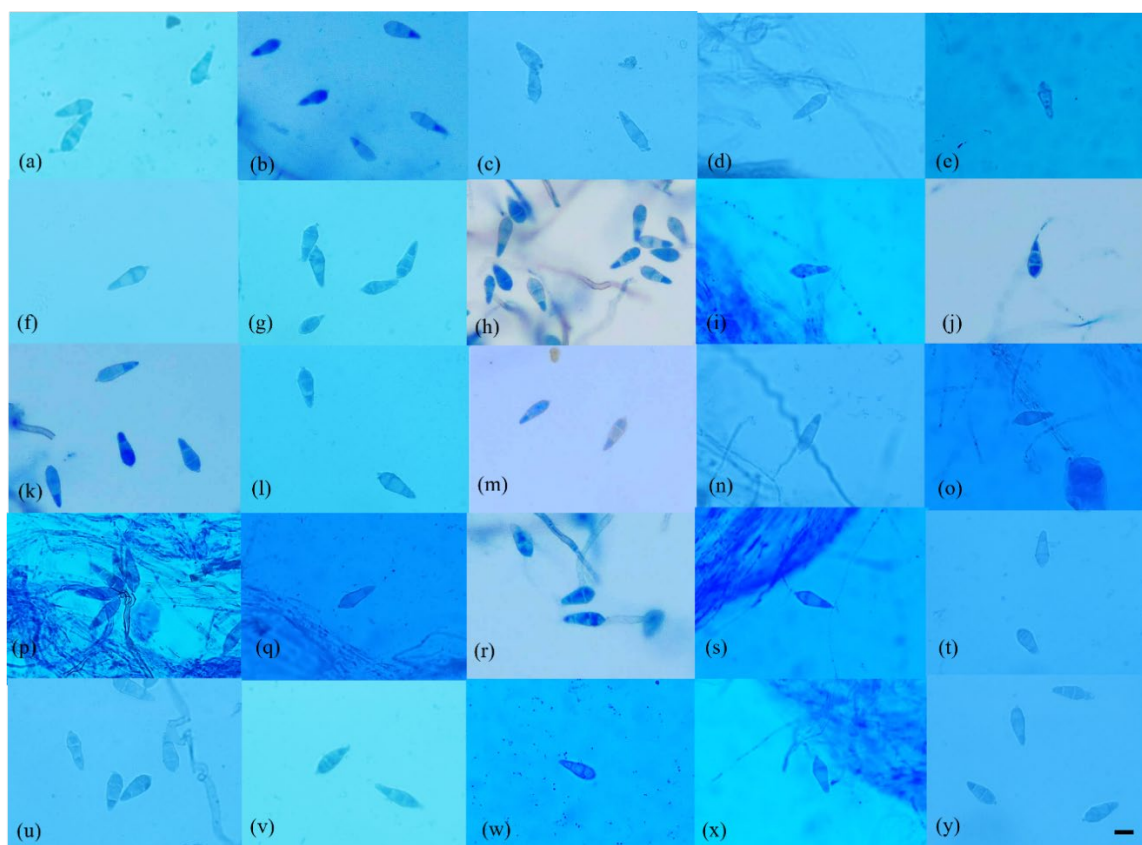


Figure 4.3: Conidia of 25 *Pyricularia oryzae* isolates from this study. (a) POB1, (b) POK9, (c) POS13, (d) POS20, (e) POS21, (f) POS22, (g) POS23, (h) POS24, (i) POS25, (j) POS26, (k) POSA13, (l) POSA14, (m) POSA15, (n) POSA16, (o) POSA17, (p) POSA18, (q) POSA19, (r) POSA20, (s) POSA21, (t) POSA22, (u) POSA23, (v) POSI3, (w) POSI5, (x) POSI6, (y) POSI7. Scale bar = 10 μ m.

Table 4.2: Summary of BLASTn result for 13 *Pyricularia oryzae* isolates obtained from this study.

Isolate	NCBI accession number	Amplicon size (bp)	Percentage of identity	E-value	NCBI accession number with the highest similarity
POB1	OR001955	487	98.97	0.0	KP144441.1
POK 9	OQ983835	490	99.80	0.0	MT093395.1
POS 13	OQ998907	322	100	4e-171	OQ913530.1
POS 22	OR001956	435	99.08	0.0	KP144441.1
POSA 14	OR001957	436	99.09	0.0	KP144441.1
POSA 18	OQ998910	485	99.59	0.0	OQ552821.1
POSA 21	OR001958	482	99.17	0.0	KP144441.1
POSA 22	OR001959	484	98.54	0.0	KP144441.1

Table 4.2: continued

POSA 23	OR001960	484	99.18	0.0	KP144441.1
POSI 3	OQ998908	470	99.58	0.0	OQ552821.1
POSI 5	OR001961	543	99.63	0.0	OQ552821.1
POSI 6	OQ998909	485	99.59	0.0	OQ552821.1
POSI 7	OR001962	469	99.79	0.0	OQ552821.1

4.2 Characterisation of *Pyricularia oryzae* Isolates on Different Media

4.2.1 Morphological Description of *Pyricularia oryzae* Isolates on Different Media

The morphological description for all 25 *P. oryzae* isolates is summarised in Table 4.3. All isolates were grouped based on form, elevation, and surface of the colony. The pigmentation and opacity of the colony were not considered in grouping of the colonies. There were nine groups of isolates for OMA (Figure 4.4) and six groups for PDA (Figure 4.5). All the isolates had entire margin for both of media.

The colony morphology of different *P. oryzae* isolates on both OMA and PDA were diverse. The colony upper surface varied from smooth, fluffy, to rough mycelia; some had wrinkled, shiny and slithery surface. The colonies' elevation (side view) in each group was either flat, raised or even both. The colony upper surface pigmentation of the colonies on both OMA and PDA were ranging from black, grey to white. Mostly were grey. For colony lower surface pigmentation, the colonies were also varied from black, different shades of grey and brown and even translucent white. Mostly were black or brown colour.

Table 4.3: Morphological description summary of 25 *Pyricularia oryzae* isolates on OMA and PDA.

Media	Group	Isolate	Pigmentation		Form	Elevation	Margin	Colony Upper Surface	Opacity
			Upper surface	Lower surface					
OMA	1	POK9	Grey	Dark grey	Circular	Flat	Entire	Smooth	Opaque
		POS13	Dark grey	Black					
		POS22	Grey	Black					
		POSA15	Grey	Pale brown at the centre, brown at the middle region.					
		POSI7	Grey	Pale brown at the centre, black middle region.					
	2	POS24	Light grey	Pale brown with dark brown patches.	Irregular	Flat			Opaque, translucent towards outer region.
		POS20	Light grey	Black					Opaque
		POS26	Light grey	Blackish brown to white edges					Opaque, translucent towards outer region.
	3	POSA14	Light grey	Black and dark brown	Circular	Raised		Smooth and cotton-like	Opaque, translucent towards outer region.
		POSI3	White	Black					
POSA21		Grey	Pale brown						

Table 4.3: continued

Media	Group	Isolate	Pigmentation		Form	Elevation	Margin	Upper Colony Surface	Opacity
			Upper colony	Lower colony					
OMA	4	POSA20	Grey with white patches	Black	Irregular	Raised	Entire	Smooth, wrinkled and cotton-like	Opaque, translucent towards outer region.
		POSA13	White	Black centre, pale grey at the middle region				Smooth and cotton-like	
		POSA22	Grey with white patches	Black				Smooth, wrinkled and cotton-like	
	5	POB1	Grey matured aerial mycelia, olive grey young mycelia	Olive grey	Circular with irregular pattern for aerial mycelia	Raised mature aerial mycelia, flat young mycelia	Cotton-like aerial mycelia	Opaque, translucent towards outer region.	
		6	POSA23	Grey matured mycelia and white young mycelia	Black centre.	Circular	Flat	Rough and fluffy aerial mycelia, smooth and shiny outer region.	Opaque centre and middle region, translucent outer region
			POSA16	Grey matured mycelia and white young mycelia	Black centre, white middle region			Rough centre, fluffy aerial mycelia at the middle region, smooth outer region.	Opaque, translucent towards outer region.
	7	POS23	Black with white and grey patches of aerial mycelia	Black	Irregular	Flat	Fluffy	Opaque	
	8	POS15	White	Pale brown	Filamentous	Raised	Rough and fuzzy	Opaque, translucent towards outer region.	
		POSA18							

Table 4.3: continued

OMA	9	POSI6	Translucent white	Translucent white	Circular	Flat	Entire	Shiny and slithery	Translucent
		POS21	White	Pale brown					
		POS25	Black with white aerial mycelia	Black					
		POSA17	Black centre with white patches of aerial mycelia	Light grey fading to light brown					Opaque, translucent towards outer region.
		POSA19	Black with scattered black patches	Light grey fades gradually to pale brown					Translucent
PDA	1	POK9	Light grey	Pale brown	Circular	Flat	Entire	Smooth and wrinkled with fluffy aerial mycelia.	Opaque
		POSI3	Light grey	Brownish grey					
		POSI6	Light grey	Pale brown					
	2	POSA16	Grey with white outer ring	Black		Raised		Fluffy aerial mycelia at the centre and outer region, smooth middle region.	Opaque
		POS20	Grey centre and white middle region towards the edge.	Creamy brown centre fading to pale brown					
		POS13	Grey with white outer ring	Black centre, with a dark brown concentric ring at the brown-coloured middle region					

Table 4.3: continued

PDA	2	POS22	White	Black centre, grey middle region, with two concentric ring: dark grey at the middle region	Circular	Raised	Entire	Smooth	Opaque
		POSA21	White	Black outline at plug area, pale brown towards the outer region.		Flat, raised aerial mycelia		Smooth	
		POSA15	Light grey	Black centre with brownish edge, pale brown middle region, and grey outer region.		Flat		Rough centre, smooth middle and outer region.	
	3	POSI5	White	Pale brown	Irregular	Flat mature aerial mycelia, raised young aerial mycelia	Entire	Rough	Translucent
		POS21	White	Pale brown				Fluffy and rough	
		POS23	Grey matured mycelia and white younger mycelia	Creamy brown centre, black middle region	Filamentous	Flat mature aerial mycelia, raised young aerial mycelia		Wrinkled centre, rough and fluffy middle and outer region	
		POSA23	White	Black centre, brown middle region	Circular and filamentous	Flat mature aerial mycelia, raised young aerial mycelia		Rough, and wrinkled centre	
		POSI7	Grey matured mycelia and white younger mycelia	Black.	Circular	Flat mature aerial mycelia, raised young mycelia		Smooth centre, rough middle, and outer region.	

Table 4.3: continued

PDA	3	POS26	Grey matured mycelia and white younger mycelia	Black centre gradually changes to pale brown	Filamentous	Flat		Rough	
		POSA17	Dark grey matured mycelia and white younger mycelia	Black centre with; grey middle region	Circular and filamentous	Flat mature mycelia. Raised young mycelia		Wrinkled, smooth centre, fluffy towards the outer region	
		POSA18	White	Pale brown		Flat		Wrinkled, rough centre, smoothened towards the outer region.	Translucent
	4	POSA19	Olive grey at matured mycelia, gradually changed to brown and then white towards younger mycelia	Light grey	Circular	Raised mature aerial mycelia, flat young mycelia	Entire	Wrinkled and dull, rough aerial mycelia in middle region.	Opaque, translucent towards outer region.
		POS24	White	Pale brown with dark brown at the middle region.	Filamentous	Raised mature aerial mycelia, flat young mycelia		Wrinkled with rough and fluffy ring of aerial mycelia at the middle region.	
		POSA13	White	Pale brown at the centre, white middle region.	Circular	Raised mature aerial mycelia, flat young mycelia		Wrinkled, smooth with fluffy aerial mycelia	Opaque, translucent towards outer region.
	5	POS25	White	Creamy pale brown				Wrinkled, rough and fluffy aerial mycelia.	Opaque, translucent towards outer region.
		POSA14	White	Grey	Circular and filamentous	Raised mature aerial mycelia, flat young mycelia	Entire	Rough aerial mycelia at the centre, smooth towards the edge.	

Table 4.3: continued

PDA	5	POSA20	White	Black centre, white middle region.		Raised mature aerial mycelia, flat young mycelia		Rough and fluffy aerial mycelia in the centre and middle region. More dense aerial mycelia in the centre.	
		POSA22	Grey matured mycelia and white younger mycelia	Black centre, brownish middle region.		Raised mature aerial mycelia, flat young mycelia		Rough and fluffy aerial mycelia, denser aerial mycelia at centre and middle region.	
	6	POB1	Olive grey	Brownish grey centre, black middle region.		Circular	Flat	Entire	Smooth and Opaque powdery

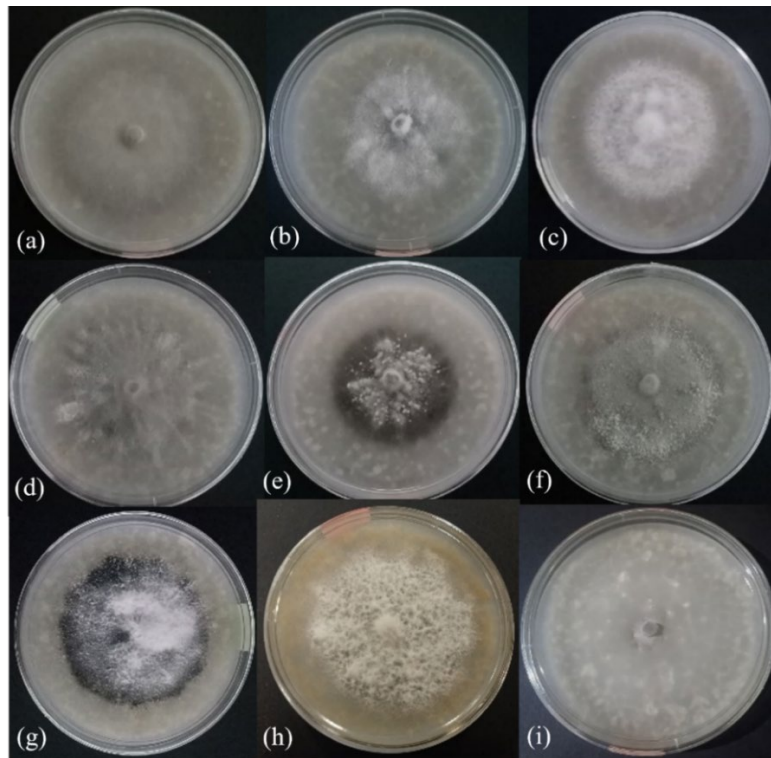


Figure 4.4: Morphological characteristics of nine groups of *Pyricularia oryzae* isolates grown on OMA at ten days after inoculation. Isolates from (a) to (i) represent Group 1 to Group 9, respectively.

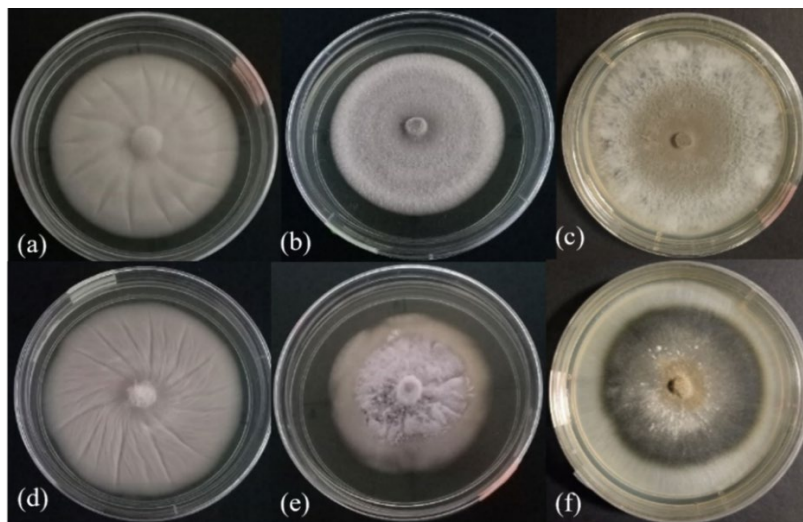


Figure 4.5: Morphological characteristics of the six groups of *Pyricularia oryzae* isolates grown on PDA at ten days after inoculation. Isolates from (a) to (f) represent Group 1 to Group 6, respectively.

4.2.2 Growth Performance and Sporulation of *P. oryzae* Isolates on Different Media

A total of 24 *P. oryzae* isolates were selected out of 25 *P. oryzae* for growth performance test on OMA and PDA. Two-way ANOVA analysis (Table 4.4) showed significant result for isolate and media interaction ($p \leq 0.05$). In the analysis to compare the growth rate of *P. oryzae* isolates on OMA and PDA, 19 out of 24 isolates had total number of samples (n) = 30 and were analysed using Student's t-test. While the remaining five isolates had $n < 30$ and were analysed using Mann-Whitney. In the analysis to compare the growth rate among isolates in each medium, one-way ANOVA was used for 19 isolates having $n=30$ and Kruskal-Wallis was used for the remaining five isolates (POS23, POS24, POSA14, POSA19 and POSA20) with $n < 30$.

Student's t-test analysis for 19 out of 24 *P. oryzae* isolates showed significant growth rate difference on OMA and PDA for some isolates (Table 4.5). Similarly, Mann-Whitney analysis carried out for (POS23, POS24, POSA14, POSA19 and POSA20) showed significant growth rate difference for some isolates when grown on OMA and PDA (Table 4.6).

One-way ANOVA analysis showed that the growth rate among isolates in each medium showed that there was significant difference among some *P. oryzae* isolates regardless the medium used (Table 4.7). Kruskal-Wallis analysis then showed that there was one isolate (POS24) that showed significant growth rate difference compared to other isolates when grown on either OMA or PDA (Table 4.8).

Growth rate of an isolate on both OMA and PDA varied where significant difference ($p < 0.05$) was observed between isolates. Some isolates had better growth on OMA compared to PDA. For example, isolate POSI3 (Table 4.5) had faster growth rate when grown on OMA ($0.30 \text{ cm/day} \pm 0.01$) compared to PDA ($0.24 \text{ cm/day} \pm 0.01$). But there were also some

isolates that grew better on PDA compared to OMA, i.e., isolate POB1 had faster growth rate on PDA ($0.31 \text{ cm/day} \pm 0.01$) compared to on OMA ($0.30 \text{ cm/day} \pm 0.01$). Hence the two-way ANOVA showed significant effect of isolate and media interaction ($p \leq 0.05$). Apart from that, the growth rate of among the isolates on a medium were also varies. There were isolates having faster growth than the others. For instance, isolate POSA18 ($0.31 \text{ cm/day} \pm 0.01$) was among the fastest growing isolate on OMA, and isolate POS21 ($0.24 \text{ cm/day} \pm 0.01$) was among the slowest growing isolates. While on PDA, isolate POS26 ($0.33 \text{ cm/day} \pm 0.02$) was among the fastest growing isolate and isolate POS21 ($0.17 \text{ cm/day} \pm 0.01$) was among the slowest (Table 4.7).

Sporulation rate of *P. oryzae* isolates on OMA and PDA is tabulated in Table 4.9. Out of 24 *P. oryzae* isolates, 19 isolates sporulated on OMA and 14 isolates sporulated on PDA. Isolates sporulating on OMA may not all sporulate on PDA, but those sporulating on PDA also sporulate on OMA (e.g.: POS24). This might suggest that OMA is a better media for sporulation compared to PDA. In average, the sporulation rate of *P. oryzae* on OMA was $4.59 \times 10^5/\text{mL}$ (range: $0 - 30.77 \times 10^5/\text{mL}$) and on PDA was $0.91 \times 10^5/\text{mL}$ (range: $0 - 6.34 \times 10^5/\text{mL}$). However, due to insufficient replication, comparison of sporulation rate between isolates on each medium was not performed. Mann-Whitney analysis to compare the sporulation of *P. oryzae* isolates grown on OMA and PDA showed no significant difference (Table 4.10).

Table 4.4: Two - way ANOVA analysis of growth rate for 24 isolates of *Pyricularia oryzae* on different media.

Source	F value	Sig. value
Isolate	142.06	0.00 [!]
Media	194.52	0.00 [!]
Isolate*Media	40.96	0.00 [!]

Note: Exclamation mark[!] indicates significant value at $p \leq 0.05$.

Table 4.5: Growth rate of 19 *Pyricularia oryzae* isolates on OMA in comparison to PDA.

Isolate	Media				Sig. value (2-tailed)
	OM)		PDA		
	Mean (cm/day)	Std. Error	Mean (cm/day)	Std. Error	
POB 1	0.30	±0.0015	0.31	±0.0021	0.00*
POK 9	0.30	±0.0015	0.31	±0.0039	0.04*
POS 13	0.29	±0.0043	0.29	±0.0063	0.55
POS 20	0.28	±0.0018	0.20	±0.0047	0.00*
POS 21	0.24	±0.0029	0.17	±0.0018	0.00*
POS 22	0.31	±0.0038	0.30	±0.0033	0.02*
POS 25	0.28	±0.0019	0.19	±0.0022	0.00*
POS 26	0.30	±0.0013	0.33	±0.0043	0.00*
POSA 13	0.30	±0.0020	0.31	±0.0033	0.18
POSA 15	0.29	±0.0022	0.30	±0.0070	0.06
POSA 16	0.26	±0.0025	0.21	±0.0053	0.00*
POSA 17	0.27	±0.0027	0.28	±0.0040	0.14
POSA 18	0.31	±0.0028	0.31	±0.0044	0.61
POSA 21	0.28	±0.0022	0.22	±0.0066	0.00*
POSA 22	0.30	±0.0036	0.29	±0.0079	0.29
POSA 23	0.31	±0.0023	0.31	±0.0046	0.31
POSI 3	0.30	±0.0029	0.24	±0.0026	0.00*
POSI 5	0.30	±0.0035	0.32	±0.0057	0.08
POSI 7	0.31	±0.0047	0.30	±0.0064	0.41
Total Mean	0.29		0.27		

Note: The data was analysed using Student's t-test. Asterisk '*' indicates significant value at $p \leq 0.05$.

Table 4.6: Growth rate of five *Pyricularia oryzae* isolates on OMA in comparison to PDA.

Isolate	OMA		PDA		Sig. value (2-tailed)
	Mean	Std. Error	Mean	Std. Error	
POS 23	0.32	±0.0031	0.29	±0.0072	0.01*
POS 24	0.28	±0.0024	0.22	±0.053	0.00*
POSA 14	0.31	±0.0086	0.30	±0.0054	0.09
POSA 19	0.31	±0.0020	0.31	±0.0058	0.84
POSA 20	0.31	±0.0026	0.32	±0.0071	0.29
Total Mean	0.31		0.29		

Note: The data was analysed using Mann-Whitney test. Asterisk '**' indicates significant $p \leq 0.05$.

Table 4.7: Comparing growth rate among the 19 *Pyricularia oryzae* isolates on OMA and PDA, respectively.

Isolate	OMA		PDA	
	Mean	Std. Error	Mean	Std. Error
POB 1	0.30 ^{defg}	±0.0015	0.31 ^{fgh}	±0.0021
POK 9	0.30 ^{efg}	±0.0015	0.31 ^{fgh}	±0.0039
POS 13	0.29 ^{bcd}	±0.0043	0.29 ^{ef}	±0.0063
POS 20	0.28 ^{bc}	±0.0018	0.20 ^{bc}	±0.0047
POS 21	0.24 ^a	±0.0029	0.17 ^a	±0.0018
POS 22	0.31 ^{fg}	±0.0038	0.30 ^{efg}	±0.0033
POS 25	0.28 ^{bc}	±0.0019	0.19 ^{ab}	±0.0022
POS 26	0.30 ^{fg}	±0.0013	0.33 ^h	±0.0043
POSA 13	0.30 ^{efg}	±0.0020	0.31 ^{fgh}	±0.0033
POSA 15	0.29 ^{cde}	±0.0022	0.30 ^{efgh}	±0.0070
POSA 16	0.26 ^a	±0.0025	0.21 ^{bc}	±0.0053
POSA 17	0.27 ^b	±0.0027	0.28 ^c	±0.0040
POSA 18	0.31 ^g	±0.0028	0.31 ^{fgh}	±0.0044
POSA 21	0.28 ^{bc}	±0.0022	0.22 ^{cd}	±0.0066
POSA 22	0.30 ^{efg}	±0.0036	0.29 ^{efg}	±0.0079
POSA 23	0.31 ^{fg}	±0.0023	0.31 ^{efgh}	±0.0046
POSI 3	0.30 ^{def}	±0.0029	0.24 ^d	±0.0026
POSI 5	0.30 ^{fg}	±0.0035	0.32 ^{gh}	±0.0057
POSI 7	0.31 ^{fg}	±0.0047	0.30 ^{efg}	±0.0064

Note: The data was analysed using one-way ANOVA followed by Tukey's HSD post-hoc test. Same letter indicates no significant difference.

Table 4.8: Comparing growth rate among five *Pyricularia oryzae* isolates on OMA and PDA, respectively.

Isolate	Media	
	OMA	PDA
POS 23	0.32 ^b	0.29 ^b
POS 24	0.28 ^a	0.22 ^a
POSA 14	0.31 ^b	0.30 ^b
POSA 19	0.31 ^b	0.31 ^b
POSA 20	0.31 ^b	0.32 ^b
Total Mean	0.31	0.29

Note: The data was analysed using Kruskal Wallis test. Same letter indicates no significant difference.

Table 4.9: Sporulation rate of *Pyricularia oryzae* isolates on OMA and PDA.

Isolate	Sporulation rate x 10 ⁵ /mL	
	Media	
	OMA	PDA
POB 1	9.25	0.84
POK 9	0.74	1.51
POS 13	0.03	0.06
POS 20	0.00	0.00
POS 21	0.00	0.00
POS 22	4.43	1.29
POS 23	0.02	0.00
POS 24	6.53	0.00
POS 25	0.00	0.00
POS 26	30.77	6.34
POSA 13	0.12	0.00
POSA 14	9.48	1.57
POSA 15	0.05	0.03
POSA 16	0.41	0.00
POSA 17	1.76	0.17
POSA 18	0.00	0.00
POSA 19	0.00	0.00
POSA 20	7.36	0.07
POSA 21	0.00	0.00
POSA 22	12.01	5.67
POSA 23	19.26	3.63
POSI 3	0.29	0.01
POSI 5	0.02	0.02
POSI 7	7.72	0.67
Total mean	4.59	0.91

Table 4.10: Sporulation rate comparison of *Pyricularia oryzae* isolates (in general) grown on OMA and PDA.

On OMA and PDA:					
Sporulation Rate	Media				Sig. value
	OMA		PDA		
	Mean	Std. Error	Mean	Std. Error	
	4.59	1.54	0.91	0.36	0.06

Note: The data was analysed using Mann-Whitney test. p-value ≤0.05.

4.3 Isolation of selected Avirulence Genes from *P. oryzae* Isolates

4.3.1 Amplification of *Avr* Genes (*AvrPiz-t*, *AvrPik* and *AvrPi-54*)

Avirulence (*Avr*) genes (*AvrPiz-t*, *AvrPik* and *AvrPi-54*) from *P. oryzae* isolates were successfully amplified from all 26 isolates for *AvrPiz-t*, 11 (out of 20) isolates for *AvrPik* and 17 (out of 20) isolates for *AvrPi-54* (Table 4.11). The *AvrPiz-t* gene nucleotide sequences ranged from 326 to 327 bp, 342 bp for all *Avr-Pik* gene sequences and 462 bp for all *AvrPi-54* gene sequences. BLASTn of the sequences showed high percentage identity (99.69-100%) with *AvrPiz-t*, *Avr-Pik* and *AvrPi-54* gene nucleotide sequences of *P. oryzae* published in the NCBI database (Table 4.12).

After translation, the length of amino acid sequence of *AvrPiz-t* for all 26 isolates was 108 amino acid, while for *Avr-Pik* and *AvrPi-54* were 114 amino acid and 154 amino acid, respectively, which were the same as previously reported *AvrPiz-t*, *AvrPik* and *AvrPi-54* amino acid sequences by Jee *et al.* (2017) for *P. oryzae* isolate of Sarawak [Accession no: KX459419 (*AvrPiz-t*); KX459420 (*AvrPik*); KY441415 (*AvrPi-54*)].

Table 4.11: Amplification of *AvrPiz-t*, *AvrPik* and *AvrPi-54* in 26 *Pyricularia oryzae* isolates of Sarawak.

Isolate	Location	Division	Presence of avirulence genes		
			<i>AvrPiz-t</i>	<i>AvrPik</i>	<i>AvrPi-54</i>
POB1	Jln Betong	Betong	✓	✗	✓
POK 9	Kpg Garung, Padawan	Kuching	✓	✗	✓
POS 13	Kpg Tema Penggal	Serian	✓	✓	✓
POS 22	Kpg Slabi Plaman	Serian	✓	✓	✓
POSA 14	Kpg Dadak B	Sri Aman	✓	✓	✗
POSA 21	Kpg Seduku	Sri Aman	✓	✗	✓
POSA 22	Engkelili	Sri Aman	✓	✓	✓
POSA 23	Lubuk Antu	Sri Aman	✓	✓	✓
POSI 5	Jambatan Igan	Sibu	✓	✗	✓
POSI 7	Kanowit	Sibu	✓	✓	✓
POK 4	Kpg Sorak Melayu	Kuching	✓	✗	✓
POK 6	Kpg Stunggang	Kuching	✓	✗	✓

Table 4.11: continued

POS 14	Kpg Riih Mawang	Serian	✓	✓	✓
POS 17	Kpg Riih Mawang	Serian	✓	✓	✓
POS 18	Kpg Riih Mawang	Serian	✓	✓	✓
POS 19	Kpg Riih Mawang	Serian	✓	✓	✓
POS 2	Kpg Remun	Serian	✓	✗	✗
POS 4	Kpg Paon Gahat	Serian	✓	✗	✗
POSA 2	Stumbin	Sri Aman	✓	✗	✓
POSI 3	Jln Kanowit-Durin	Sibu	✓	✓	✓
POK 7	Kpg Bengoh	Kuching	✓	N/A	N/A
POMI 1	Bario	Miri	✓	N/A	N/A
POS 1	Kpg Remun	Serian	✓	N/A	N/A
POS 8	Kpg Tarat	Serian	✓	N/A	N/A
POSA 1	Blok A, Stumbin	Sri Aman	✓	N/A	N/A
POSA 5	Kpg Semulong Ulu	Sri Aman	✓	N/A	N/A

Note: ✓ - amplification successful, ✗ - amplification unsuccessful, N/A - isolate not available.

Table 4.12: Result of nucleotide BLASTn result of *Avr* genes (*AvrPiz-t*, *AvrPik* and *AvrPi-54*) isolated from *P. oryzae* isolates.

Isolate	Accession number	Amplicon size (bp)	Percentage of identity	E-value	NCBI accession number with the highest similarity
<i>AvrPiz-t</i> gene					
POB1	OQ943791	327	100	3e-168	MN072468.1
POK 4	OQ943797	326	99.69	5e-166	MN072468.1
POK 6	OQ943792	327	100	3e-168	HE650811.1
POK 7	OQ943798	326	99.69	5e-166	MN072468.1
POK 9	OQ943793	327	100	3e-168	MN072468.1
POMI 1	OQ943794	327	100	3e-168	MN072468.1
POS 1	OQ943799	326	99.69	5e-166	MN072468.1
POS 13	OQ943802	326	99.69	5e-166	MN072468.1
POS 14	OQ943803	326	99.69	5e-166	MN072468.1
POS 17	OQ943804	326	99.69	5e-166	MN072468.1
POS 18	OQ943805	326	99.69	5e-166	MN072468.1
POS 19	OQ943806	326	99.69	5e-166	MN072468.1
POS 2	OQ943800	326	99.69	5e-166	MN072468.1
POS 22	OQ943807	326	99.69	5e-166	MN072468.1
POS 4	OQ943801	326	99.69	5e-166	MN072468.1
POS 8	OQ943795	327	100	3e-168	MN072468.1
POSA 1	OQ943808	326	99.69	5e-166	MN072468.1
POSA 14	OQ943811	326	99.69	5e-166	MN072468.1
POSA 2	OQ943809	326	99.69	5e-166	MN072468.1

Table 4.12: continued

POSA 21	OQ943812	326	99.69	5e-166	MN072468.1
POSA 22	OQ943796	327	100	3e-168	MN072468.1
POSA 23	OQ943813	326	99.69	5e-166	MN072468.1
POSA 5	OQ943810	326	99.69	5e-166	MN072468.1
POSI 3	OQ943814	326	99.69	5e-166	MN072468.1
POSI 5	OQ943815	326	99.69	5e-166	MN072468.1
POSI 7	OQ943816	326	99.69	5e-166	MN072468.1
<i>AvrPik</i> gene					
POS 13	N/A	342	100	1e-176	OP750463.1
POS 14	N/A	342	100	1e-176	MK327187.1
POS 17	N/A	342	100	1e-176	OP750463.1
POS 18	N/A	342	100	1e-176	OP750463.1
POS 19	N/A	342	100	1e-176	OP750463.1
POS 22	N/A	342	100	1e-176	MK327187.1
POSA 14	N/A	342	100	1e-176	MK327187.1
POSA 22	N/A	342	100	1e-176	OP750463.1
POSA 23	N/A	342	100	1e-176	MK327190.1
POSI 3	N/A	342	100	1e-176	OP750463.1
POSI 7	N/A	342	100	1e-176	OP750463.1
<i>AvrPi-54</i> gene					
POB1	N/A	462	100	0.0	HF545677.2
POK 4	N/A	462	100	0.0	HF545677.2
POK 6	N/A	462	100	0.0	HF545677.2
POK 9	N/A	462	100	0.0	HF545677.2
POS 13	N/A	462	100	0.0	HF545677.2
POS17	N/A	462	100	0.0	HF545677.2
POS 18	N/A	462	100	0.0	HF545677.2
POS 19	N/A	462	100	0.0	HF545677.2
POS 22	N/A	462	100	0.0	HF545677.2
POSA 2	N/A	462	100	0.0	HF545677.2
POSA 21	N/A	462	100	0.0	HF545677.2
POSA 22	N/A	462	100	0.0	HF545677.2
POSA 23	N/A	462	100	0.0	HF545677.2
POSI 3	N/A	462	100	0.0	HF545677.2
POSI 5	N/A	462	100	0.0	MN072491.1
POSI 7	N/A	462	100	0.0	HF545677.2

N/A- accession number is not available.

4.3.2 Haplotype analysis of *AvrPiz-t* genes

A total of 224 *AvrPiz-t* open reading frame (ORF) sequences available in National Centre of Biotechnology Information (NCBI) database were downloaded and aligned with 26 *AvrPiz-t* ORF sequences from *P. oryzae* of Malaysia (Sarawak). A total of 11 haplotypes were identified from the alignment of 250 *AvrPiz-t* ORF sequences amplified from *P. oryzae* originating from Malaysia (Sarawak), Thailand, India and China. The ORF sequence of the 11 haplotypes were translated into amino acid sequence and aligned with the amino acid sequence of the eight *AvrPiz-t* haplotypes of Chen *et al.* (2014). Based on this alignment, a total of 19 haplotypes were observed (Figure 4.6). They were designated as H1 - H19 continuing the haplotype numbering of Chen *et al.* (2014). Among these 19 haplotypes, H1 to H8 were the eight haplotypes of Chen *et al.* (2014). H9 to H11 were the haplotypes reported by Wang *et al.* (2020). Haplotype VII of Chen *et al.* (2014) (now H7 in this study) was the same haplotype of H7 reported by Wang *et al.* (2020). H9, H10 and H11 were corresponding to the haplotype H3, H6 and H10 of Wang *et al.* (2020), respectively. In addition to the *AvrPiz-t* haplotypes reported by Chen *et al.* (2014) and Wang *et al.* (2020), there were eight new haplotypes identified. There were three new haplotypes found in the *P. oryzae* of Malaysia (Sarawak), three from Thailand and three from India. These new eight haplotypes were designated as H12 to H19.

The *AvrPiz-t* ORF sequence of haplotype H12 to H19 had mutations that were non-synonymous except for one synonymous mutation occur in H15 (base substitution from T to C occur at 231bp) and four in H19 (base substitution from A to C occur at 240bp and from C to A at 261bp, 270bp and 291bp). Base deletion occurs in H14 (G deletion at 300bp) causing frameshift and led to the missing of stop codon in the ORF region. To sum up, there were nine non-synonymous and five synonymous mutations occurred, with 15 segregating

sites at the single nucleotide position (SNPs) within the ORF sequence of the eight new *AvrPiz-t* haplotypes (Table 4.13 and Table 4.14).

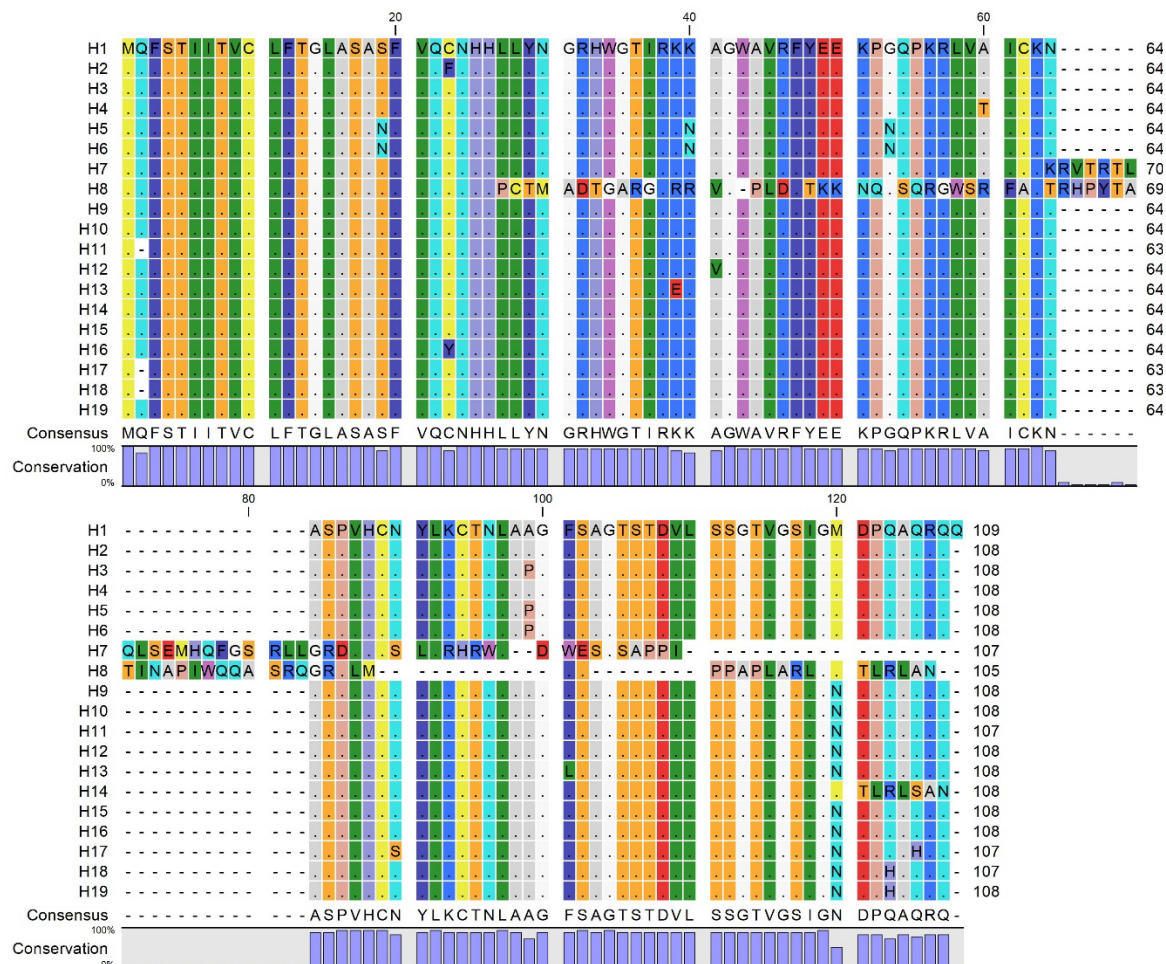


Figure 4.6: *AvrPiz-t* haplotype alignment based on amino acid sequences from the present study, H1 – H8: Chen *et al.* (2014); H9 – H11 : Wang *et al.* (2020); H12 - H19 : new *AvrPiz-t* haplotypes..

Table 4.13: Single nucleotide polymorphisms (SNPs) occurs in *AvrPiz-t* open reading frame (ORF) for the eight new haplotypes identified in this study.

Haplotype	Representative isolate	Nucleotide position (codon number)														
		4 (2)	6 (2)	68 (23)	115 (39)	122 (41)	212 (71)	231 (77)	240 (80)	243 (82)	261 (87)	270 (90)	291 (97)	300 (100)	312 (104)	318 (106)
H9	EU837058	C	G	G	A	C	A	T	A	T	C	C	C	G	G	G
H12	POK6	C	G	G	A	T**	A	T	A	T	C	C	C	G	G	G
H13	KX459419	C	G	G	G**	C	A	T	A	C**	C	C	C	G	G	G
H14	POS14	C	G	G	A	C	A	T	A	T	C	C	C	-	G	G
H15	HE578830	C	G	G	A	C	A	C*	A	T	C	C	C	G	G	G
H16	HE584662	C	G	A**	A	C	A	T	A	T	C	C	C	G	G	G
H17	LC175911	T**	A**	G	A	C	G**	T	A	T	C	C	C	G	G	T**
H18	LC175915	T**	A**	G	A	C	A	T	A	T	C	C	C	G	T**	G
H19	LC175919	C	G	G	A	C	A	T	C*	T	A*	A*	A*	G	T**	G

AvrPiz-t ORF sequence of accession EU837058 was used as the reference sequence in this table following Wang *et al.* (2020). *synonymous mutation **non-synonymous mutation

Table 4.14: Summary of polymorphic sites of amino acids sequence of the eight new *AvrPiz-t* haplotypes identified in this study.

Haplotype number	Codon number																	
	2	23	39	41	71	77	80	82	87	90	97	100	101	102	103	104	105	106
H9	Q	C	K	A	N	N	A	F	S	V	G	G	N	D	P	Q	A	Q
H12	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H13	-	-	E	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-
H14	-	-	-	-	-	-	-	-	-	-	-	-	M	T	L	R	L	S
H15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H16	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H17	*	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	H
H18	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-
H19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-

The amino acid sequence of H9 was used as reference sequence following Wang *et al.* (2020). H14 had a deletion at nucleotide 300 bp, which resulted in a frameshift and changes the subsequence amino acids. * stop codon; - no change of amino acid.

Based on the origin of the *P. oryzae* isolates, all the 19 haplotypes were distributed across 14 countries [Malaysia (Sarawak), Thailand, India, China, Philippines, Vietnam, Japan, Gabon, Colombia, Kenya, United States of America (USA), Egypt, Brazil, and Burundi]. There were four haplotypes (H3, H6, H9, H12) present in more than two countries and fifteen haplotypes (H1, H2, H4, H5, H7, H8, H10, H11, H13, H14, H15, H16, H17, H18, H19) were found in isolates of *P. oryzae* from one country but not the others (Figure 4.7). The dominant haplotype was H9 as it was found in 116 *P. oryzae* isolates from Malaysia (Sarawak; 5 isolates), India (71 isolates), Thailand (39 isolates), and China (1 isolate).

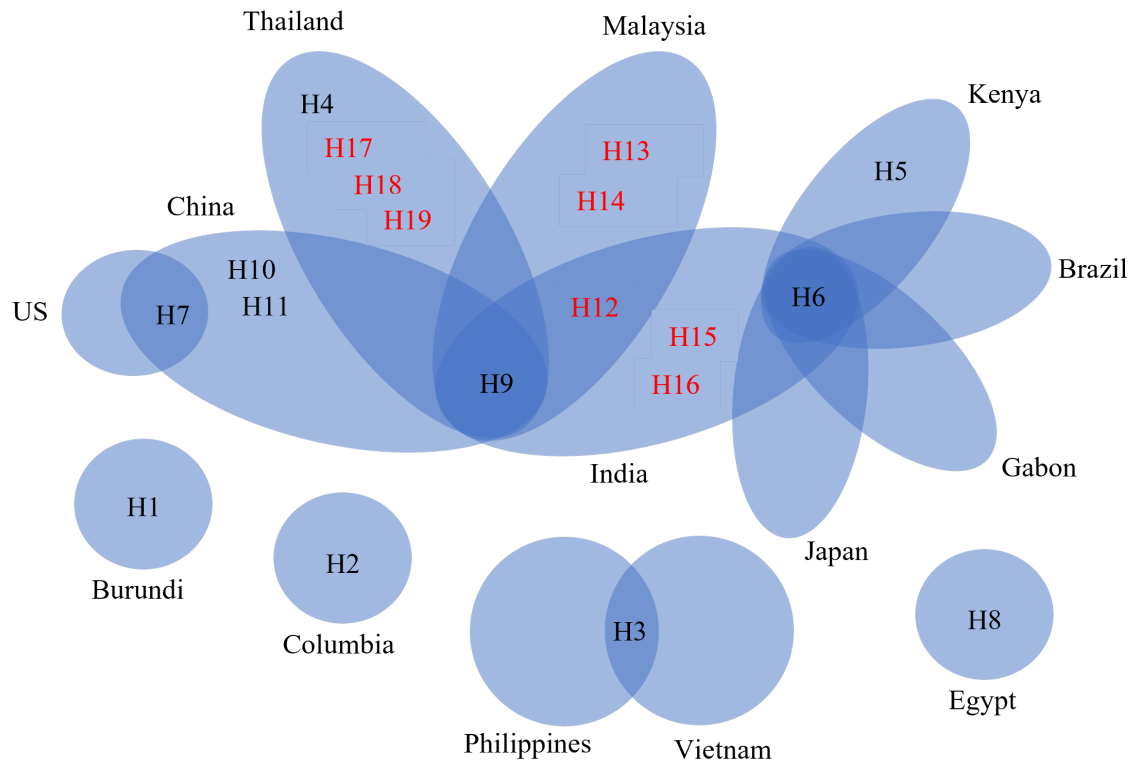


Figure 4.7: Venn diagram showing the presence of *AvrPiz-t* haplotypes based on the new haplotypes, Chen *et al.* (2014) and Wang *et al.* (2020) in 14 countries.

4.3.3 Haplotype Analysis of *AvrPik* Gene

A total of 169 *AvrPik* ORF sequences available in NCBI database were downloaded and aligned with 11 *AvrPik* ORF from *P. oryzae* of Malaysia (Sarawak). From the alignment of 181 *AvrPik* gene ORF from Malaysia (Sarawak), Thailand, China, Japan and Korea, 12 haplotypes were observed.

The 12 *Avr-Pik* haplotype amino acid sequence were then aligned with five *AvrPik* amino acid sequence from Zhang *et al.* (2020) revealing a total of 13 *AvrPik* haplotypes (Figure 4.8). All 13 *AvrPik* haplotypes were designated as *AvrPik-A*, *AvrPik-B*, *AvrPik-C*, *AvrPik-D*, *AvrPik-E*, *AvrPik-F*, *AvrPik-G*, *AvrPik-H*, *AvrPik-I*, *AvrPik-J*, *AvrPik-K*, *AvrPik-L* and *AvrPik-M*. Notably, the *AvrPik-A* to *AvrPik-E* classifications were previously documented by Yoshida *et al.* (2019), whereas *AvrPik-F* was identified by Longya *et al.* (2020). The *AvrPik-G* through *AvrPik-K* variants were reported in the study by Zhang *et al.* (2020). *AvrPik-L* was previously known as H8 in Li *et al.* (2019) and *AvrPik-M* was the newly identified *AvrPik* haplotype in this study. All substitution mutations that occurred among all 12 *AvrPik* haplotypes (excluding *AvrPik-J*) in this study were non-synonymous, with seven SNPs (Table 4.15 and Table 4.16). *AvrPik-J* was excluded because the ORF sequence was not made available in NCBI. Only amino acid sequence was available based on Zhang *et al.* (2020)

Based on the origin of the *P. oryzae* isolates, all the 13 *AvrPik* alleles were distributed across 6 countries [Malaysia (Sarawak), Thailand, China, Japan, Korea and United States (USA)] (Figure 4.9). Most of the alleles were present in two or more countries except for *AvrPik-E* which was unique to Japan isolates, *AvrPik-L* unique to China isolates, *AvrPik-M* to Korea isolates and *AvrPik-J* to USA isolates.

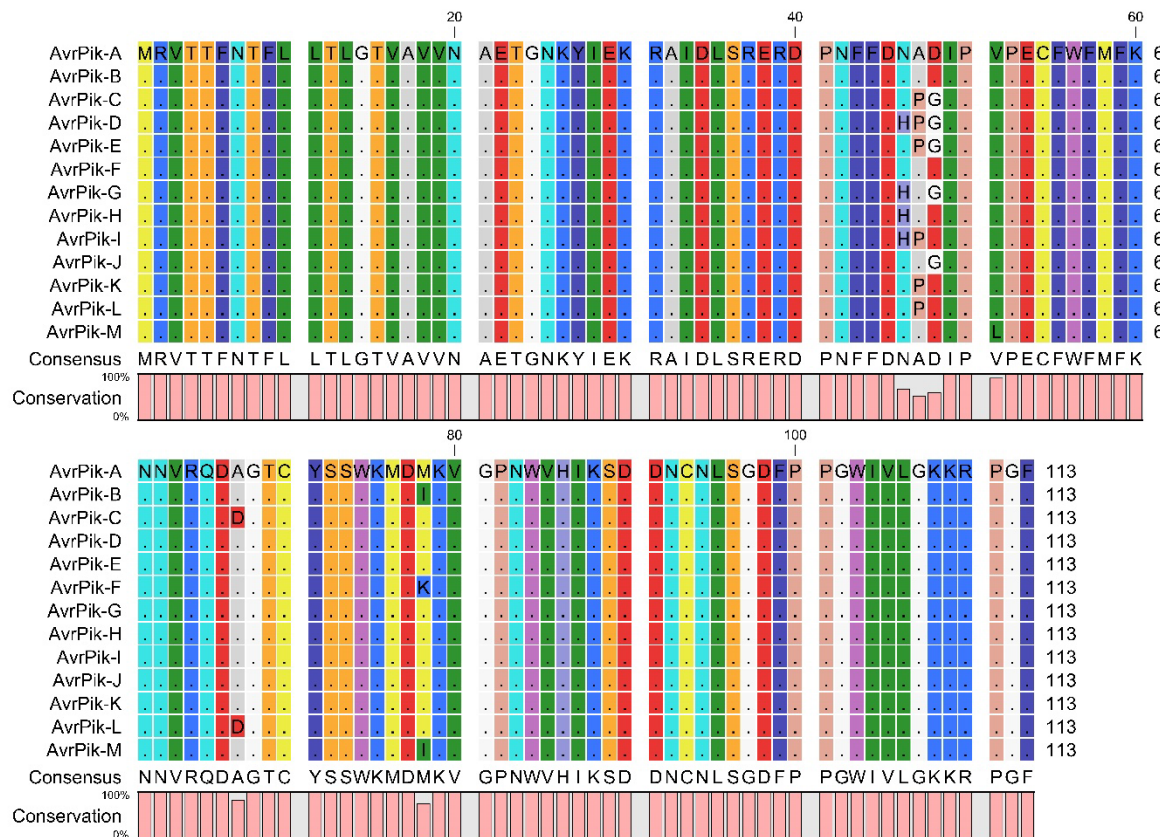


Figure 4.8: Amino acid sequence alignment of 13 *AvrPik* haplotypes from present study. *AvrPik-A* - *AvrPik-E* : Yoshida *et al.* (2019); *AvrPik-F* : Longya *et al.* (2020); *AvrPik-G* - *AvrPik-K* : Zhang *et al.* (2020); *AvrPik-L* : Li *et al.* (2019). *AvrPik-M* : new *AvrPik* haplotype.

Table 4.15: Summary of single nucleotide polymorphisms (SNPs) occurs in *AvrPik* ORF of the 12 haplotypes identified in this study.

Avr-Pik alleles	Representative isolate	Nucleotide position (codon number)						
		136 (46)	139 (47)	143 (48)	151 (51)	200 (67)	233 (78)	234 (78)
<i>AvrPik-A</i>	AB498876.1 ^a	A	G	A	G	C	T	G
<i>AvrPik-B</i>	AB498877.1	A	G	A	G	C	T	A**
<i>AvrPik-C</i>	AB498878.1	A	C**	G**	G	A**	T	G
<i>AvrPik-D</i>	POS13	C**	C**	G**	G	C	T	G
<i>AvrPik-E</i>	AB498879.1	A	C**	G**	G	C	T	G
<i>AvrPik-F</i>	POSA23	A	G	A	G	C	A**	G
<i>AvrPik-G</i>	KY432944.1	C**	G	G**	G	C	T	G
<i>AvrPik-H</i>	KY432943.1	C**	G	A	G	C	T	G
<i>AvrPik-I</i>	POS14	C**	C**	A	G	C	T	G
<i>AvrPik-K</i>	MK327186.1	A	C**	A	G	C	T	G
<i>AvrPik-L</i>	MK327188.1	A	C**	A	G	A**	T	G
<i>AvrPik-M</i>	ON721121.1	A	G	A	T**	C	T	A**

Note: ^a-reference sequence. *AvrPik-J* from Zhang *et al.* (2020) was not included in this *AvrPik* ORF alignment. **non-synonymous mutation.

Table 4.16: Summary of polymorphic sites in amino acids sequence of the 13 *AvrPik* haplotypes identified in this study.

Avr-Pik alleles	Representative isolate	Codon position					
		46	47	48	51	67	78
<i>AvrPik-A</i>	AB498876.1 ^a	N	A	D	V	A	M
<i>AvrPik-B</i>	AB498877.1	-	-	-	-	-	I
<i>AvrPik-C</i>	AB498878.1	-	P	G	-	D	-
<i>AvrPik-D</i>	POS13	H	P	G	-	-	-
<i>AvrPik-E</i>	AB498879.1	-	P	G	-	-	-
<i>AvrPik-F</i>	POSA23	-	-	-	-	-	K
<i>AvrPik-G</i>	KY432944.1	H	-	G	-	-	-
<i>AvrPik-H</i>	KY432943.1	H	-	-	-	-	-
<i>AvrPik-I</i>	POS14	H	P	-	-	-	-
<i>AvrPik-J</i>	MK327186.1	-	-	G	-	-	-
<i>AvrPik-K</i>	MK327188.1	-	P	-	-	-	-
<i>AvrPik-L</i>	ON721121.1	-	P	-	-	D	-
<i>AvrPik-M</i>	AB498876.1 ^a	-	-	-	L	-	I

Note: ^a - reference sequence. *AvrPik-J* is from Zhang *et al.* (2020).

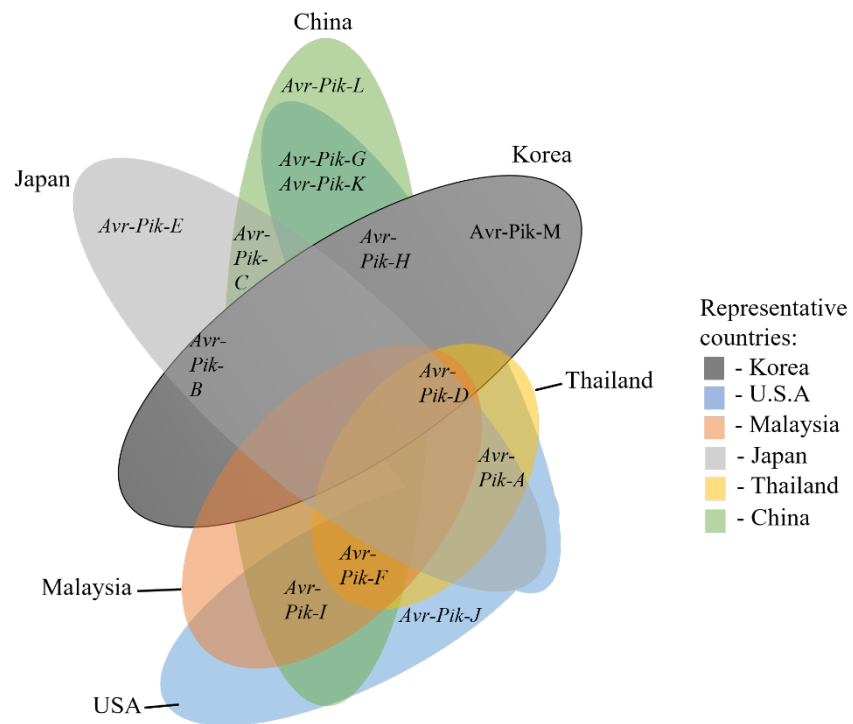


Figure 4.9: Venn diagram of *Avr-Pik* haplotypes distribution in six countries.

4.3.4 Haplotype Analysis of *AvrPi-54* Gene

A total of 78 *AvrPi-54* ORF sequences available in NCBI database were downloaded and aligned with 17 *AvrPi-54* ORF sequences from *P. oryzae* of Malaysia (Sarawak). From the alignment of 95 *AvrPi-54* ORF sequences amplified from *P. oryzae* of Malaysia (Sarawak) and India, seven haplotypes were observed. These seven haplotypes were designated as haplotype H1 to H7 (Figure 4.3.5). Both synonymous and non-synonymous mutations occurred among the *AvrPi54* haplotypes with seven segregating sites at the SNPs (Table 4.17).

At position 27bp of *AvrPi-54* ORF of haplotype H2 to H5, a synonymous mutation that caused by C to T substitution occurred which encodes for the same amino acid. for haplotype H1 to H7 (except for H5 due to another non-synonymous base mutation at 26bp). This has resulted in similar amino acid sequence for haplotype H1 and H2. Early stop codon was observed at haplotype H3 and H7 at codon number 153 which resulted in shorter amino acid. sequence compared to the other *AvrPi-54* haplotypes in this study (Table 4.18; Figure 4.10).

Based on the origin of the *P. oryzae* isolates, all the seven haplotypes from this study were distributed across Malaysia (Sarawak) and India. Most of the haplotypes were present in India (H2-H7) except for H6 which was present only in Malaysia (Sarawak). Both countries had haplotype H1 (Figure 4.11).

Table 4.17: Single nucleotide polymorphisms (SNPs) occurs in *AvrPi-54* ORF for the 7 haplotypes identified in this study.

Haplotype number	Representative NCBI sequence	Nucleotide position (codon number)						
		22 (8)	23 (8)	26 (9)	27 (9)	343 (115)	458 (153)	459 (153)
H1	POB1 ^a	A	T	C	C	T	G	C
H2	HF545750.1	A	T	C	T**	T	G	C
H3	HF545751.1	A	C*	C	T**	T	A**	A**
H4	HF545753.1	A	C*	C	T**	T	G	C
H5	HF545752.1	C**	C**	T**	T**	T	G	C
H6	KY441415.1	A	T	C	C	C**	G	C
H7	HF545749.1	A	T	C	C	T	G	A**

^a-Reference sequence, *-synonymous mutation **-non-synonymous mutation.

Table 4.18: Summary of polymorphic sites in amino acids sequence the 7 *AvrPi-54* haplotypes identified in this study.

Haplotype number	Representative NCBI sequence	Codon position			
		8	9	115	153
H1	POB1 ^a	I	S	S	C
H2	HF545750.1	-	-	-	-
H3	HF545751.1	T	-	-	*
H4	HF545753.1	T	-	-	-
H5	HF545752.1	P	F	-	-
H6	KY441415.1	-	-	P	-
H7	HF545749.1	-	-	-	*

^a-Reference sequence, *-stop codon. H1 and H2 had the same amino acid sequence due to synonymous mutation occurred at codon number 9.

4.3.5 Genetic Diversity of *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes

In general, among all three *Avr* genes in this study, *AvrPik* gene had the highest haplotype diversity and number of segregating sites followed by *AvrPiz-t* and *AvrPi-54* gene (Table 4.19, Table 4.20 & Table 4.21). From the total of 250 *AvrPiz-t* ORF sequences from Malaysia (Sarawak), Thailand, India, and China (including sequences from Wang *et al.* 2020), isolates from Thailand had the highest nucleotide polymorphism value ($\pi = 0.0173$) as compared to isolates from other countries (Table 4.19). Among the 169 *AvrPik* ORF sequences from Malaysia (Sarawak), Thailand, China, Japan and Korea, isolates from China had the highest nucleotide polymorphism value ($\pi = 0.00682$) compared to isolates from other countries (Table 4.20). For *AvrPi-54*, from a total of 95 *AvrPi-54* ORF sequences from Malaysia (Sarawak) and India, isolates from India had the highest π value (0.00066) compared to isolates from Malaysia (Sarawak) (Table 4.21).

Sliding window analysis of π show sites with high nucleotide diversity in *Avr* gene ORF sequences which indicated by presence of peaks in the graph. For *AvrPiz-t* gene, sliding window analysis of π (Figure 4.12a) showed two peaks, one at 100 - 140 bp and the highest peak at 250 - 260 bp. For *AvrPik* (Figure 4.12b) sliding window analysis of π also showed two peaks, where the highest was at 140 bp while the second peak at 240 bp. For *AvrPi-54*, sliding window analysis of π showed peak at 320 - 360 bp (Figure 4.12c).

Neutrality test based on three parameters, Tajima's D (Tajima, 1989) , Fu and Li's D and F (Fu & Li, 1993) showed that the *AvrPiz-t* and *AvrPi-54* genes from overall population used in this study had negative and significant values ($p < 0.01$) for Tajima's D and Fu and Li's F, except for Fu and Li's D statistic for *AvrPi-54* (Table 4.21). This result suggests that *AvrPiz-t* and *AvrPi-54* of the *P. oryzae* population are deviated from neutrality. While for

AvrPik from overall population showed positive value but was insignificant for all three parameters (Table 4.22).

Value of π_{non}/π_{syn} was calculated for the three *Avr* genes (Table 4.22). The π_{non}/π_{syn} ratio for *AvrPiz-t* (0.95946) and *AvrPi-54* (0.8088) were less than 1, which suggest both genes were under negative selection while for *AvrPik* (60.8) the value was more than 1, showing the gene is under positive selection.

Table 4.19: Result from polymorphism test and neutrality test of *AvrPiz-t* gene.

Population	N	S	H'	Hd	π	Θ	Tajima's D	Fu and Li's D	Fu and Li's F
All	250	15	12	0.146	0.00071	0.00760	-2.2531**	-3.48458**	-3.62215**
China	101	2	4	0.096	0.00030	0.00118	-1.1467	0.68464	0.13991
India	80	3	4	0.21	0.00066	0.00185	-1.1995	0.84631	0.24476
Malaysia	27	3	4	0.145	0.00068	0.00239	-1.7336	-2.61242*	-2.73186*
Thailand	42	9	4	0.139	0.00173	0.00640	-2.1130*	-2.44491	-2.74768*

Abbreviations: N number of sequences, S Number of segregating sites, H' Number of haplotypes (based on the alignment of amino acid), Hd Haplotype diversity π nucleotide diversity, Θ Watterson's estimator, asterisk indicates significant statistics * P < 0.05 and **P < 0.01.

Table 4.20: Result summary from test and neutrality test of *AvrPik* gene.

Population	N	S	H'	Hd	π	Θ	Tajimas' D	Fu and Li's D	Fu and Li's F
All	181	7	12	0.508	0.00475	0.00355	0.73080	0.20648	0.46298
Thailand	144	4	3	0.338	0.00384	0.00211	1.52838	0.91024	1.31411
China	9	5	7	0.944	0.00682	0.00538	1.13663	0.68670	0.88347
Japan	5	4	4	0.900	0.00585	0.00561	0.27345	0.27345	0.27834
Korea	11	5	4	0.673	0.00457	0.00499	-0.32197	-0.76384	-0.73647
Malaysia	12	4	3	0.621	0.00421	0.00387	0.30492	1.19483	1.09650

Abbreviations: N number of sequences, S Number of segregating sites, H' Number of haplotypes (based on the alignment of amino acid), Hd Haplotype diversity π nucleotide diversity, Θ Watterson's estimator, asterisk indicates significant statistics.

Table 4.21: Result from polymorphism test and neutrality test of *AvrPi-54* gene.

Populatio n	N	S	H	Hd	π	Θ	Tajimas' D	Fu and Li's D	Fu and Li's F
All	95	7	7	0.123	0.00059	0.00298	-1.89728*	-2.27259	-2.53745*
India	77	6	6	0.126	0.00066	0.00266	-1.76141	-1.62609	-1.96713
Malaysia	18	1	2	0.111	0.00024	0.00063	-1.16467	-1.49949	-1.61172

Abbreviations: N number of sequences, S Number of segregating sites, H Number of haplotypes, Hd Haplotype diversity π nucleotide diversity, Θ Watterson's estimator, asterisk indicates significant statistics * $P < 0.05$,

Table 4.22: Ratio of nonsynonymous over synonymous polymorphic site diversity of *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes from *Pyricularia oryzae*.

Avirulence genes	π_{non}	π_{syn}	π_{non}/π_{syn}^a
<i>AvrPiz-t</i>	0.00071	0.00074	0.95946
<i>AvrPik</i>	0.00608	0.00010	60.8
<i>AvrPi-54</i>	0.00055	0.00068	0.8088

^a The ratio of nonsynonymous (amino acid altering) site diversity over synonymous site diversity of avirulence genes.

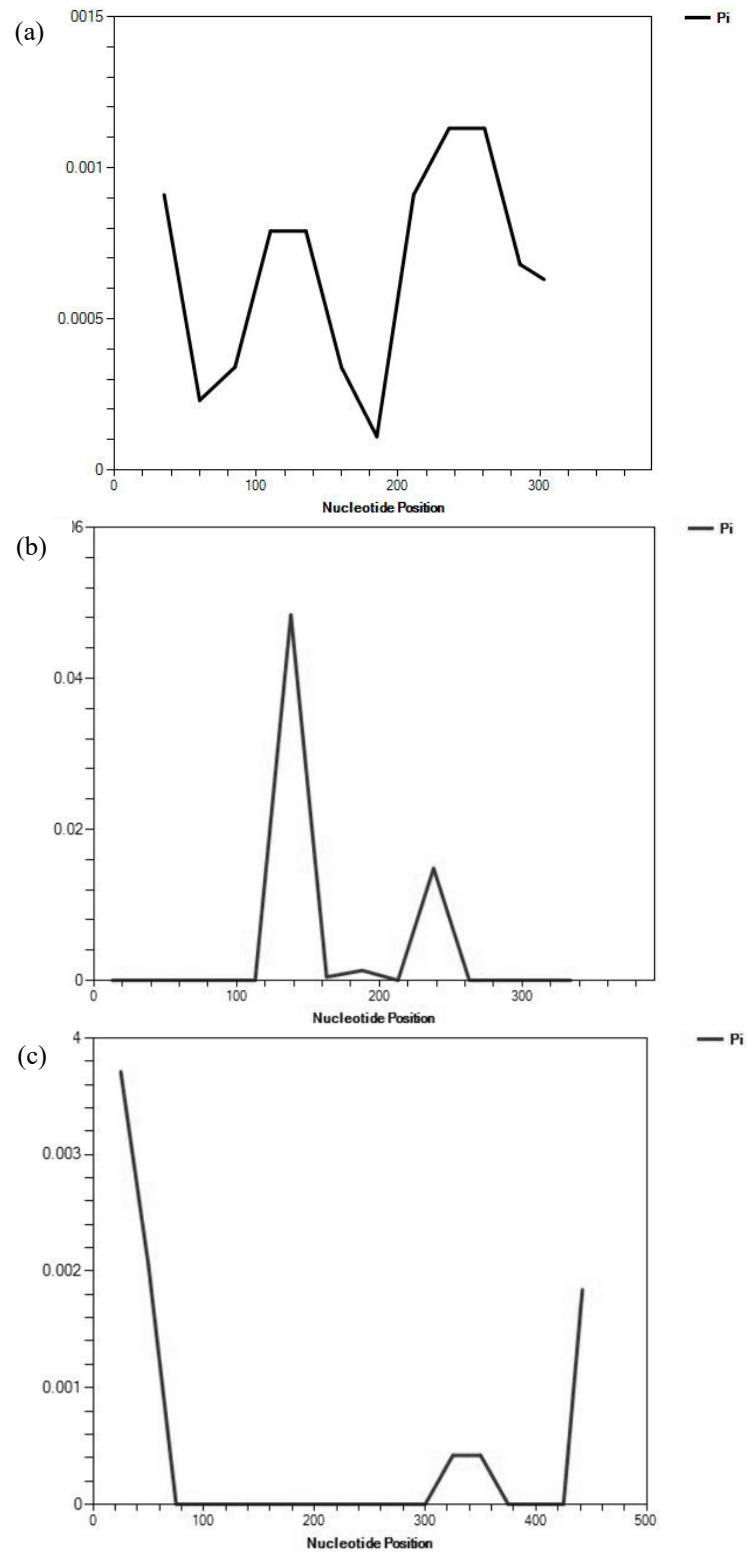


Figure 4.12: Sliding window analysis of π shows nucleotide variation along (a) *AvrPiz-t*, (b) *AvrPik*, and (c) *AvrPi-54* sequences. X-axis indicates nucleotide position while Y-axis indicates nucleotide diversity (π) value.

4.4 Pathogenicity Test of Selected Sarawak Rice Landraces Against *Pyricularia oryzae*

Pathogenicity test was successfully performed with two repetitions for five selected *P. oryzae* isolates (POS22, POS24, POSA20, POSA23 and POSI7). However, pathogenicity test for Merah landrace against POSI7 and for all landraces against POSA20 was conducted only one time.. The conidia germination rate after incubation for all five selected *P. oryzae* isolates was above 95% on each repetition.

Leaf symptom typically develop at 2 days post-inoculation (dpi) which started as small circular pin size spot on the inoculated area, then later developed into larger necrotic lesions on 3 dpi onwards. Some leaves had increased in number of small circular pin size spot which later turned into necrotic-freckles-like appearance on the leaf. Most of the symptoms fully developed by 5 or 6 dpi, however the symptom is not the typical diamond-shaped lesion observed in the field (Figure 4.4.1).

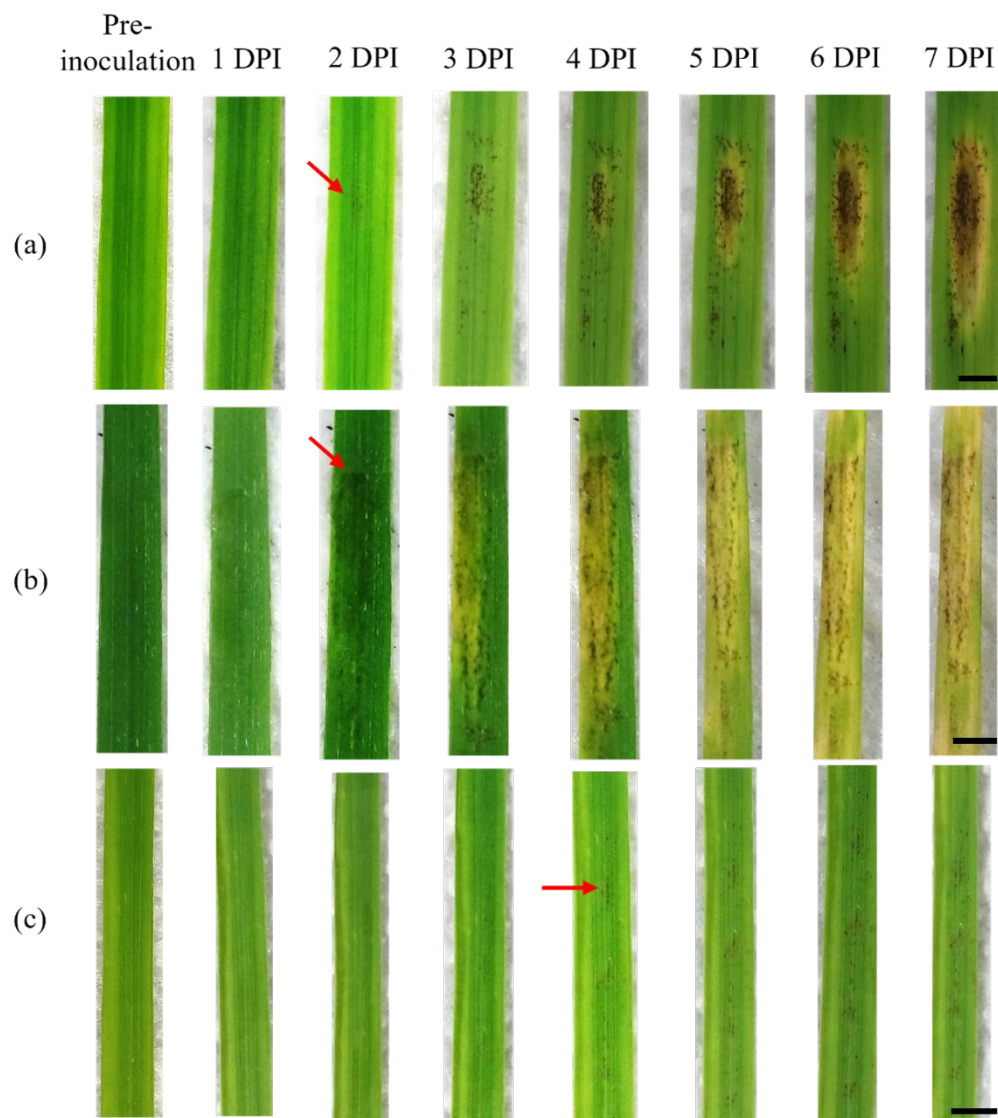


Figure 4.13: Rice blast symptom development observed from pre-inoculation to 7th day post-inoculation (DPI) on Sarawak rice landraces inoculated with *Pyricularia oryzae* isolate POSA23. Symptom (red arrow) appeared earlier on (a) Selasih landrace and (b) Merawi landrace (at 2nd DPI) as compared to (c) Pandan Wangi landrace (at 4th DPI). Scale bar = 1cm.

Re-isolation of *P. oryzae* following Koch's postulate were successful for all isolates and each repetition (Figure 4.15). There was no contamination of water from 3rd rinse of surface-sterilisation step cultured on PDA which further confirms that the single conidium of *P. oryzae* obtained was indeed from the infected leaf itself and not from any other possible contaminants (Figure 4.14).

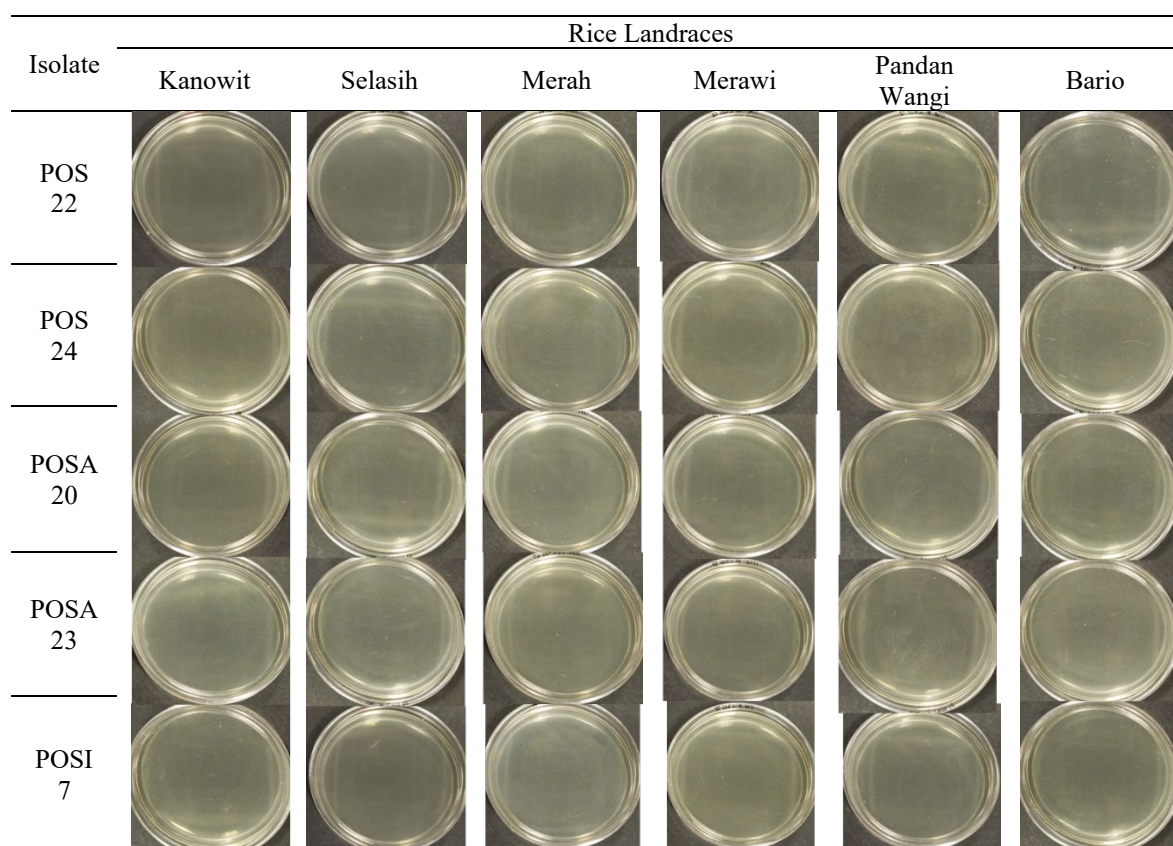


Figure 4.14: Water from 3rd rinse of surface-sterilisation from re-isolation of six Sarawak's rice landrace leaves inoculated with five *Pyricularia oryzae* isolates cultured on PDA after 3 days.

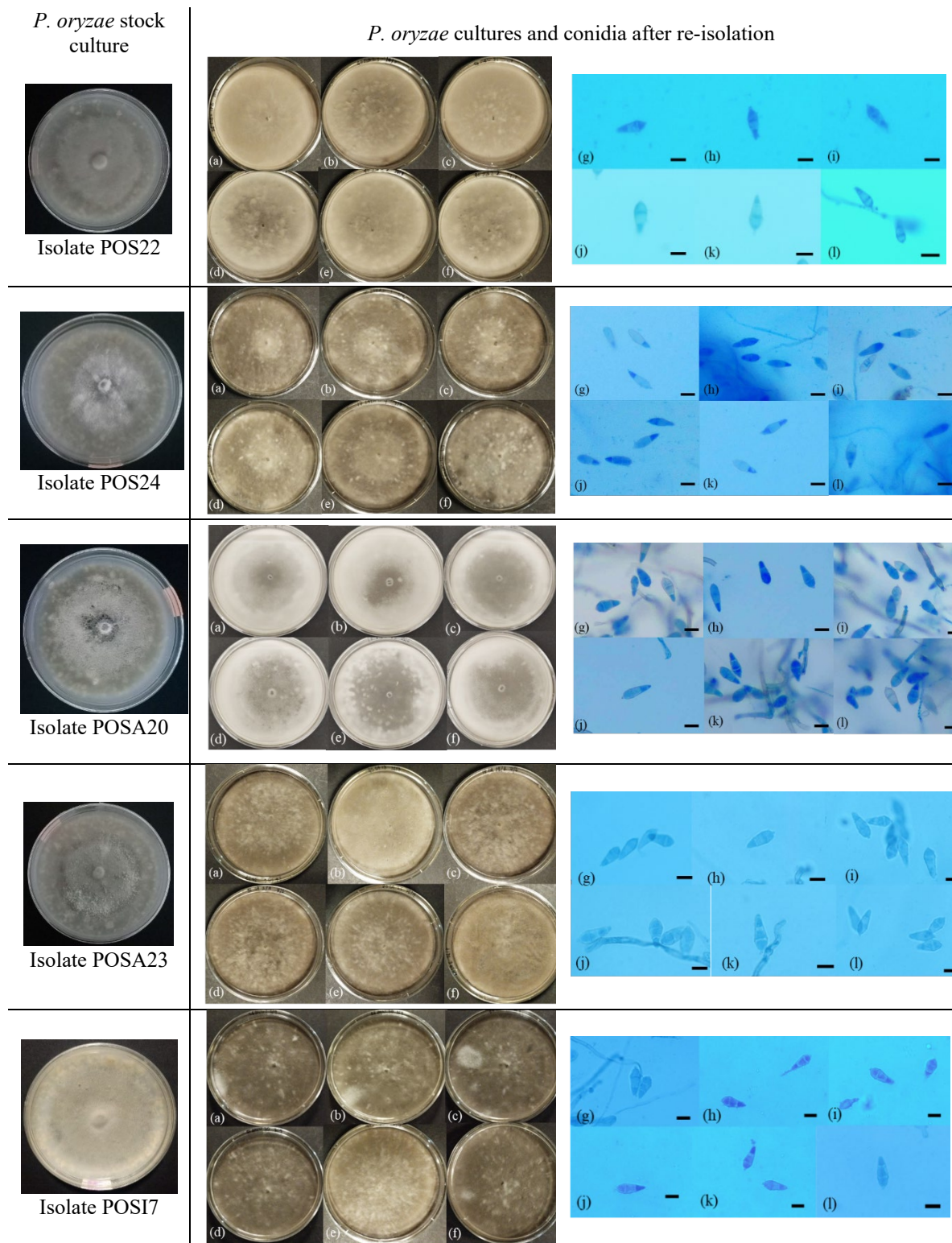


Figure 4.15: *Pyricularia oryzae* re-isolated from blast lesion on (a) Kanowit landrace (b) Selasih landrace (c) Merah landrace (d) Merawi landrace (e) Pandan Wangi landrace (f) Bario landrace. (g) – (l) Observed fungal conidia from the respective (a) – (f) culture plates under compound microscope at 40X magnification. Scale bar = 10 μ m.

Table 4.4.1 shows the result for susceptibility of six different Sarawak rice landraces (Kanowit, Selasih, Merah, Merawi, Pandan Wangi and Bario) against *P. oryzae* isolate POS22, POS24, POSA20, POSA23 and POSI7 on 7 dpi. The six rice landraces showed different levels of susceptibility towards the five *P. oryzae* isolates (Table 4.23). For example, Merah landrace was susceptible (S) towards isolate POS22, moderately resistant (MR) towards POSA23 and resistant (R) towards POSI7. These scoring are based on the rating scale adapted and modified from Challagulla *et al.* (2015) and Takahashi *et al.* (2009, 2014) (Table 3.4). This score may not reflect by statistical analysis where landrace scored as MR may statistically insignificantly different from landrace scored as S (Table 4.25). Isolate POS22 was observed as the most virulent isolate as all landraces were susceptible (score $\geq$ 3.0) towards the isolate.

Apart from that, the development of symptom on the different rice landraces was at different rate. For example, the symptom on Selasih landrace and Merawi had consistently appeared earlier (at 2dpi) than Pandan Wangi landrace (at 4 dpi) when inoculated with isolate POSA23 (Table 4.26; Figure 4.13). There were also differences in disease severity index (DSI) (Table 4.24) of rice landrace when inoculated with different isolates. For example, Merawi landrace had 33% DSI differences when inoculated with isolate POS22 (DSI = 78%) and POS24 (DSI = 45%).

Table 4.23: Disease score of six Sarawak rice landraces inoculated with five *Pyricularia oryzae* isolates from Sarawak using detached-leaf filter paper method.

Isolate	Score	Disease score frequency on 7 th day post inoculation (dpi)					
		Kanowit	Selasih	Merah	Merawi	Pandan Wangi	Bario
POS22	0	0	0	0	0	0	0
	1	6	0	0	0	0	0
	2	2	0	0	0	1	0
	3	0	0	0	22	12	13
	4	17	25	25	3	12	12
Total leaves		25	25	25	25	25	25
Score range		1-4	4	4	3-4	2-4	3-4
Average score		3.1	4.0	4.0	3.1	3.4	3.5
Susceptibility		S	S	S	S	S	S
POS24	0	0	0	0	0	0	0
	1	3	4	13	13	9	9
	2	9	3	2	5	5	12
	3	7	8	6	6	11	4
	4	6	10	4	1	0	0
Total leaves		25	25	25	25	25	25
Score range		1-4	1-4	1-4	1-4	1-3	1-3
Average score		2.6	3.0	2.0	1.8	2.1	1.8
Susceptibility		MR	S	MR	R	MR	R
POSA20	0	1	1	0	0	0	0
	1	1	9	1	9	14	7
	2	5	3	17	10	0	6
	3	11	4	4	3	11	2
	4	7	8	3	3	0	0
Total leaves		25	25	25	25	25	15
Score range		0-4	0-4	1-4	1-4	1-3	1-3
Average score		2.9	2.4	2.4	2.0	1.9	1.7
Susceptibility		MR	MR	MR	MR	R	R
POSA23	0	0	1	0	1	2	0
	1	0	1	2	4	9	5
	2	7	10	15	6	2	6
	3	10	4	4	7	3	2
	4	8	9	4	7	9	12
Total leaves		25	25	25	25	25	25
Score range		2-4	0-4	1-4	0-4	0-4	1-4
Average score		3.0	2.8	2.4	2.6	2.3	2.8
Susceptibility		S	MR	MR	MR	MR	MR
POSI7	0	0	0	0	0	0	0
	1	0	9	10	2	0	5
	2	0	9	0	9	6	6
	3	25	5	0	14	14	9
	4	0	2	0	0	11	5
Total leaves		25	25	10	25	25	25
Score range		3	1-4	1	1-3	2-4	1-4
Average score		3.0	2.0	1.0	2.5	3.4	2.6
Susceptibility		S	MR	R	MR	S	MR

Note: S- susceptible, MR-moderately resistant, R-resistant. Average score >3.0 (R), 3.0 < (MR), <2.0 (S).

Table 4.24: Disease severity index (DSI) of symptoms caused by *Pyricularia oryzae* inoculated on six different Sarawak rice landraces.

Isolate	Disease Severity Index (DSI) (%)					
	Kanowit	Selasih	Merah	Merawi	Pandan Wangi	Bario
POS22	78	100	100	78	86	87
POS24	66	74	51	45	69	60
POSA20	72	59	59	50	63	56
POSA23	76	69	60	65	58	71
POSI7	100	50	100	83	86	64
Average DSI	78	70	74	64	72	68

Disease severity index (DSI) following McKinney (1923)

Table 4.25: Disease score of six selected Sarawak rice landraces challenged with five *Pyricularia oryzae* isolates using detached-leaf filter paper method.

Rice Landraces	Mean Disease Score (0 – 4 scale)									
	Isolates									
	POS22		POS24		POSA20		POSA23		POSI7	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Kanowit	3.12 ^a	0.27	2.64 ^{bc}	0.20	2.88 ^b	0.20	3.04 ^a	0.16	2.20 ^a	0.17
Selasih	4.00 ^b	0.00	2.96 ^c	0.22	2.36 ^{ab}	0.28	2.76 ^a	0.23	2.00 ^a	0.19
Merah	4.00 ^b	0.00	2.04 ^{ab}	0.24	2.36 ^{ab}	0.15	2.40 ^a	0.17	N/A	
Merawi	3.12 ^a	0.07	1.80 ^a	0.19	2.00 ^a	0.20	2.60 ^a	0.24	2.48 ^a	0.13
Pandan Wangi	3.44 ^a	0.12	2.08 ^{ab}	0.18	1.88 ^a	0.20	2.32 ^a	0.30	3.44 ^b	0.10
Bario	3.48 ^{ab}	0.10	1.80 ^a	0.14	N/A		2.84 ^a	0.09	2.56 ^a	0.21
Total	3.53	0.06	2.22	0.09	2.30	0.10	2.66	0.09	2.65	0.21

Note: Same letter indicates no significant difference between rice landraces challenged with the respective *P. oryzae* isolates, based on one-way ANOVA followed by Tukey's HSD post-hoc test.

Table 4.26: The average on first appearance of rice blast symptoms on six different Sarawak rice landraces after inoculated with five *Pyricularia oryzae* isolates.

Rice Landraces	Day of the first appearance of rice blast symptom									
	Isolates									
	POS22		POS24		POSA20		POSA23		POSI7	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Kanowit	3.00 ^a	0.00	2.48 ^b	0.11	2.00 ^a	0.00	3.00 ^{ab}	0.00	2.96 ^b	0.22
Selasih	2.48 ^b	0.11	2.43 ^b	0.11	2.35 ^b	0.10	2.61 ^a	0.10	2.78 ^b	0.21
Merah	2.70 ^b	0.12	2.00 ^a	0.00	2.00 ^a	0.00	3.00 ^{ab}	0.09	resistant	
Merawi	2.00 ^a	0.00	2.00 ^a	0.00	2.52 ^b	0.11	2.52 ^a	0.14	2.52 ^{ab}	0.12
Pandan Wangi	2.00 ^a	0.00	2.65 ^b	0.10	2.00 ^a	0.00	3.52 ^b	0.36	2.00 ^a	0.00
Bario	2.00 ^a	0.00	2.00 ^a	0.00	resistant		2.87 ^{ab}	0.23	2.00 ^a	0.00
Total	2.36	0.04	2.26	0.04	2.17	0.04	2.92	0.08	2.45	0.07

Note: Same letter indicates no significant difference between rice landraces challenged with the respective *P. oryzae* isolates, based on one-way ANOVA followed by Tukey's HSD post-hoc test

4.5 Isolation of *Pyricularia oryzae* from Alternative Host

4.5.1 *Pyricularia oryzae* Isolates from Alternative Host

A total of seven *P. oryzae* isolates were successfully isolated from *Eleusine indica* at rice fields of four divisions of Sarawak (Kuching, Serian, Sri Aman and Sibü) (Table 4.27). All seven isolates were pre-identified based on the morphology of the conidia (Figure 4.16) reported as described by Hussin *et al.* (2020). Amplification of ITS region was done for five isolates, POWK2, POWS1, POWSA1, POWSA2 and POWSI1. The result of BLASTn showed that all five isolates were identified as *P. oryzae* with 97.79 – 100% of percentage identity. The ITS sequences ranged from 464 bp to 634 bp (Table 4.28).

Table 4.27: List of *Pyricularia oryzae* isolated from weed host.

Isolate	Location	District	Division	Coordinate	Host species
POWK1	Kpg Siru Melayu	Sematan	Kuching	N1°49'51.9", E109°43'38.7"	<i>Eleusine indica</i>
POWK2	Kpg Karu	Padawan	Kuching	N1°15'59.46", E110°17'1.86"	<i>Eleusine indica</i>
POWK3	Kpg Karu	Padawan	Kuching	N1°15'59.46", E110°17'1.86"	<i>Eleusine indica</i>
POWS1	Kpg Slabi Plaman	Serian	Serian	N1°8'26", E110°33'59"	<i>Eleusine indica</i>
POWSA1	Kpg Dadak B	Pantu	Sri Aman	N1°4'58.26", E111°2'28.20"	<i>Eleusine indica</i>
POWSA2	Along roadside	Engkelili	Sri Aman	N1°8'2.28", E111°38'50.64"	<i>Eleusine indica</i>
POWSI1	Near Igan Bridge	Sibu	Sibu	N2°20'54.42", E111°48'30.54"	<i>Eleusine indica</i>

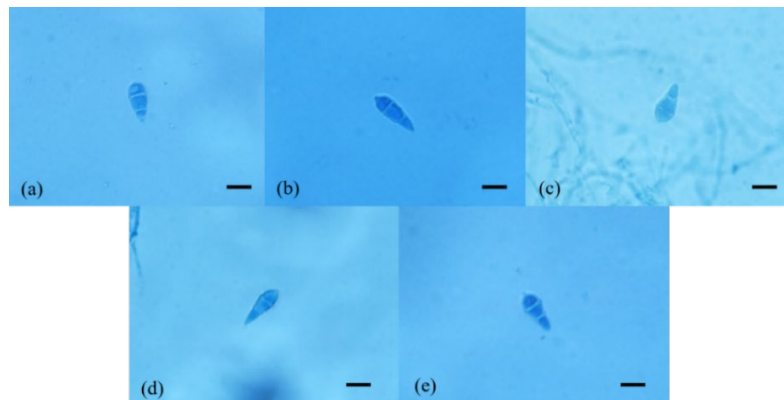


Figure 4.16: Conidia of *Pyricularia oryzae* isolates isolated from *Eleusine indica*. (a) POWK1 (b) POWK2 (c) POWK3 (d) POWSA1 (e) POWSA2.

Table 4.28: BLASTn result of five *Pyricularia oryzae* isolates from *Eleusine indica*.

Isolate	Amplicon size	Percentage identity	E - value	Accession number with highest similarity
POWK2	472	100	0.0	KP144443.1
POWS1	619	98.05	0.0	LK932254.1
POWSA1	634	97.79	0.0	LK932254.1
POWSA2	472	100	0.0	KP144443.1
POWSI1	464	100	0.0	KP144443.1

4.5.2 Morphological Description of *P. oryzae* isolated from Alternative Host on Different Media.

Morphological description of all seven *P. oryzae* isolates from weed host is summarised in Table 4.29. All isolates were grouped based on similar characteristics (form, elevation, and surface of the colony) as mentioned in section 4.2.1. The pigmentation and opacity of the colony were not considered in grouping of the colonies. There were four groups of colony morphology grown on OMA (Figure 4.17) and three on PDA (Figure 4.18). The colony morphology of different *P. oryzae* isolates from *E. indica* on OMA and PDA were diverse. The upper colony surface of different groups varied from rough to smooth, and fluffy to flat mycelia, some had wrinkled surface, powdery surface and some with presence of concentric ring. The elevation of the colonies was either flat or raised, or even both elevations in one isolate. Most of the isolates had entire margin except for isolates POWSI1 and POWK1 on PDA (Table 4.29).

The pigmentation on lower surface of the colonies on both OMA and PDA varied from black, grey, light grey and white. Most of the colonies had black or greyish front pigmentation. While for the reverse-view pigmentation, the colonies varied from black, brown, light brown, creamy brown, and white. Most colonies had black or brownish shade.

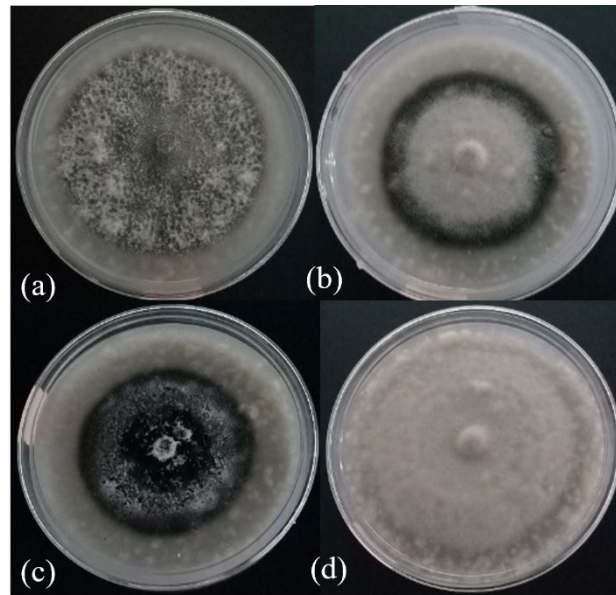


Figure 4.17: Morphological characteristics of *Pyricularia oryzae* isolates from *Eleusine indica* grown on OMA. Isolate from (a) to (d) represents Group 1 to Group 4, respectively.

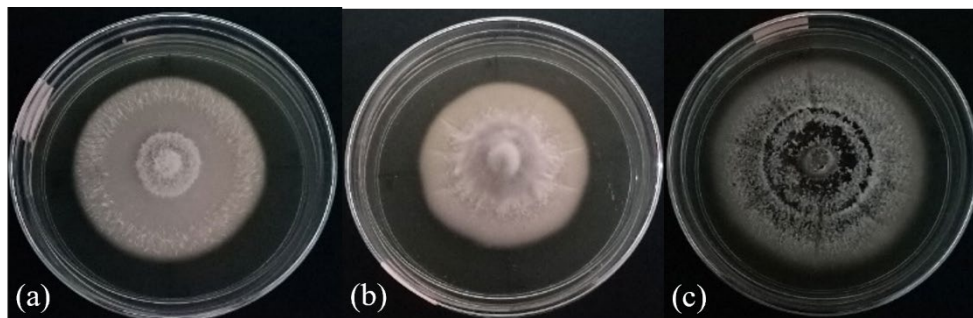


Figure 4.18: Morphological characteristics of *Pyricularia oryzae* isolates from *Eleusine indica* grown on PDA. Isolate from (a) to (c) represents Group 1 to Group 3, respectively.

Table 4.29: Morphological description summary of *Pyricularia oryzae* isolates from *Eleusine indica* on OMA and PDA.

Media	Group	Isolate	Pigmentation		Form	Elevation	Margin	Upper Colony Surface		Opacity
			Upper colony	Lower colony						
OMA	1	POWK2	Grey with white patches.	Black	Circular	Raised aerial mycelia	Entire	Rough mycelia, edges	aerial smooth	Opaque, translucent edges
	2	POWSA1	Black with white-greyish patches of aerial mycelia.	Black	Circular	Flat with raised aerial mycelia.	Entire	Rough patches of aerial mycelia in the centre, smooth and shiny middle and outer region.		Opaque centre and middle region, translucent outer region.
		POWSA2	Light grey aerial mycelia, black middle region,	Black		Raised aerial mycelia, flat middle and outer region.		Smooth and fluffy aerial mycelia, shiny and flat outer region.		Opaque and translucent outer region.
		POWS1	Grey with white patches of aerial mycelia	Black with brown middle region,		Flat		Smooth aerial mycelia with patches		Opaque centre, translucent outer region.
	3	POWK3	Black with white aerial mycelia	Black	Circular	Flat, raised aerial mycelia	Entire	Rough and powdery aerial mycelia, smooth and shiny towards the edge.		Opaque, translucent outer region.
		POWSI1	Black with white patches in the middle region,	Black		Flat		Smooth and dull		Opaque middle region, translucent outer region.
	4	POWK1	Grey	Black centre, pale brown at the middle region	Circular	Raised aerial mycelia, flat edges	Entire	Smooth and fluffy aerial mycelia		Opaque, translucent outer region

Table 4.29: continued

PDA	1	POWK3	White	Light brown centre, white middle	Circular	Flat and raised aerial mycelia	Entire	Rough centre and outer region, smooth middle region.	Opaque
		POWS1	White	Creamy pale brown			Entire	Rough, fuzzy outer region.	
		POWS11	Grey centre, light grey middle region	Creamy pale brown.		Raised	Undulated	Presence of concentric rings, fluffy aerial mycelia at middle and outer region.	
	2	POWK1	Light grey at the centre and middle region	Light brown	Circular	Raised	Undulate	Wrinkled, dense aerial mycelia at the centre.	Opaque
		POWK2	White with greyish pigmentation at the centre.	Black with brown middle region			Entire	Rough, dense aerial mycelia in the centre.	
	3	POWSA1	Black base with greyish white aerial mycelia	Light brown with white edge. White wrinkled area.	Circular	Flat	Entire	Wrinkled, concentric ring in middle region, denser aerial mycelia at the outer region.	Opaque
		POWSA2	Greyish aerial mycelia at the centre, dark grey with white aerial mycelia outline at middle region	Pale brown centre, brown middle region, creamy pale brown at outer region.				Wrinkled, presence of concentric ring in middle region	

4.5.3 Growth Performance and Sporulation of *P. oryzae* Isolated from Weed Host on Different Media.

Growth rate of the seven *P. oryzae* isolates isolated from weed is tabulated in Table 4.30. The growth rate on both OMA and PDA were varied, where most of the isolates had significant difference ($p < 0.05$) in mean growth rate between media. All the isolates had higher growth rate on OMA compared to PDA except for isolate POWSA2 which had the same mean growth rate on both media.

Isolate POWSA1 was among the fastest growing isolates [OMA ($0.33 \text{ cm/day} \pm 0.00$) and PDA ($0.30 \text{ cm/day} \pm 0.01$)] as compared to POWK3 and POWSI1 for example. Apart from that, some isolates had slower growth on OMA but faster growth on PDA. For example, isolate POWK3 was among the isolates having slow growth on OMA ($0.27 \text{ cm/day} \pm 0.01$) but was among the isolates having intermediate growth on PDA ($0.24 \text{ cm/day} \pm 0.01$) (Table 4.31). Sporulation rate of the *P. oryzae* isolates on OMA and PDA are tabulated in Table 4.32. Due to insufficient replication, statistical analysis for sporulation rate of *P. oryzae* isolates cannot be performed. Most isolates grew on OMA seemed to have higher sporulation rate compared to on PDA.

Table 4.30: Growth rate of seven *Pyricularia oryzae* isolates from *Eleusine indica* grown on OMA and PDA.

Isolate	Oatmeal Agar (OMA)		Potato Dextrose Agar (PDA)		p-value
	Mean	Std. Error	Mean	Std. Error	
POWK1	0.32	0.00	0.24	0.00	0.008*
POWK2	0.33	0.00	0.27	0.00	0.008*
POWK3	0.27	0.00	0.24	0.00	0.008*
POWS1	0.29	0.01	0.19	0.00	0.008*
POWSA1	0.33	0.01	0.30	0.00	0.008*
POWSA2	0.30	0.00	0.30	0.00	0.151
POWSI1	0.30	0.00	0.22	0.00	0.008*

Note: The data was analysed using Mann-Whitney test. Asterisk '*' indicates significant $p \leq 0.05$.

Table 4.31: Growth rate comparison of seven *Pyricularia oryzae* isolates from *Eleusine indica* grown on OMA and PDA.

Isolate	OMA	PDA
	Mean	Mean
POWK1	0.32 ^{cd}	0.24 ^c
POWK2	0.33 ^d	0.27 ^d
POWK3	0.27 ^a	0.24 ^c
POWS1	0.29 ^{ab}	0.19 ^a
POWSA1	0.33 ^d	0.30 ^e
POWSA2	0.30 ^{bc}	0.30 ^e
POWSI1	0.30 ^{bc}	0.22 ^b

Note: The data was analysed using Kruskal Wallis test. Same letter indicates no significant difference of growth rate between *Pyricularia oryzae* weed isolates.

Table 4.32: Sporulation rate of *Pyricularia oryzae* isolates from *Eleusine indica* on OMA and PDA.

Isolate	Sporulation rate x 10 ⁵ /mL	
	Media	
	OMA	PDA
POB 1	9.25	0.84
POWK1	0.00	0.26
POWK2	20.4	0.02
POWK3	0.30	0.00
POWS1	0.06	0.00
POWSA1	0.14	0.14
POWSA2	20.40	40.90
POWSI1	0.02	0.02
Total mean	5.91	5.90

CHAPTER 5

DISCUSSION

5.1 New isolates of *Pyricularia oryzae* for Northwestern region of Sarawak

A total of 25 new *P. oryzae* isolates obtained from five different divisions in Sarawak (Kuching, Serian, Sri Aman, Betong and Sibu) from this study, in addition to 15 isolates from Hussin (2021). Thus, there are a total of 40 isolates collected from Sarawak. There were distinctive morphological characteristics of colony for *P. oryzae* isolates from this study with some similar features compared to other *P. oryzae* isolates from Sarawak reported by Hussin *et al.*, (2020). For example, the colony upper surface for some isolates grown on OMA and PDA in this study were varied from smooth, fluffy, to rough mycelia; some had wrinkled, shiny and slithery surface. Some of these characteristics such as smooth and fluffy colony surface when grown on either OMA or PDA were also observed in some isolates from Hussin *et al.* (2020). The characteristic of shiny and slithery colony upper surface of isolates from this study when grown on OMA were not found in colony upper surfaces of *P. oryzae* isolates from Hussin *et al.* (2020).

There were also differences in colony morphology and some similar features of *P. oryzae* isolates from Sarawak compared to isolates from other countries. For instance, *P. oryzae* isolates from Dhaka, Bangladesh when grown on PDA, the isolates had either regular or irregular shape (Nazifa *et al.*, 2021). While for *P. oryzae* from Sarawak, some isolates on PDA had either irregular, circular, filamentous or even a combination of circular with filamentous shape. In this study, the irregular colony shape was not observed in 17 out of 25 total isolates. Nazifa *et al.* (2021) also mentioned that the surface of some *P. oryzae* colony in their study had velvety surface while the rest were either rough, cottony or smooth when grown on PDA. Isolates from Sarawak did not have velvety surface on the surface of the

colony when grown on PDA. The colony differences between the present study and from previous studies might be due to different isolate genotype which might had different metabolic requirements or optimal condition required.

Most of the isolates in this study had pigmented mycelia and some were not. There were isolates that displayed distinct pigmentation patterns when grown on both OMA and PDA; for example, isolate POS24 showed darker pigmentation (light grey) on OMA compared to white pigmentation on PDA (Appendix B). Isolates of *P. oryzae* from Bangladesh also exhibited similar upper colony colour ranging from light grey, brown, greenish, to white (Nazifa *et al.*, 2021). Another study by Kulmitra *et al.* (2017) from India, also exhibited *P. oryzae* colony colour varies from black, greyish white, to white colour with smooth to irregular margin. Apart from that, study by Ashrafi *et al.* (2021), revealed that some of the *Magnaporthe oryzae triticum* isolates used in their study which originated from Bangladesh, had whitish to greyish upper colony with flat surface when grown on OMA which was similar to some of the isolates characterised in this study. While grown on PDA, some of the isolates of Ashrafi *et al.* (2021) had whitish upper colony with smooth surface which was also similar to some of the isolates characterised in this study.

All the 25 isolates obtained from five divisions in Sarawak (Kuching, Serian, Sri Aman, Betong and Sibu) exhibited varying morphological traits, indicating potential genetic diversity among them. Rice blast isolates from the same district or division does not necessarily share the same colony morphology. For instance, all rice blast isolates from Serian having different morphology, hence they were grouped based on their morphological characteristics (Table 4.2.1). This finding aligns with Hussin *et al.* (2020) which also stated for their isolates, there was no distinctive variation unique to a particular location, where isolates with similar morphology were classified into different groups regardless of their

origin. Another study by Srivastava *et al.* (2014), indicated that there is no correlation between geographical distribution and morphological traits among *P. oryzae* isolates. However, this does not imply that isolates from the same location are genetically unrelated. In fact, Abed-ashtiani *et al.* (2016) conducted a study reporting a positive correlation between molecular data and the geographical origin of *P. oryzae* isolates from Peninsular Malaysia. In their research, isolates from the same location tended to cluster together based on two different markers, suggesting a genetic relationship among those isolates despite their geographic dispersion.

The growth rate of *P. oryzae* isolates from this study ranged from 0.24 - 0.32 cm/day on OMA. While on PDA, the growth rate ranged from 0.17 - 0.33 cm/day. Hussin *et al.* (2020) observed the growth rate of *P. oryzae* isolates ranged from 0.32 – 0.38 cm/day on OMA and 0.33 – 0.44 cm/day on PDA. Growth rate of *P. oryzae* isolates from Bangladesh cultured on PDA ranged from 0.32 – 0.41 cm/day (Nazifa *et al.*, 2021). Variation in range of growth rate of isolates from this study and from Hussin *et al.* (2020) and Nazifa *et al.* (2021) although using the same type of media might be due to genetic differences of the isolate from different origin and due to differences in experimental setup such as temperature, pH level of media, humidity, light exposure etc. In general, the *P. oryzae* isolates in the present study seems to have a slower growth rate compared to Sarawak isolates from the study by Hussin *et al.* (2020) on OMA as well as on PDA.

The change of growth and sporulation rate pattern of each *P. oryzae* isolate on different media in this study shows that different media affect the physiology of isolates differently. For instance, isolate POS23 displayed a faster growth rate on OMA (0.32 cm/day $\pm$ 0.01) compared to PDA (0.29 cm/day $\pm$ 0.02). On the other hand, isolate POS26 showed a faster growth rate on PDA (0.33 cm/day $\pm$ 0.02) compared to OMA (0.30 cm/day $\pm$ 0.01) (Table

4.2.5 & Table 4.2.6). In contrast, isolate POS21 exhibited the slowest growth rate on both media (OMA: 0.24 cm/day $\pm$ 0.01 and PDA: 0.17 cm/day $\pm$ 0.01) among all the isolates. These variations of growth rate on different media may indicate that the different isolates have distinct nutritional needs or preferences, or they might have different metabolic rates (Mohsen *et al.*, 2016). The growth and conidium formation of *P. oryzae* are influenced by the type of carbon and nitrogen sources available. A study by Gohel and Chauhan (2015) reported that starch is the best carbon source for supporting the growth and sporulation of *P. oryzae* followed by sucrose, glucose, and mannitol, whereas lactose and galactose result in poor growth and sporulation. Another study by Pandey (2014) found that maltose was the most effective carbon source for maximum mycelial growth of *P. oryzae*, followed by sucrose and fructose. Starch is the main source of carbon for both OMA and PDA. Oatmeal which is an ingredient for OMA contains 60% of starch (Zhang *et al.*, 2021) while potato which is an ingredient for PDA contain 66-80% of starch (Liu *et al.*, 2017). The high content of starch for both OMA and PDA are ideal for the growth and sporulation of *P. oryzae*. Additionally, Gohel and Chauhan (2015) reported that ammoniacal nitrogenous sources are unfavourable for the fungal growth and sporulation of *P. oryzae* compared to non-ammoniacal nitrogenous sources.

Previous findings indicated that OMA was the most favourable medium for mycelial growth (Gad *et al.*, 2011; Kulmitra *et al.*, 2017; Manjunatha & Krishnappa, 2019, Ashrafi *et al.*, 2021) and sporulation of *P. oryzae* (Gad *et al.*, 2011; Gohel & Chauhan, 2015; Hussin *et al.*, 2020). Whilst, PDA has also been reported as the best medium for *P. oryzae* mycelial growth (Pandey, 2014; Hussin *et al.*, 2020). So far, there was no report stating that PDA as the best medium for sporulation of *P. oryzae* in comparison to OMA. These earlier findings are consistent with the results obtained in this study. For studying the mycelial growth rate,

the translucent PDA would be a good choice. This is because the opaqueness of OMA hampers the observation (Hussin *et al.*, 2020) making it hard to observe the mycelial growth of *P. oryzae* especially when the isolate had not developed any pigmentation.

The sporulation rate of *P. oryzae* isolates cannot be analysed statistically due to insufficient data. Nevertheless, it can be seen, generally, that *P. oryzae* isolates in this study sporulate better on OMA than PDA (Table 4.2.8), but no assumption can be made from this as there was not enough replicates to give a reliable data. For future recommendations, study on the sporulation rate of these *P. oryzae* isolates on OMA and PDA with more replications would give a more reliable observation on their sporulation rate.

Prior studies have linked dark pigmentation to isolate pathogenicity (Lujan *et al.*, 2016; Oh *et al.*, 2017), due to the presence of melanin pigment, which plays a crucial role in forming melanized appressoria, enabling successful penetration into the host cell wall (Motoyama, 2020). It has been reported that melanin promotes sporulation of *P. oryzae* (Huang *et al.*, 2022). However, whether the pigmentation of upper colony surface of the *P. oryzae* isolates from this study correlates with sporulation rate cannot be determined, which can be investigated in the future. The future findings might underscore the significance of melanin pigment in both pathogenicity and sporulation of *P. oryzae*, providing valuable insights into potential mechanisms underlying its virulence and reproductive capabilities.

5.2 Pathogenicity of *Pyricularia oryzae* isolates towards Sarawak's rice landraces

Pathogenicity assay had shown that all five *P. oryzae* isolates had different levels of pathogenic variability towards all six different Sarawak's rice landraces, whereas all six Sarawak rice landraces had different susceptibility level towards the five isolates ranging from resistant (R), moderately resistant (MR) to susceptible (S) (Table 4.4.1). In this study, isolate POS22 was observed as the most virulent isolate compared to the other four isolates,

as four out of six Sarawak rice landrace (Kanowit, Selasih, Merah and Pandan Wangi landrace) were susceptible (S) towards the isolate. While Kanowit landrace was observed as the most susceptible landrace compared to the other five landraces (susceptible to three out of five isolates). Isolate POS22 was isolated from Serian division while Kanowit landrace is only planted by smallholder farmers. Based on this information, it is recommended to have a continuous monitoring of rice blast status especially in Serian division and in areas where Kanowit landrace are planted to avoid any possible outbreak and to minimise the impact of the disease towards Sarawak's rice farmers.

Some isolates showed different pathogenic variability towards different rice landrace. For example, Merah landrace was susceptible (S) towards isolate POS22 but was resistant (R) towards isolate POSI7. It was also observed that Bario landrace was resistant (R) against isolate POS24 and POSA20 but was susceptible (S) against isolate POS22. It is unclear whether the pathogenic variability of *P. oryzae* isolates in this study correlates with geographical origin or not. Merah landrace, originating from the Padawan division, was susceptible to isolate POS22 from Serian but resistant to isolate POSI7 from Sibul. Geographically, Serian is closer to Padawan compared to Sibul. On the other hand, Bario landrace, which was originated from Sri Aman division, was resistant and moderately resistant to isolates from its own division, POSA20 and POSA23 respectively.

5.2.1 Partial resistance of Sarawak's rice landrace towards *Pyricularia oryzae*

Differences in the symptom development rate (latency period) in this study (Table 4.4.4) might indicate that there is a possibility of weaker form of *R-gene* present (weak qualitative resistance) or quantitative resistance gene which may suggest partial resistance (Lai *et al.*, 2019; Yeo *et al.*, 2017; Niks *et al.*, 2011; Niks & Marcel., 2009; Poland *et al.*, 2009; Yeo, 2014) among Sarawak's rice landraces towards *P. oryzae* isolates. Partial resistance is a form

of quantitative resistance which involves multiple genes (polygenic) that contributes a small degree of protection towards the host. Unlike qualitative resistance, which is controlled by a single or a few major genes and typically results in complete resistance or susceptibility, quantitative resistance is more complex and offers a range of intermediate responses (Saito *et al.*, 2022). This type of resistance often leads to a reduction in disease severity, slower pathogen growth, and decreased disease spread, rather than a complete prevention of infection. Quantitative resistance is considered more durable and stable over time compared to qualitative resistance, as the pathogen is less likely to overcome the combined effects of multiple genetic factors (Niks *et al.*, 2015).

Previous study by Hussin (2021) had suggested that variation in latency period among Sarawak rice landraces with disease score 3 and above, might indicate the presence of partial resistance among the landraces. Evidence of partial resistance among Sarawak rice landraces also further supported by Lai *et al.* (2018) also reported that two Sarawak rice landraces; Hitam and Entangor landraces might show partial resistance. Investigating the presence of partial resistance gene and characterise them (if any) can be done in the future to further confirms the presence of partial resistance among Sarawak's rice landraces.

5.2.2 Hypersensitivity response (HR) of Sarawak's rice blast towards *Pyricularia oryzae* isolates

The host plant initiates a hypersensitivity response (HR) as a defence mechanism to prevent the infection from spreading. This response involves programmed cell death, where infected area of the plant rapidly undergo self-destruction. This not only confines the infection but also serves to halt the pathogen from advancing into other parts of the plant. This response can be seen by the appearance of small lesion on the leaf surface of the plant. A successful infection of the pathogen will cause the lesions to merge into a bigger lesion

(Jones & Dangl, 2006). In this study, the pathogenicity assay revealed HR on all six Sarawak landraces when inoculated with all five *P. oryzae* isolates, where most of the lesions expanded over time while some were not. However, the appearance of rice blast symptom was not the same as the typical diamond-shaped lesions observed in the field (Appendix G). Challagula *et al.* (2015) also reported poor rice blast disease expression using artificial inoculation (spot inoculation and filter paper inoculation). This could be influenced by many factors such as humidity, time exposure and temperature which cause it to be impossible to imitate the natural condition for development of typical diamond-shaped lesion of rice blast.

5.3 Haplotype diversity of *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes from *Pyricularia oryzae*

Avirulence (*Avr*) genes play a vital role in determining the pathogenicity of *P. oryzae* towards rice. *Avr* genes are known to be unstable and has high rate of adaptation due to different mechanisms such as genetic mutation, recombination, and mating type, which may result in polymorphism and genetic variation of *Avr* genes (Hu *et al.*, 2022; Huang *et al.*, 2014). This study investigated the presence of *AvrPiz-t*, *AvrPik* and *AvrPi-54* haplotypes from Sarawak *P. oryzae* isolates.

5.3.1 Haplotypes of *AvrPiz-t*

This study reports a total of 19 *AvrPiz-t* haplotypes of which, eight haplotypes (H12 - H19) are newly identified. The newly identified haplotypes, H12 - H19 exhibited two types of mutations in their ORF sequences: substitutions and deletion (specifically observed in H14). The substitution mutations resulted in both non-synonymous (nine sites) and synonymous (five sites) changes, as shown in Table 4.3.3 and Table 4.3.4. It is worth noting that H14 had a deletion, causing a frameshift in the sequence and resulting in the absence of

a stop codon in the ORF sequence. Consequently, the amino acid sequence of haplotypes H12 to H19 exhibited eight polymorphic sites (Table 4.3.4), not accounting for H14.

Among the 19 *AvrPiz-t* haplotypes, four haplotypes (H1, H7, H8, and H14) were found to have a missing stop codon, while three haplotypes (H11, H17, and H18) had a premature stop codon. In a study by Wang *et al.* (2020), their Haplotype 10, now renamed as H11 in this study, was reported to have a premature stop codon at the second amino acid position. This premature stop codon was also observed in haplotypes H17 and H18 in the current study. The distinction between these three haplotypes lies in the fact that H11 has a start codon (TTG; non-ATG start codon) immediately after the premature stop codon. In contrast, H17 and H18 had a nonsense premature stop codon. Notably, all three haplotypes possessed another stop codon at the end of the amino acid sequence, situated at the same site as the other haplotypes. Wang *et al.* (2020) proposed that the phenomenon observed in H11 might represent a potential new mechanism to maintain the avirulent function of an *Avr* gene.

Haplotypes H1 and H7 of *AvrPiz-t* have been identified as capable of triggering resistance in the *Piz-t* harbouring rice cultivar (Toride). On the other hand, haplotypes H2 and H8 have been shown not to induce resistance (Chen *et al.*, 2014; Wang *et al.*, 2020). As for the remaining 15 haplotypes, their functional roles are yet to be confirmed. Merely having a complete ORF sequence of *AvrPiz-t* does not guarantee the gene's functionality. Mutations in the ORF sequence, as well as the promoter region (was not analysed in this study), may affect the functionality of the protein. Therefore, further investigation and characterization of the gene sequence are essential in the future. To gain a better understanding of the gene's functionality, it is recommended to assess the pathogenicity of isolates carrying each respective *AvrPiz-t* haplotype against the *Piz-t* harbouring rice cultivar. This evaluation will

provide valuable insights into the specific effects of each haplotype and its potential role in triggering or evading resistance in the host plant.

Haplotype H9 is observed as the dominant haplotype as it was present in 116 out of 150 *AvrPiz-t* ORF sequences from Malaysia (Sarawak), India, Thailand and China. Haplotype H9 which was known as H3 by Wang *et al.* (2020), was also reported as the most dominant haplotype (69% of the total isolates) spread across 13 prefectures in Yunnan Province, China. It is unlikely to conclude that haplotype H9 as the major haplotype of *P. oryzae* globally, as well as locally in Malaysia if solely in the present study as limited number of *AvrPiz-t* sequences representing different countries are available.

5.3.2 Haplotypes of *Avr-Pik*

Previous studies have reported the presence of *Avr-Pik* alleles; *Avr-Pik-A*, *Avr-Pik-B*, *Avr-Pik-C*, *Avr-Pik-D*, *Avr-Pik-E*, *Avr-Pik-F*, *Avr-Pik-G*, *Avr-Pik-H*, *Avr-Pik-I*, *Avr-Pik-J* and *Avr-Pik-K* (Yoshida *et al.*, 2009; Longya *et al.*, 2019; Zhang *et al.*, 2020). In this study, isolates from Malaysia (Sarawak) were found to harbour *Avr-Pik-D*, *Avr-Pik-F* and *Avr-Pik-I* alleles. *Avr-Pik-D* was reported as an ancestral allele (Yoshida *et al.*, 2009) which might be the reason why it is the dominant allele present among the 181 *P. oryzae* isolates used in this study. While *AvrPik-F* allele was reported as newly emerging aggressive *AvrPik* allele by Longya *et al.* (2019) as none of the *Pik* alleles were able to recognise it. Longya *et al.* (2019) reported that the emergence of new novel *AvrPik-F* allele was due to the implementation of *Pik* based resistance in Thailand rice variety. This is an example of co-arms evolution pattern between *R-gene* and *Avr-gene* in rice and rice blast pathosystem. In Malaysia (Sarawak), two isolates, POK1 and POSA23 which originated from Kuching and Sri Aman division, respectively, were also found to harbour *AvrPik-F* allele. Since *AvrPik-*

F allele is deemed to be aggressive, this might be putting some precaution for *Pik* based rice resistance implementation in Malaysia especially in Sarawak.

Interestingly, gene duplication happened for *AvrPik* gene where two copies of *AvrPik* alleles were present in one *P. oryzae* isolate were previously reported by Longya *et al.* (2019). In their findings, 28 out of 60 Thai's *P. oryzae* isolates had two copies of *AvrPik* alleles, *AvrPik-D* (avirulent) and *AvrPik-F* (virulent). Their study was further supported by Sutthiphai *et al.* (2021) of which reported 14 of Thai's blast isolates had both *AvrPik-D* and *AvrPik-F* allele in each isolate. Another study by Hu *et al.* (2022) also reported the presence of *AvrPik* gene duplication in their isolates as some of their isolates contained either both *AvrPik-D* and *AvrPik-A* alleles or both *AvrPik-D* and *AvrPik-E* alleles in each isolate. These findings suggests that *P. oryzae* isolates having avirulent *AvrPik* alleles (*AvrPik-D*) gain its virulence by pairing with aggressive or less avirulent *AvrPik* alleles (in this case, *AvrPik-F*, *AvrPik-A* and *AvrPik-E*).

Since both *Avr-Pik-D* and *Avr-Pik-F* alleles were present in Malaysia (Sarawak) *P. oryzae* isolates (seven isolates with *AvrPik-D* allele and two isolates with *AvrPik-F* allele), there is a possibility that gene duplication might be present among *P. oryzae* population in Malaysia (Sarawak) too which can be investigated in the future.

5.3.3 Haplotypes of *AvrPi-54*

This study is the first to report of *AvrPi-54* haplotype variations from 96 *P. oryzae* isolates coming from India and Malaysia. A total of seven *AvrPi-54* haplotypes were observed and designated as H1 to H7. All seven haplotypes exhibited substitution mutation within their ORF sequences. This created early stop codon for H3 and H7 at codon number 153 resulting in shorter amino acid sequence compared to the rest of the *AvrPi-54* haplotypes. Since the virulent function of all *AvrPi-54* haplotypes identified in this study

have not been determined, it remains unclear whether isolates carrying haplotypes H3 or H7 have lost their function.

Previous study by Ray *et al.* (2016) revealed that a virulent *P. oryzae* isolate, MG-79 was transformed with *AvrPi-54* gene which originated from isolate RML-29 harbouring *AvrPi-54* gene. When the transformed MG-79 isolate was inoculated on rice line TP2 (contains *Pi-54* gene), the isolate becomes avirulent. Isolate RML-29 was included in *AvrPi-54* ORF sequence alignment in this study and was designated as haplotype H1. This observation suggests that *P. oryzae* isolates carrying *AvrPi-54* of haplotype H1 probably have functioning avirulent gene.

5.3.4 Population genetic analysis based on *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes

Neutrality test to show the direction of selection for *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes for *P. oryzae* isolates in this study indicates that both *AvrPiz-t* and *AvrPi-54* genes of *P. oryzae* isolates used in this study were under negative selection or known as purifying selection. The neutrality test result for *AvrPiz-t* gene in this study was not in accordance with previous studies. Previous study by Sirisathaworn *et al.* (2017) reported that *AvrPiz-t* gene of 42 *P. oryzae* isolates from Thailand were not under any selection pressure. A study from Wang *et al.* (2020) also reported that *AvrPiz-t* gene of 100 *P. oryzae* isolates from Yunnan, China was not under any selection pressure. While *AvrPiz-t* gene of *P. oryzae* isolates from Chen *et al.* (2014) were under positive selection.

As the non-synonymous diversity (π_{non}) value was lower than synonymous diversity (π_{syn}) value, suggested that negative selection or known as purifying selection reduces the frequency of non-synonymous mutations which might be non-beneficial to the population. Purifying selection functions to remove harmful non-synonymous mutations, yet it is assumed to have minimal or negligible impact on synonymous mutations that evolve

neutrally (Hahn *et al.*, 2002). However, in this study whether the negative selection is strong enough to reduce the diversity of *AvrPiz-t* gene of *P. oryzae* population is not known. Further investigation can be done in the future to see the dynamic change of *AvrPiz-t* gene in *P. oryzae* population especially in Malaysia (Sarawak).

On the other hand, *Avr-Pik* gene of *P. oryzae* isolates used in this study showed positive values for all three neutrality parameters (Tajima's D, Fu and Li's D and Fu and Li's F). This result was also in agreement with Sutthipai *et al.* (2021) where the neutrality test for *Avr-Pik* gene among Thai's blast isolates showed positive values. The value of π_{non}/π_{syn} ratio in this study also showed that the *Avr-Pik* gene of *P. oryzae* isolates in this study is under positive selection. This result is further supported by Li *et al.* (2019) which also reported that *Avr-Pik* gene of *P. oryzae* isolates from Yunnan, China were under positive selection. Positive selection signifies a reduction in population size and/or the presence of balancing selection (Sutthipai *et al.*, 2021). Balancing selection takes place when several advantageous alleles are preserved at a particular locus (Charlesworth, 2006). This can increase the levels of polymorphism around the balanced locus and result in a corresponding decrease in substitutions (Siewert & Voight, 2017). Hence, the result in the current study indicates that different *Avr*-genes undergo different selection pressure which might cause different variation on *Avr*-gene.

5.3.5 Genetic diversity of *Avr* genes of *Pyricularia oryzae* isolates

Previous studies by Chen *et al.* (2014) and Sirisathaworn *et al.* (2017) had mentioned that *P. oryzae* isolates have low genetic variation in *AvrPiz-t* gene. Chen *et al.* (2014) found out that there is higher variation in the promoter region of *AvrPiz-t* compared to ORF region itself. Whilst Sirisathaworn *et al.* (2017), stated that the low diversity is probably because the *P. oryzae* isolates consists of only one mating type where recombination may not occur

between the isolates. It was also reported by Sutthipai *et al.* (2021) that Thailand isolates had low genetic variation for *AvrPi-9*, and *AvrPik* gene, but high genetic variation for *AvrPita-1* gene. In Malaysia, especially Sarawak, there are not enough isolates collected to suggest the population in the country has high or low variation. Study on the mating type of *P. oryzae* isolates in Malaysia (Sarawak) would be an interesting area for future study.

5.3.6 Pathogenicity assessment of *Pyricularia oryzae* isolates carrying *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes against Sarawak rice landraces

Pathogenicity assay was carried out on six Sarawak rice landraces with unknown presence of *R-gene* against five *P. oryzae* isolates from this study. Out of five isolates, three isolates (POS22, POSA23 and POSI7) had *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes. The *Avr* genes haplotype for each isolate are presented in Table 5.1. All six Sarawak rice landraces were either susceptible or moderately resistant towards isolate POS22, POSA23 and POSI7 except for Merah landrace which was resistant towards isolate POSI7. Merah landrace may have the *R-gene(s)* corresponding the haplotypes of *AvrPiz-t*, *AvrPik* and *AvrPi-54* (assuming functioning *Avr* gene). Or *R-gene(s)* that recognize *Avr* genes which have yet to be identified in isolate POSI7. Kanowit landrace was observed as the most susceptible. Suggesting Kanowit has no *R-gene(s)* recognizing any of the *Avr* genes of isolates POS22, POSA23 and POSI7. Merawi landrace was observed as the least susceptible compared to other landraces, suggesting presence of corresponding *R-gene(s)*. Based on gene-for-gene hypothesis (Flor, 1971), it is suggested to use the corresponding *R-gene* for development of blast resistant rice variety in Sarawak. Investigating the presence of *R-gene* in Merah (resistant towards isolate POSI7) and Merawi (the least susceptible landrace) landraces would be beneficial.

Table 5.1: The presence of *Avr* haplotypes for isolate POS22, POSA23 and POSI7 based on *AvrPiz-t*, *AvrPik* and *AvrPi-54* genes.

Isolate	Haplotype of <i>Avr</i> -genes		
	<i>AvrPiz-t</i>	<i>AvrPik</i>	<i>AvrPi-54</i>
POS22		<i>AvrPik-I</i>	
POSA23	H14	<i>AvrPik-F</i>	H1
POSI7		<i>AvrPik-D</i>	

5.4 Alternative Host for *Pyricularia oryzae* in Sarawak

Plant pathogens have developed the ability to switch hosts and expand their range, allowing them to transition from one host to another and establish themselves in new hosts to ensure their survival (Zhong *et al.*, 2016). *P. oryzae* was observed to have high adaptability and able to infect more than 50 *Poaceae* species (Ou, 1985). The strains of *P. oryzae* are divided into a several pathotypes; *Oryza* pathotype (isolated from *O. sativa*), *Panicum* pathotype (isolated from *P. miliaceum*), *Eleusine* pathotype (isolated from *E. coracana*), *Triticum* pathotype (isolated from *T. aestivum*), *Avena* pathotype (isolated from *A. sativa*), and *Lolium* pathotype (isolated from *L. perenne*) (Castroagudin *et al.*, 2016).

Previous study from Mackill and Bonman, (1986) had reported cross-infectivity of *P. oryzae* isolates between rice host and weed host. From a total of 100 *P. oryzae* isolates from rice host, *Oryza sativa* and weed hosts (*Brachiaria mutica*, *Eleusine indica*, *Panicum repens*, *Pennisetum purpureum*, *Dactyloctenium aegyptium*, *Digitaria ciliaris*, *Echinochloa colona*, *Leersia hexandra*, *Rottboellia exaltata* and *Brachiaria distachya*) in cross-inoculation assay. The cross-pathogenicity result showed that *B. distachya*, *E. colona*, *L. hexandra*, *Leptochloa chinensis* and *R. exaltata* were susceptible to *P. oryzae* infection from rice host. While *P. oryzae* isolates from *B. distachya*, *E. colona*, *L. hexandra*, and *R. exaltata* were able to infect rice.

In Malaysia, *E. indica*, *Echinochloa* spp., *L. chinensis* and *D. ciliaris* were reported as common weed that were pre-dominant in rice planting area (Dilipkumar *et al.*, 2017; Hakim *et al.*, 2013). In this study, *P. oryzae* isolates were successfully isolated from *E. indica* coming from different divisions of Sarawak (Table 4.5.1). Similar morphological characteristics (conidia and colony appearance) were observed between *P. oryzae* isolates from *E. indica* and rice (Appendix B & Appendix C). Apart from that, *P. oryzae* isolates from *E. indica* have higher growth rate when cultured on OMA compared to PDA. However due to insufficient data, the sporulation rate of *P. oryzae* from *E. indica* were inconclusive as there was no statistical analysis carried out. However, based on general observation, isolates grown on OMA sporulate better compared to PDA. The isolation of *P. oryzae* from *E. indica* suggest that to control *P. oryzae* one must also control *E. indica*.

Eleusine indica, commonly referred to as goosegrass, is recognized as a problematic weed in agricultural area and has earned its place as one of the world's top five most notorious weeds (Takano *et al.*, 2016). This could be attributed to the strong resilience of goosegrass seeds, which exhibit a high rate of seedling germination as the seed can germinate under a wide range of temperature and moisture stress conditions (Seng *et al.*, 2023; Shekoofa *et al.*, 2020). In Malaysia, goosegrass has been documented as showing resistance to herbicides (Dilipkumar *et al.*, 2017). In agricultural ecosystems, goosegrass was reported as a common weed in rain-fed planting area (Chauhan *et al.*, 2008). This invasive species has infiltrated agricultural landscapes in as many as 46 different crops across approximately 60 countries (Ma *et al.*, 2015). Goosegrass was reported as frequent weed found in upland, dry-seeded and wet-seeded rice fields in the Philippines (Moody *et al.*, 1989).

In Sarawak, rice is planted follows a seasonal pattern. Depending on the type of rice being grown, rice is typically planted once or twice annually. Following harvest, the planting

area remains untouched until the next planting season, leading to the growth of weeds on the planting plot. These weeds could potentially serve as a reservoir for *P. oryzae* to persist during the periods when rice is not being cultivated. Therefore, it is prudent to observe the weeds occurred in Sarawak rice planting area especially for smallholder farmers and remove any weed species that has the potential to act as alternative host for *P. oryzae* during and also between rice planting seasons.

Pathogenicity tests aimed at observing potential cross-infection between *P. oryzae* originating from rice and weed hosts, have not been conducted. These investigations would help shed light on the potential host range and the pathogenic ability of Sarawak's *P. oryzae* isolates from both rice and weed hosts.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the objectives of the present study has been achieved. A total of 25 *P. oryzae* isolates were successfully isolated from infected rice in Kuching, Serian, Sri Aman, Betong and Sibu division using spore-drop isolation. Two different media (OMA and PDA) affects the morphological and physiological (growth rate and sporulation rate) characteristics of *P. oryzae* isolates. There was no correlation between geographical distribution of isolates with the morphological characteristic. The analysis of *Avr* genes (*AvrPiz-t*, *AvrPik* and *AvrPi-54*) sequences of *P. oryzae* isolates from Sarawak Malaysia and isolates from various countries revealed new *Avr* haplotypes. There were eight new *AvrPiz-t* haplotypes, one new *AvrPik* haplotype and seven *AvrPi-54* haplotypes. Neutrality tests suggested that *AvrPiz-t* and *AvrPi-54* gene were under negative selection while *AvrPik* was under positive selection. Pathogenicity assay revealed that isolate POS22 was the most virulent among five tested *P. oryzae* isolates. Kanowit landrace was the most susceptible landrace while Merawi landrace was the least susceptible. A total of seven *P. oryzae* isolates coming from Kuching, Serian, Sri Aman and Sibu division were successfully isolated from *E. indica* by using spore-drop isolation method. Both OMA and PDA affects the morphological and physiological (growth rate and sporulation rate) characteristics of *P. oryzae* isolates from *E. indica*. There was also no correlation between geographical distribution of isolates with the morphological characteristics.

6.2 Recommendations

The following recommendations are proposed for further research. Firstly, it is suggested to conduct a study on the sporulation rate of the 25 *P. oryzae* isolates obtained in this study on OMA and PDA media with an increased number of replications, ensuring a more robust and reliable observation of their sporulation rates on each medium. Secondly, investigating the correlation between the pigmentation of the colony surface of the *P. oryzae* isolates and their sporulation rates can be undertaken. Additionally, the pathogenicity of Sarawak isolates, each carrying the *AvrPiz-t*, *AvrPik*, and *AvrPi-54* haplotypes, against the *Piz-t*, *Pik*, and *Pi-54* harboring differential rice lines, respectively, should be assessed. Furthermore, it is recommended to investigate the presence of *R*-genes in Merah (resistant to isolate POSI7) and Merawi (the least susceptible landrace) landraces. Another important avenue of exploration involves investigating the presence of partial resistance genes among Sarawak's rice landraces and characterizing them (if present). Finally, conducting pathogenicity tests to observe potential cross-infection between *P. oryzae* originating from rice and weed hosts in Sarawak will contribute to a more comprehensive understanding of the pathogen's dynamics in the region.

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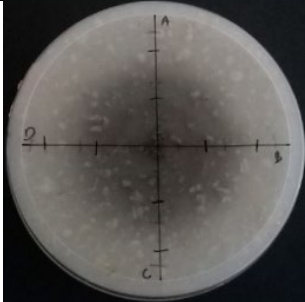
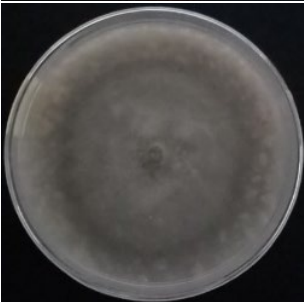
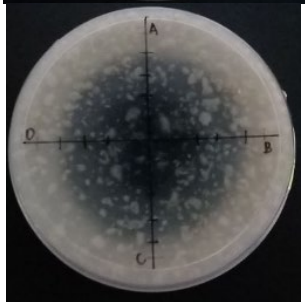
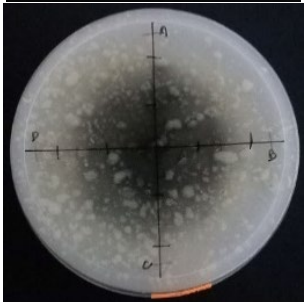
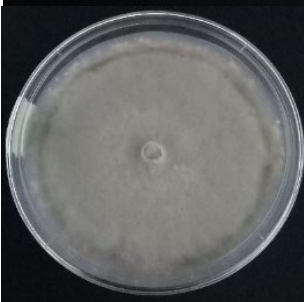
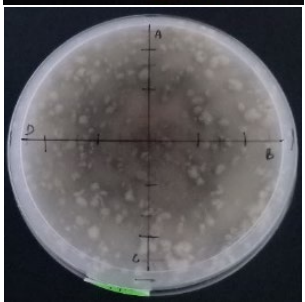
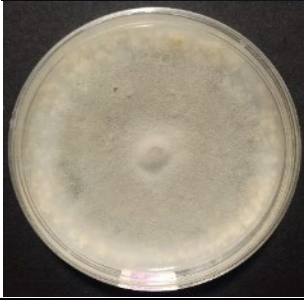
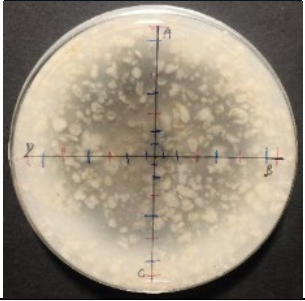
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APPENDICES

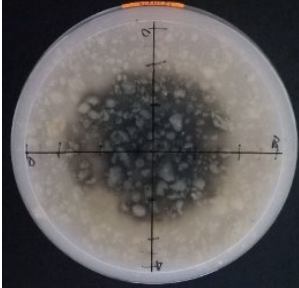
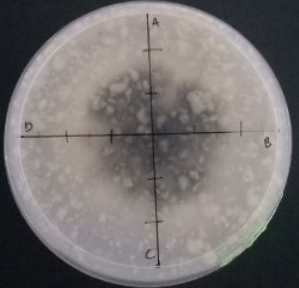
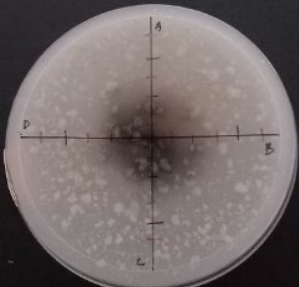
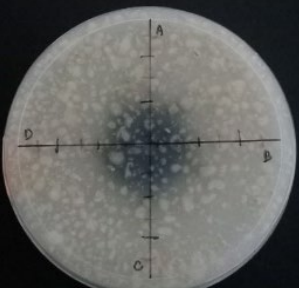
Appendix A: Journal Publications

1. **Rafael, E. S.**, Yeo, F. K. S., Hussin, N. A., Vu, T. T. A., Ang, P. S., Ewe, Z. H., & Lai, L. S. (2023). Morphological and Physiological Development of *Pyricularia oryzae* Isolates from North-western Region of Sarawak on Different Media under Laboratory Conditions. *Borneo Journal of Resource Science and Technology*, 13(1), 81-92. DOI: 10.33736/bjrst.5258.2023
2. Yeo, F. K. S., **Rafael, E. S.**, Ewe, Z. H., Ang, P. S., Vu T. T. A., Chung, H. H., Lai, L. S., Ting, H. M., & Bao, Y. (2024). New variants of AvrPiz-t Identified in *Pyricularia oryzae* from Malaysia. *Plant Stress*, 11(100322), 1-11. DOI: 10.1016/j.stress.2023.100322

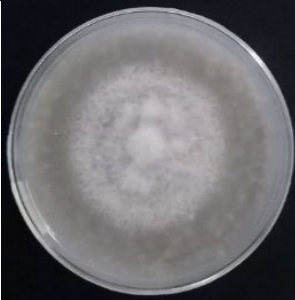
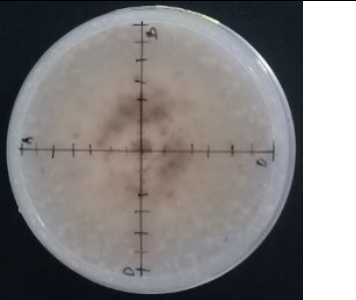
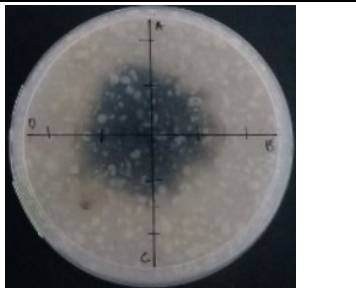
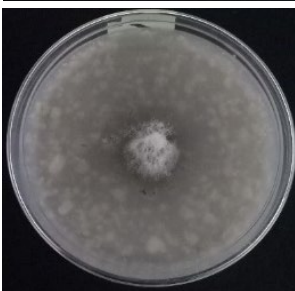
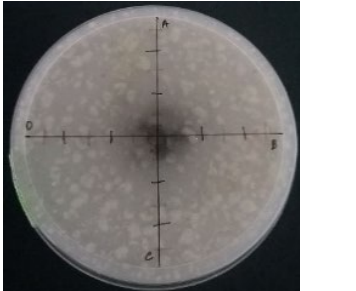
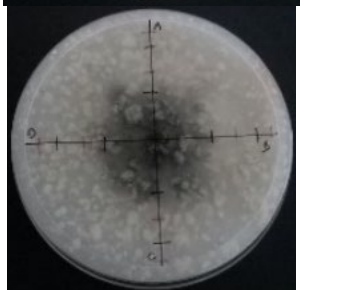
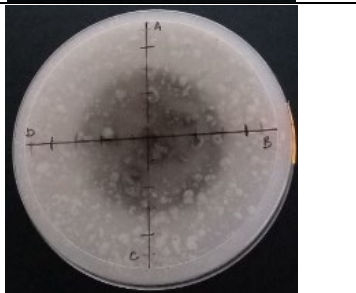
Appendix B: Morphological groupings of *Pyricularia oryzae* isolates from Sarawak collected from rice hosts on different media, OMA and PDA.

Media: Oatmeal Agar (OMA)			
Group	Isolate	Front-view	Reverse-view
1	POK9		
	POS13		
	POS22		
	POSA15		
	POSI7		

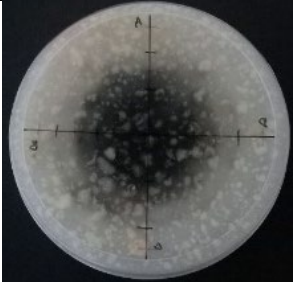
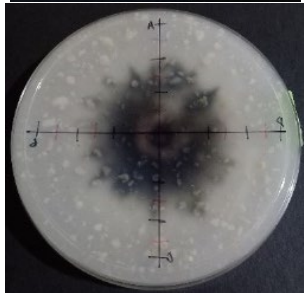
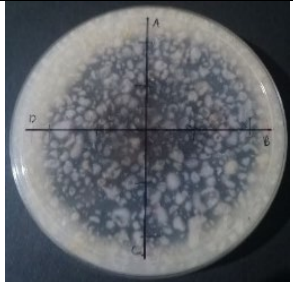
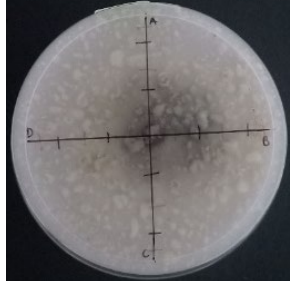
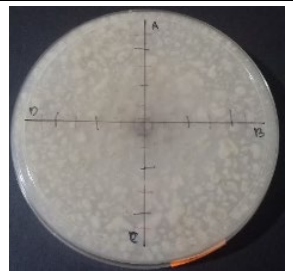
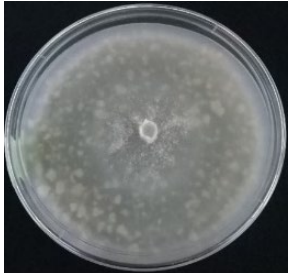
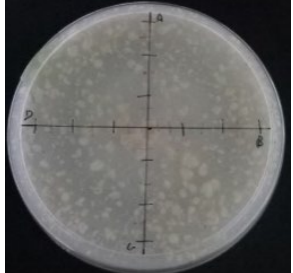
Appendix B: continued

2	POS24		
	POS20		
	POS26		
3	POSA14		
	POSI3		

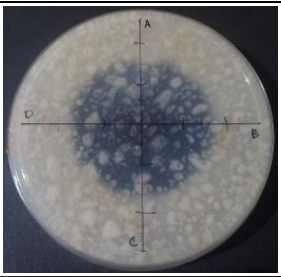
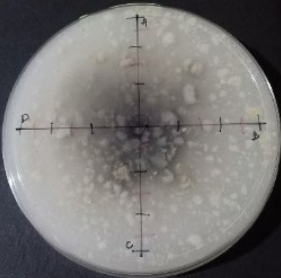
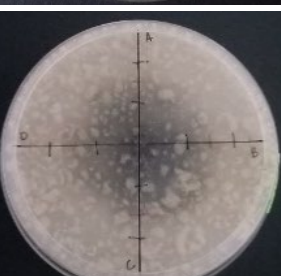
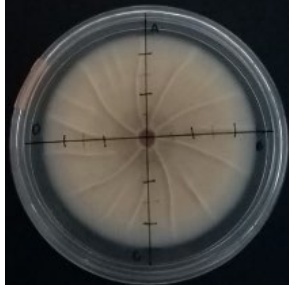
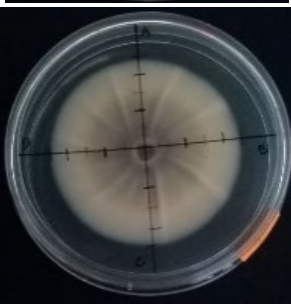
Appendix B: continued

3	POSA21		
	POSA20		
4	POSA13		
	POSA22		
5	POB1		

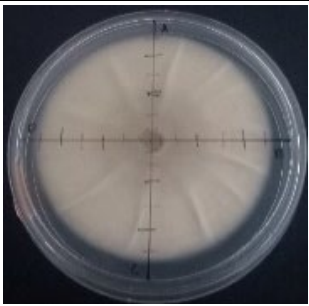
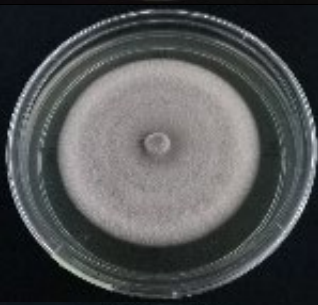
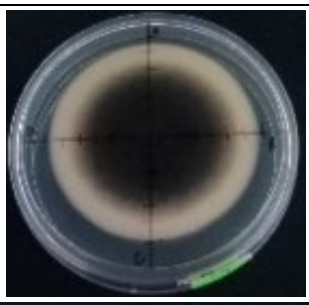
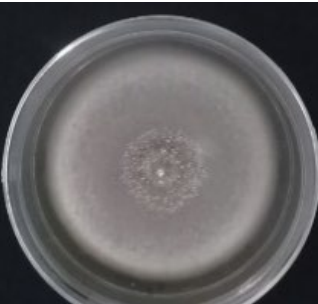
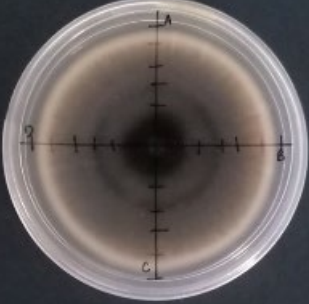
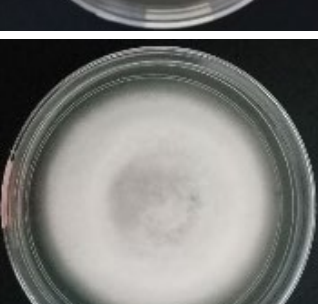
Appendix B: continued

6	POSA23		
	POSA16		
7	POS23		
8	POSA18		
9	POSI6		
	POS21		

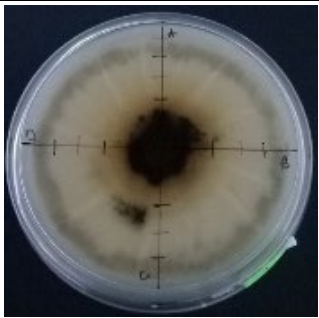
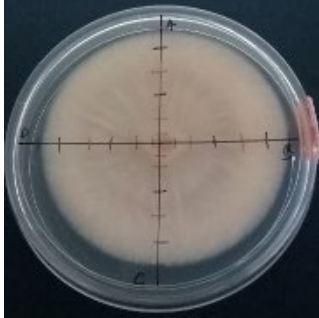
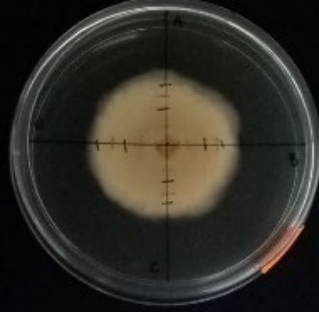
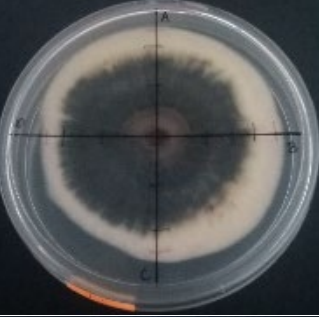
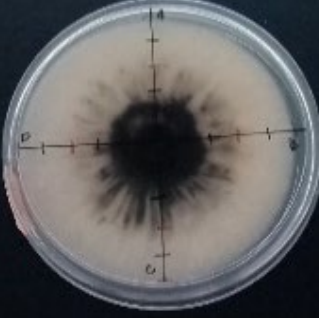
Appendix B: continued

9	POS25		
	POSA17		
	POSA19		
Media: Potato Dextrose Agar (PDA)			
1	POK9		
	POSI3		

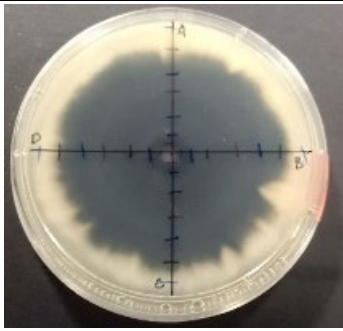
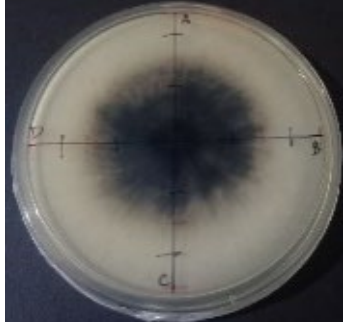
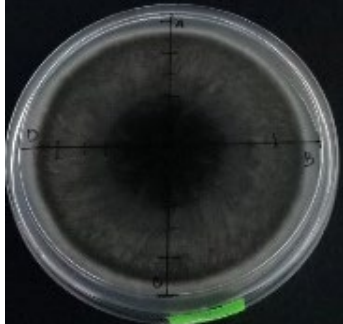
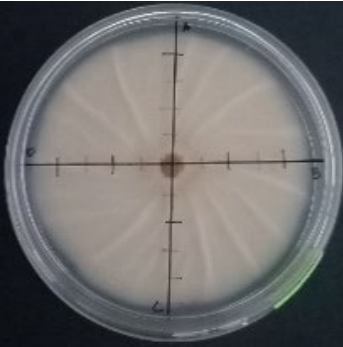
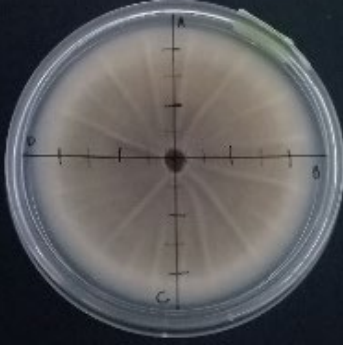
Appendix B: continued

1	POSI6		
			
2	POSA16		
	POS20		
	POS13		
	POS22		

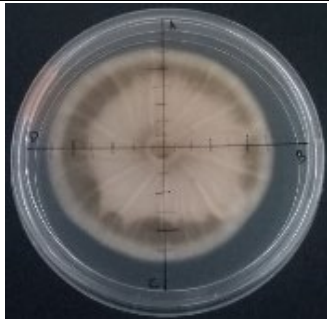
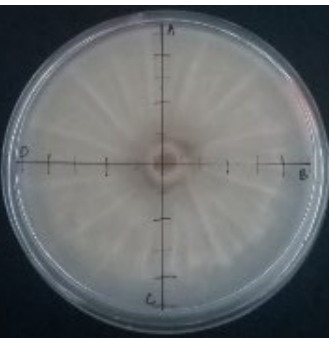
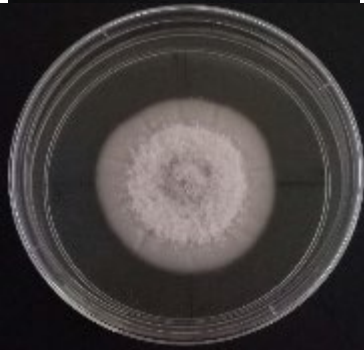
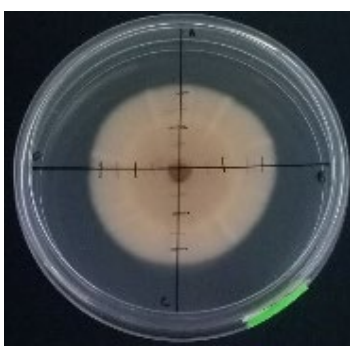
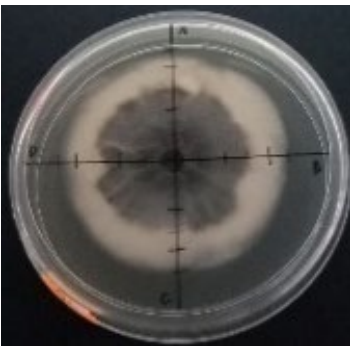
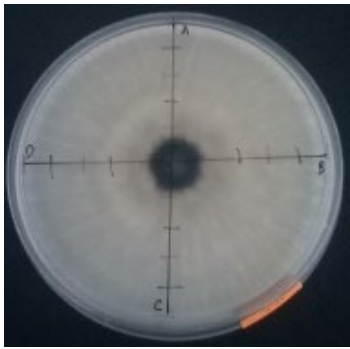
Appendix B: continued

2	POSA15		
3	POS15		
	POS21		
	POS23		
	POSA23		

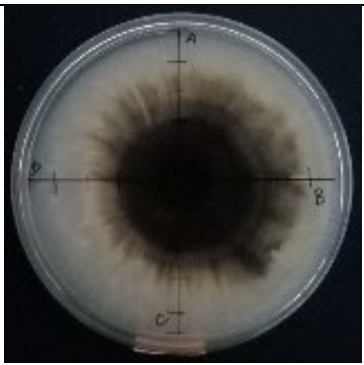
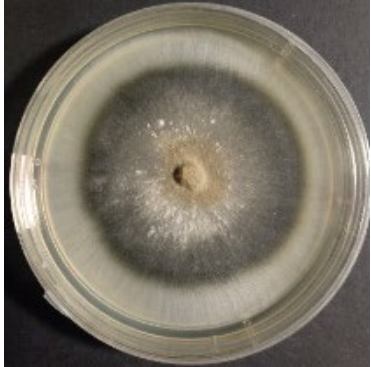
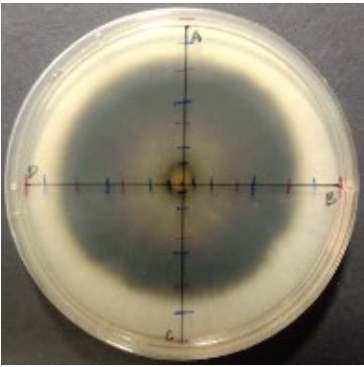
Appendix B: continued

3	POSI7		
	POS26		
	POSA17		
4	POSA18		
	POSA19		

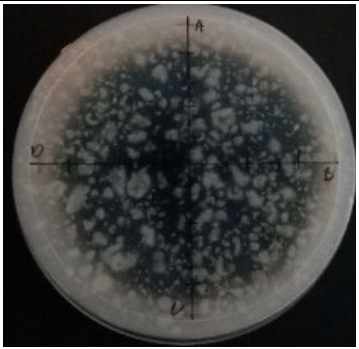
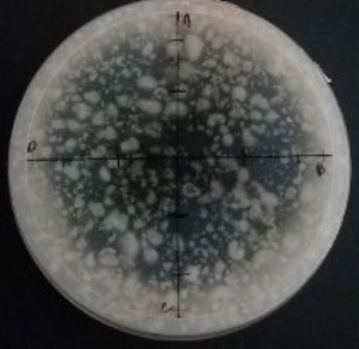
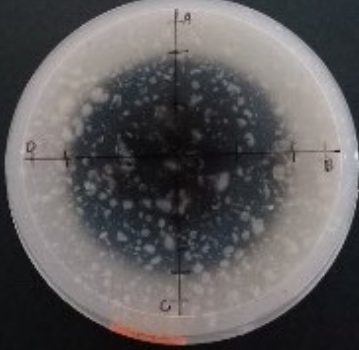
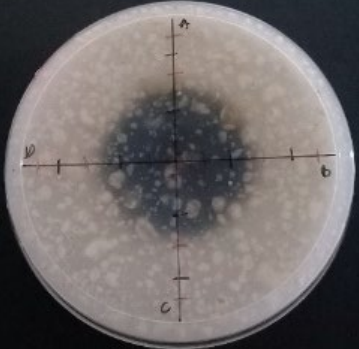
Appendix B: continued

4	POS24		
	POSA13		
5	POS25		
	POSA14		
	POSA20		

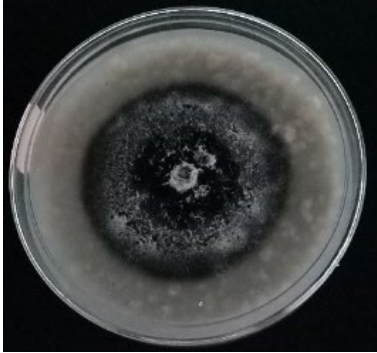
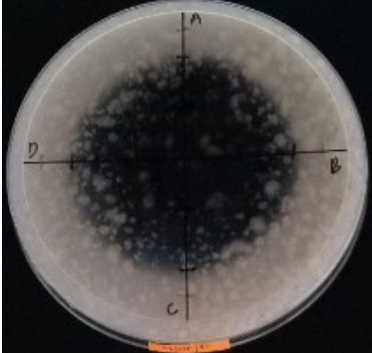
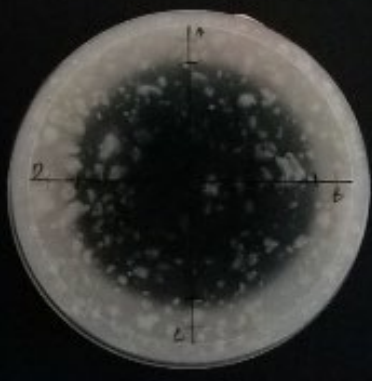
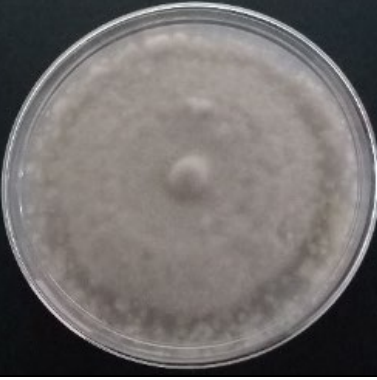
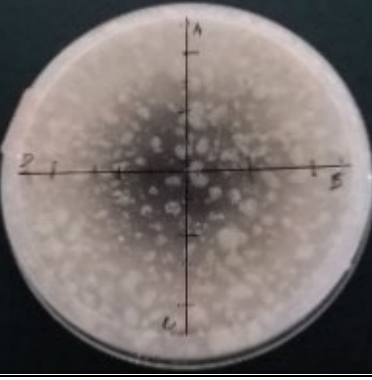
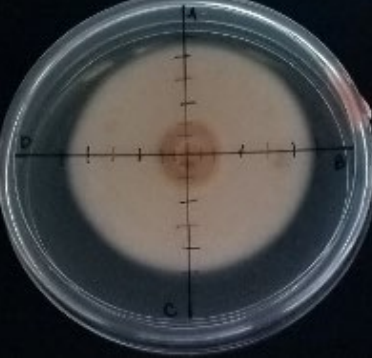
Appendix B: continued

5	POSA22		
6	POB1		

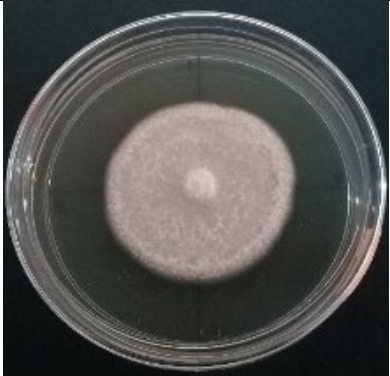
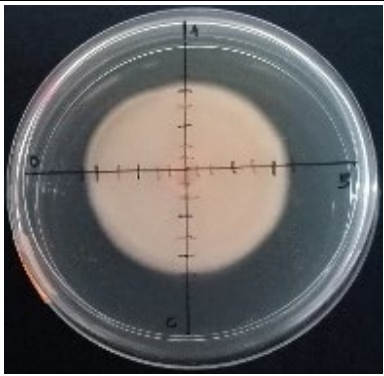
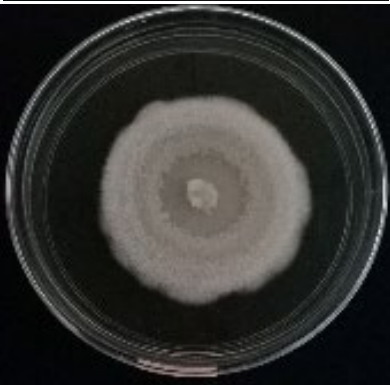
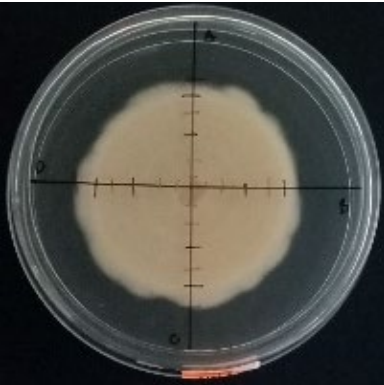
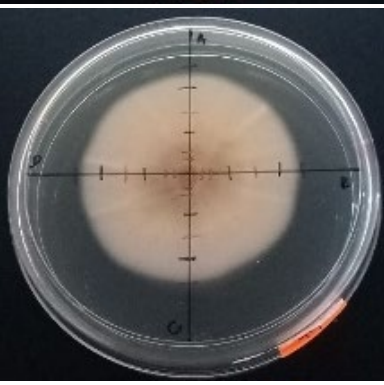
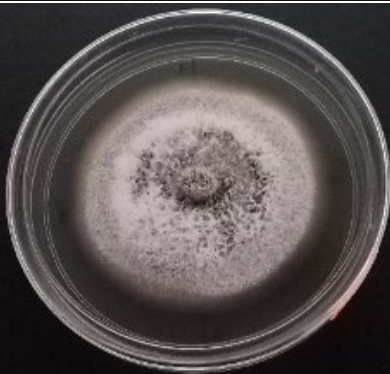
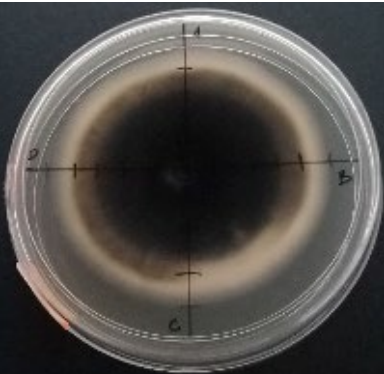
Appendix C: Morphological groupings of *Pyricularia oryzae* isolates from Sarawak collected from *Eleusine indica* on different media, OMA and PDA.

		Media: Oatmeal Agar (OMA)	
Group	Isolate	Front-view	Reverse-view
1	POWK2		
	POWSA1		
2	POWSA2		
	POWS1		

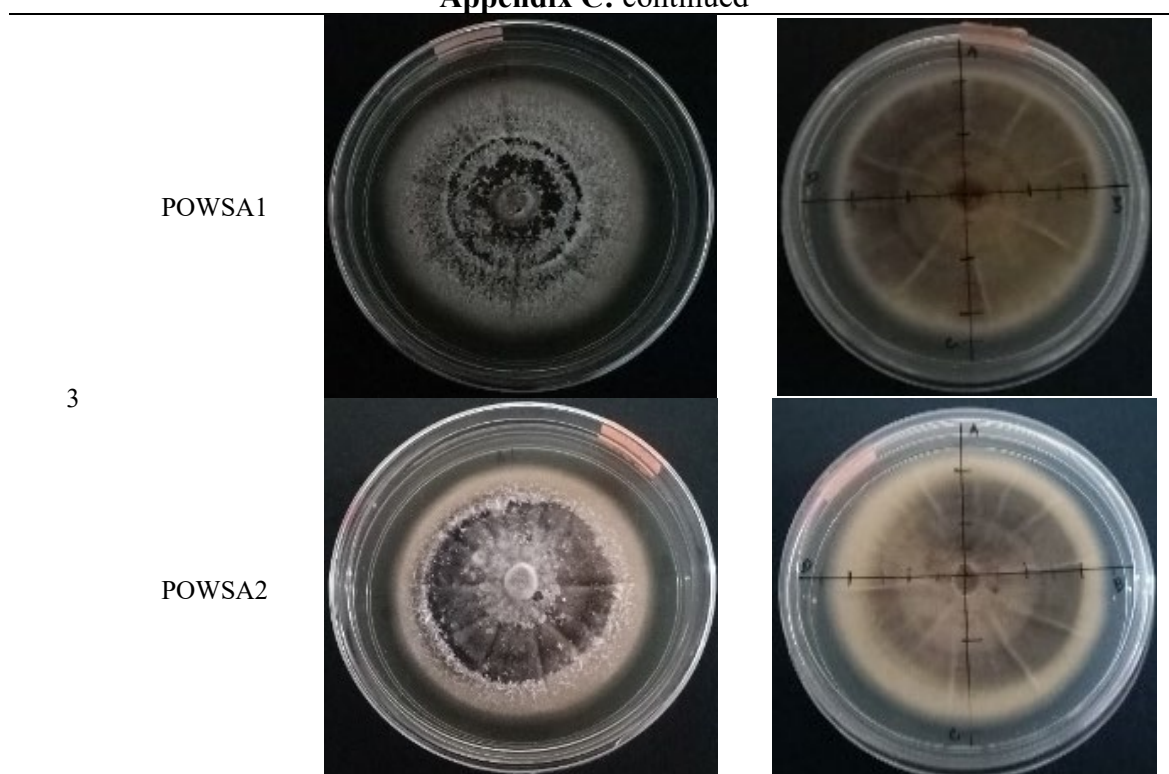
Appendix C: continued

3	POWK3	 
	POWSI1	 
4	POWK1	 
Media : Potato Dextrose Agar (PDA)		
1	POWK3	 

Appendix C: continued

1	POWS1		
	POWS11		
2	POWK1		
	POWK2		

Appendix C: continued



Appendix D: List of 123 *AvrPiz-t* sequences of *Pyricularia oryzae* downloaded from National Centre for Biotechnology Information (NCBI) GenBank website.

NCBI Accession number	Isolate	Country	Sequence Authors
EU837058.1	81278ZB15	China	Li <i>et al.</i> , 2009
HE565704.1	Mo-si-63	India	Singh <i>et al.</i> , unpublished;
HE578813.1	Mo-ni-025	India	Singh <i>et al.</i> , unpublished
HE578814.1	Mo-si-12	India	Singh <i>et al.</i> , unpublished
HE578817.1	Mo-ei-26	India	Singh <i>et al.</i> , unpublished
HE578819.1	Mo-ni-027	India	Singh <i>et al.</i> , unpublished
HE578820.1	Mo-ni-028	India	Singh <i>et al.</i> , unpublished
HE578822.1	Mo-ei-27	India	Singh <i>et al.</i> , unpublished
HE578824.1	Mo-ei-28	India	Singh <i>et al.</i> , unpublished
HE578826.1	Mo-nwi-31	India	Singh <i>et al.</i> , unpublished
HE578828.1	Mo-ei-40	India	Singh <i>et al.</i> , unpublished
HE578830.1	Mo-ei-48	India	Singh <i>et al.</i> , unpublished
HE578833.1	Mo-nwi-60	India	Singh <i>et al.</i> , unpublished
HE578834.1	Mo-nwi-75	India	Singh <i>et al.</i> , unpublished
HE578835.1	Mo-ei-79	India	Singh <i>et al.</i> , unpublished
HE578837.1	Mo-nwi-85	India	Singh <i>et al.</i> , unpublished
HE578839.1	Mo-nwi-103	India	Singh <i>et al.</i> , unpublished
HE578841.1	Mo-ei-3	India	Singh <i>et al.</i> , unpublished
HE578843.1	Mo-ei-5	India	Singh <i>et al.</i> , unpublished
HE578845.1	Mo-ei-7	India	Singh <i>et al.</i> , unpublished
HE578847.1	Mo-ei-8	India	Singh <i>et al.</i> , unpublished
HE584658.1	Mo-ei-11	India	Singh <i>et al.</i> , unpublished
HE584659.1	Mo-ei-15	India	Singh <i>et al.</i> , unpublished
HE584660.1	Mo-ei-16	India	Singh <i>et al.</i> , unpublished
HE584661.1	Mo-ei-20	India	Singh <i>et al.</i> , unpublished
HE584662.1	Mo-ei-22	India	Singh <i>et al.</i> , unpublished
HE584663.1	Mo-ei-23	India	Singh <i>et al.</i> , unpublished
HE584664.1	Mo-ei-24	India	Singh <i>et al.</i> , unpublished
HE584665.1	Mo-ei-114	India	Singh <i>et al.</i> , unpublished
HE584666.1	Mo-ni-001	India	Singh <i>et al.</i> , unpublished
HE584667.1	Mo-ni-007	India	Singh <i>et al.</i> , unpublished
HE584668.1	Mo-ni-008	India	Singh <i>et al.</i> , unpublished
HE584669.1	Mo-ni-015	India	Singh <i>et al.</i> , unpublished
HE584670.1	Mo-ni-016	India	Singh <i>et al.</i> , unpublished
HE584671.1	Mo-ni-022	India	Singh <i>et al.</i> , unpublished
HE584672.1	Mo-ni-024	India	Singh <i>et al.</i> , unpublished
HE584673.1	Mo-nwi-204	India	Singh <i>et al.</i> , unpublished
HE584674.1	Mo-nwi-205	India	Singh <i>et al.</i> , unpublished
HE584675.1	Mo-nwi-46	India	Singh <i>et al.</i> , unpublished
HE584676.1	Mo-nwi-47	India	Singh <i>et al.</i> , unpublished
HE584677.1	Mo-nwi-29	India	Singh <i>et al.</i> , unpublished

Appendix D: continued

HE584678.1	Mo-nwi-203	India	Singh <i>et al.</i> , unpublished
HE584679.1	Mo-nwi-16	India	Singh <i>et al.</i> , unpublished
HE584680.1	Mo-ni-026	India	Singh <i>et al.</i> , unpublished
HE584681.1	Mo-nwi-48	India	Singh <i>et al.</i> , unpublished
HE584682.1	Mo-nwi-50	India	Singh <i>et al.</i> , unpublished
HE584683.1	Mo-nwi-208	India	Singh <i>et al.</i> , unpublished
HE584684.1	Mo-nwi-51	India	Singh <i>et al.</i> , unpublished
HE584685.1	Mo-nwi-54	India	Singh <i>et al.</i> , unpublished
HE584686.1	Mo-nwi-55	India	Singh <i>et al.</i> , unpublished
HE584687.1	Mo-nwi-42	India	Singh <i>et al.</i> , unpublished
HE584688.1	Mo-ei-2	India	Singh <i>et al.</i> , unpublished
HE584689.1	Mo-ei-4	India	Singh <i>et al.</i> , unpublished
HE584690.1	Mo-ei-10	India	Singh <i>et al.</i> , unpublished
HE584691.1	Mo-ei-12	India	Singh <i>et al.</i> , unpublished
HE584692.1	Mo-ei-21	India	Singh <i>et al.</i> , unpublished
HE584693.1	Mo-ni-002	India	Singh <i>et al.</i> , unpublished
HE584694.1	Mo-ni-004	India	Singh <i>et al.</i> , unpublished
HE584695.1	Mo-si-23	India	Singh <i>et al.</i> , unpublished
HE584696.1	Mo-si-55	India	Singh <i>et al.</i> , unpublished
HE650799.1	Mo-ei-6	India	Singh <i>et al.</i> , unpublished
HE650800.1	Mo-ei-9	India	Singh <i>et al.</i> , unpublished
HE650801.1	Mo-ei-13	India	Singh <i>et al.</i> , unpublished
HE650802.1	Mo-ei-14	India	Singh <i>et al.</i> , unpublished
HE650803.1	Mo-ei-18	India	Singh <i>et al.</i> , unpublished
HE650804.1	Mo-ei-19	India	Singh <i>et al.</i> , unpublished
HE650805.1	Mo-ei-25	India	Singh <i>et al.</i> , unpublished
HE650806.1	Mo-ni-010	India	Singh <i>et al.</i> , unpublished
HE650807.1	Mo-nwi-28	India	Singh <i>et al.</i> , unpublished
HE650808.1	Mo-si-38	India	Singh <i>et al.</i> , unpublished
HE650809.1	Mo-si-42	India	Singh <i>et al.</i> , unpublished
HE650810.1	Mo-si-47	India	Singh <i>et al.</i> , unpublished
HE650811.1	Mo-si-56	India	Singh <i>et al.</i> , unpublished
HE650812.1	Mo-si-60	India	Singh <i>et al.</i> , unpublished
HE650813.1	Mo-si-62	India	Singh <i>et al.</i> , unpublished
HE650814.1	Mo-si-67	India	Singh <i>et al.</i> , unpublished
HE650815.1	Mo-si-68	India	Singh <i>et al.</i> , unpublished
HE650816.1	Mo-si-72	India	Singh <i>et al.</i> , unpublished
HE650817.1	Mo-si-74	India	Singh <i>et al.</i> , unpublished
HE651026.1	Mo-nwi-209	India	Singh <i>et al.</i> , unpublished
HE651027.1	Mo-ni-019	India	Singh <i>et al.</i> , unpublished
LC175910.1	10302	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175911.1	10459	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175912.1	10552	Thailand	Sirisathaworn <i>et al.</i> , 2017

Appendix D: continued

LC175913.1	10578	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175914.1	10732	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175915.1	10873	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175916.1	10985	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175917.1	11100	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175918.1	BAG1-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175919.1	BAG2-3	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175920.1	BAG3-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175921.1	BAG4-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175922.1	BAG5-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175923.1	BAG6-5	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175924.1	BAG8-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175925.1	BAG9-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175926.1	BAG11-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175927.1	BAG14-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175928.1	BAG15-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175929.1	BAG16-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175930.1	BAG17-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175931.1	BAG18-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175932.1	BAG19-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175933.1	BAG20-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175934.1	BAG21-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175935.1	BAG22-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175936.1	BAG23-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175937.1	BAG24-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175938.1	BAG25-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175939.1	BAG26-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175940.1	BAG27-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175941.1	BAG28-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175942.1	BAG29-2	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175943.1	BAG32-1	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175944.1	BKK55001	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175945.1	BKK55002	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175946.1	BKK55003	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175947.1	CCOO56001	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175948.1	CCOO56002	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175949.1	RBR55001	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175950.1	RBR55002	Thailand	Sirisathaworn <i>et al.</i> , 2017
LC175951.1	SRN54002	Thailand	Sirisathaworn <i>et al.</i> , 2017

Appendix E: List of 169 *AvrPik* sequences of *Pyricularia oryzae* downloaded from National Centre for Biotechnology Information (NCBI) GenBank website.

NCBI Accession Number	Isolate	Country	Sequence Authors
AB498875.1	Ina86-137	Japan	Yoshida <i>et al.</i> , 2009
AB498876.1	Ina-168	Japan	Yoshida <i>et al.</i> , 2009
AB498877.1	9505-3	Japan	Yoshida <i>et al.</i> , 2009
AB498878.1	Y93-245c-2	Japan	Yoshida <i>et al.</i> , 2009
AB498879.1	P-2b	Japan	Yoshida <i>et al.</i> , 2009
KY432942.1	FQ-77	China	Li <i>et al.</i> , 2017
KY432943.1	FQ-78	China	Li <i>et al.</i> , 2017
KY432944.1	DF-18	China	Li <i>et al.</i> , 2017
LC636471.1	Ina168	Japan	Yoshida <i>et al.</i> , 2009
MH078516.1	10100	Thailand	Longya <i>et al.</i> , 2019
MK327186.1	Y72391a	China	Li <i>et al.</i> , 2019
MK327187.1	YK97691A	China	Li <i>et al.</i> , 2019
MK327188.1	YK97611A	China	Li <i>et al.</i> , 2019
MK327189.1	Y96162a	China	Li <i>et al.</i> , 2019
MK327190.1	YN95692A	China	Li <i>et al.</i> , 2019
MW259148.1	PNB61008	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259149.1	YST61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259150.1	UTI61102	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259151.1	STI61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259152.1	STI61004	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259153.1	PYO61008	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259154.1	PRE61014	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259155.1	PYO61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259156.1	PRE61021	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259157.1	TAK61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259158.1	TAK61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259159.1	SSK61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259160.1	SSK61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259161.1	SRN61004	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259162.1	RBR61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259163.1	PRE61016	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259164.1	PYO61005	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259165.1	STI61005	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259166.1	LPG61011	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259167.1	LPG61011	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259168.1	LPG61005	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259169.1	PL61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259170.1	PL61009	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259171.1	KSN61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259172.1	KSN61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259173.1	NST61103	Thailand	Sutthipai & Jantasuriyarat, 2022

Appendix E: continued

MW259174.1	NKI61010	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259175.1	NBP61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259176.1	LPG61004	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259177.1	CRI61007	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259178.1	CRI61007	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259179.1	CRI61010	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259180.1	MSN61008	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259181.1	KPT61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259182.1	TAK61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259183.1	TAK61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259184.1	YST61004	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259185.1	UBN61011	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259186.1	KKN61005	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259187.1	KKN61005	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259188.1	LRI61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259189.1	PL61017	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259190.1	CMI61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259191.1	CMI61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259192.1	CMI61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259193.1	CRI61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259194.1	CRI61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259195.1	PYO61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259196.1	PYO61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259197.1	LRI61104	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259198.1	PRE61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259199.1	PRE61002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259200.1	SRN61007	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259201.1	MSN61005	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259202.1	PL61121	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259203.1	LPG61007	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259204.1	LPG61007	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259205.1	NMA61004	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259206.1	UDN61008	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259207.1	NKI61013	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259208.1	PL61006	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259209.1	PL61006	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259210.1	SKN61009	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259211.1	RBR61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259212.1	CMI61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259213.1	CMI61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259214.1	BRM61010	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259215.1	PCT61003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259216.1	UBN61017	Thailand	Sutthipai & Jantasuriyarat, 2022

Appendix E: continued

MW259217.1	PL61019	Thailand	Sutthipai & Jantasuriyarat, 2022
MW259218.1	NPM61001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335055.1	BRM60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335056.1	BRM60003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335057.1	BRM60006.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335058.1	BRM60011.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335059.1	BRM60012.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335060.1	CRI60001.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335061.1	CRI60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335062.1	CRI60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335063.1	CRI60004.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335064.1	CRI60005.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335065.1	CRI60005.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335066.1	CRI60005.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335067.1	CRI60006.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335068.1	CRI60007	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335069.1	KBI60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335070.1	MSN60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335071.1	MSN60002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335072.1	MSN60003.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335073.1	MSN60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335074.1	MSN60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335075.1	MSN60004.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335076.1	MSN60004.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335077.1	MSN60005.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335078.1	MSN60006.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335079.1	MSN60008.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335080.1	MSN60009.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335081.1	MSN60011.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335082.1	MSN60011.3	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335083.1	MSN60012	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335084.1	MSN60023	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335085.1	NBP60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335086.1	NBP60002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335087.1	NBP60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335088.1	NBP60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335089.1	NBP60003.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335090.1	NK60001.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335091.1	NKI-60001.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335092.1	NKI-60001.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335093.1	NMA60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335094.1	NRT60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335095.1	NRT60002	Thailand	Sutthipai & Jantasuriyarat, 2022

Appendix E: continued

MW335096.1	NRT60002	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335097.1	PL60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335098.1	PL60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335099.1	PL60004	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335100.1	PL60009	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335101.1	PNB60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335102.1	PRE60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335103.1	SKN60001.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335104.1	SKN60001.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335105.1	SKN60001.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335106.1	SKN60002.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335107.1	SKN60002.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335108.1	SKN60003	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335109.1	SPB60009	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335110.1	SPB60010	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335111.1	SPB60012	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335112.1	SPB60012	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335113.1	SPB60013	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335114.1	SPB60013	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335115.1	SRN60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335116.1	SRN60001	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335117.1	SRN60005.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335118.1	SRN60005.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335119.1	UDN60001.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335120.1	UDN60001.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335121.1	UDN60002.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335122.1	UDN60003.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335123.1	UDN60003.2	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335124.1	UDN60003.3	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335125.1	UDN60004.1	Thailand	Sutthipai & Jantasuriyarat, 2022
MW335126.1	UDN60005	Thailand	Sutthipai & Jantasuriyarat, 2022
ON721120.1	NC16-160	Korea	Chung <i>et al.</i> , 2023
ON721121.1	NC16-165	Korea	Chung <i>et al.</i> , 2023
ON721122.1	NC16-172	Korea	Chung <i>et al.</i> , 2023
ON721123.1	NC16-052	Korea	Chung <i>et al.</i> , 2023
ON721124.1	NC16-057	Korea	Chung <i>et al.</i> , 2023
OP750463.1	NC17-433	Korea	Chung <i>et al.</i> , 2023
OP750464.1	NC17-026	Korea	Chung <i>et al.</i> , 2023
OP750465.1	NC17-102	Korea	Chung <i>et al.</i> , 2023
OP750466.1	NC17-018	Korea	Chung <i>et al.</i> , 2023
OP750467.1	NC16-098	Korea	Chung <i>et al.</i> , 2023
OP750468.1	NC16-115	Korea	Chung <i>et al.</i> , 2023

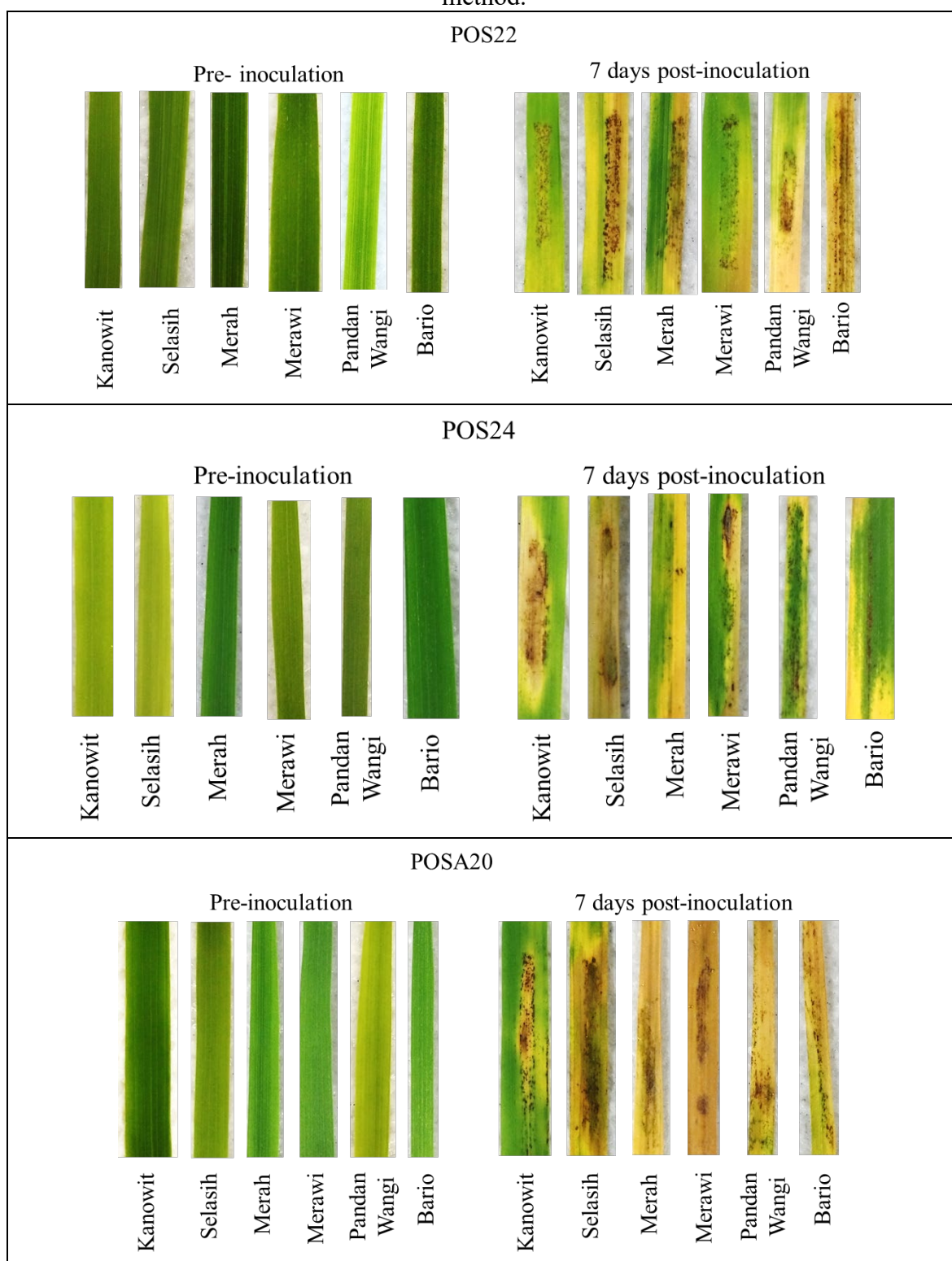
Appendix F: List of 77 *AvrPi-54* sequences of *Pyricularia oryzae* downloaded from National Centre for Biotechnology Information (NCBI) GenBank website.

NCBI Accession Number	Isolate	Country	Sequence Authors
HF545677.2	Mo-nwi-55	India	Ray <i>et al.</i> , 2013
HF545678.1	Mo-nwi-209	India	Ray <i>et al.</i> , 2013
HF545679.1	Mo-nwi-29	India	Ray <i>et al.</i> , 2013
HF545680.1	Mo-nwi-16	India	Ray <i>et al.</i> , 2013
HF545681.1	Mo-nwi-48	India	Ray <i>et al.</i> , 2013
HF545682.1	Mo-nwi-50	India	Ray <i>et al.</i> , 2013
HF545683.1	Mo-nwi-51	India	Ray <i>et al.</i> , 2013
HF545684.1	Mo-nwi-54	India	Ray <i>et al.</i> , 2013
HF545685.1	Mo-nwi-46	India	Ray <i>et al.</i> , 2013
HF545686.1	Mo-nwi-47	India	Ray <i>et al.</i> , 2013
HF545687.1	Mo-nwi-31	India	Ray <i>et al.</i> , 2013
HF545688.1	Mo-nwi-42	India	Ray <i>et al.</i> , 2013
HF545689.1	Mo-nwi-205	India	Ray <i>et al.</i> , 2013
HF545690.1	Mo-nwi-203	India	Ray <i>et al.</i> , 2013
HF545691.1	Mo-nwi-204	India	Ray <i>et al.</i> , 2013
HF545692.1	Mo-nwi-208	India	Ray <i>et al.</i> , 2013
HF545693.1	Mo-nwi-75	India	Ray <i>et al.</i> , 2013
HF545694.1	Mo-nwi-85	India	Ray <i>et al.</i> , 2013
HF545695.1	Mo-nwi-28	India	Ray <i>et al.</i> , 2013
HF545696.1	Mo-nwi-103	India	Ray <i>et al.</i> , 2013
HF545697.1	Mo-ei-26	India	Ray <i>et al.</i> , 2013
HF545698.1	Mo-ei-28	India	Ray <i>et al.</i> , 2013
HF545699.1	Mo-ei-40	India	Ray <i>et al.</i> , 2013
HF545700.1	Mo-ei-48	India	Ray <i>et al.</i> , 2013
HF545701.1	Mo-ei-79	India	Ray <i>et al.</i> , 2013
HF545702.1	Mo-ei-2	India	Ray <i>et al.</i> , 2013
HF545703.1	Mo-ei-3	India	Ray <i>et al.</i> , 2013
HF545704.1	Mo-ei-4	India	Ray <i>et al.</i> , 2013
HF545705.1	Mo-ei-5	India	Ray <i>et al.</i> , 2013
HF545706.1	Mo-ei-6	India	Ray <i>et al.</i> , 2013
HF545707.1	Mo-ei-7	India	Ray <i>et al.</i> , 2013
HF545708.1	Mo-ei-8	India	Ray <i>et al.</i> , 2013
HF545709.1	Mo-ei-10	India	Ray <i>et al.</i> , 2013
HF545710.1	Mo-ei-11	India	Ray <i>et al.</i> , 2013
HF545711.1	Mo-ei-13	India	Ray <i>et al.</i> , 2013
HF545712.1	Mo-ei-14	India	Ray <i>et al.</i> , 2013
HF545713.1	Mo-ei-15	India	Ray <i>et al.</i> , 2013
HF545714.1	Mo-ei-16	India	Ray <i>et al.</i> , 2013
HF545715.1	Mo-ei-20	India	Ray <i>et al.</i> , 2013
HF545716.1	Mo-ei-21	India	Ray <i>et al.</i> , 2013
HF545717.1	Mo-ei-22	India	Ray <i>et al.</i> , 2013

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HF545718.1	Mo-ei-23	India	Ray <i>et al.</i> , 2013
HF545719.1	Mo-ei-25	India	Ray <i>et al.</i> , 2013
HF545720.1	Mo-ni-026	India	Ray <i>et al.</i> , 2013
HF545721.1	Mo-ni-028	India	Ray <i>et al.</i> , 2013
HF545722.1	Mo-ei-12	India	Ray <i>et al.</i> , 2013
HF545723.1	Mo-ni-025	India	Ray <i>et al.</i> , 2013
HF545724.1	Mo-ni-027	India	Ray <i>et al.</i> , 2013
HF545725.1	Mo-ni-001	India	Ray <i>et al.</i> , 2013
HF545726.1	Mo-ni-002	India	Ray <i>et al.</i> , 2013
HF545727.1	Mo-ni-004	India	Ray <i>et al.</i> , 2013
HF545728.1	Mo-ni-007	India	Ray <i>et al.</i> , 2013
HF545729.1	Mo-ni-010	India	Ray <i>et al.</i> , 2013
HF545730.1	Mo-ni-015	India	Ray <i>et al.</i> , 2013
HF545731.1	Mo-ni-016	India	Ray <i>et al.</i> , 2013
HF545732.1	Mo-ni-019	India	Ray <i>et al.</i> , 2013
HF545733.1	Mo-ni-022	India	Ray <i>et al.</i> , 2013
HF545734.1	Mo-ni-024	India	Ray <i>et al.</i> , 2013
HF545735.1	Mo-si-63	India	Ray <i>et al.</i> , 2013
HF545736.1	Mo-si-12	India	Ray <i>et al.</i> , 2013
HF545737.1	Mo-si-23	India	Ray <i>et al.</i> , 2013
HF545738.1	Mo-si-38	India	Ray <i>et al.</i> , 2013
HF545739.1	Mo-si-42	India	Ray <i>et al.</i> , 2013
HF545740.1	Mo-si-55	India	Ray <i>et al.</i> , 2013
HF545741.1	Mo-si-60	India	Ray <i>et al.</i> , 2013
HF545742.1	Mo-si-56	India	Ray <i>et al.</i> , 2013
HF545743.1	Mo-si-62	India	Ray <i>et al.</i> , 2013
HF545744.1	Mo-si-67	India	Ray <i>et al.</i> , 2013
HF545745.1	Mo-si-68	India	Ray <i>et al.</i> , 2013
HF545746.1	Mo-si-72	India	Ray <i>et al.</i> , 2013
HF545747.1	Mo-si-74	India	Ray <i>et al.</i> , 2013
HF545748.1	Mo-ni-008	India	Ray <i>et al.</i> , 2013
HF545749.1	Mo-ei-9	India	Ray <i>et al.</i> , 2013
HF545750.1	Mo-ei-18	India	Ray <i>et al.</i> , 2013
HF545751.1	Mo-ei-19	India	Ray <i>et al.</i> , 2013
HF545752.1	Mo-ei-24	India	Ray <i>et al.</i> , 2013
HF545753.1	Mo-nwi-60	India	Ray <i>et al.</i> , 2013

Appendix G: Appearance of rice blast symptoms on six Sarawak landraces inoculated with five *Pyricularia oryzae* isolates after 7 days post-inoculation using detached leaf method.



Appendix G: continued

