



Faculty of Resource Science and Technology

**Genetic Variation and Aggressiveness of *Phytophthora capsici*, Foot Rot
Pathogen of Pepper (*Piper nigrum* L.) in Sarawak**

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Genetic Variation and Aggressiveness of *Phytophthora capsici*, Foot Rot
Pathogen of Pepper (*Piper nigrum* L.) in 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

Phytophthora capsici is the causal agent of *Phytophthora* foot rot disease in black pepper (*Piper nigrum* L.) and given its devastating impact on black pepper's growth stages, leading to significant yield reductions, an in-depth understanding of its diversity is crucial. Hence, this study aims (i) to isolate *P. capsici* from infected black pepper plants in Sarawak and characterise the isolates based on their morphology; (ii) to examine the aggressiveness of *P. capsici* isolates on common black pepper cultivars planted in Sarawak; (iii) and to investigate the genetic variation of *P. capsici* isolated from infected black pepper plants in Sarawak. Firstly, sampling was conducted in four major black pepper-growing divisions in Sarawak: Sarikei, Betong, Serian, and Kuching. Samples were collected from black pepper vines displaying clear symptoms of the disease. In total, 153 samples were gathered from 28 farms. For isolation, two techniques were utilised: tissue plating and leaf-baiting. Overall, 52 isolates were retrieved across 10 farms originating from Sarikei, Betong and Serian. Through the sequence of Internal Transcribed Spacers region, 30 selected isolates were confirmed as *P. capsici*. The isolates exhibited distinct colony growth patterns, sporangia morphologies, and radial growth rates on different media (Corn Meal Agar, V8 Juice Agar, and Potato Dextrose Agar). All isolates consistently exhibited torulose hyphae structure, and the presence of oogonia when pairing isolates amongst one another revealed the existence of opposite mating types. Black pepper cuttings from Kuching and Semenggok Aman varieties were inoculated with *P. capsici* to assess their aggressiveness. The time taken for the vines to die post-inoculation was recorded and analysed using the Kaplan–Meier estimator to determine disease severity and plot survival curves. Across both sets of aggressiveness tests, IJ1 stood out as the most virulent isolate while EBK1 was the least virulent isolate overall. To assess the population's genetic diversity, 52 isolates retrieved from this study were

genotyped using three Random Amplified Microsatellites (RAMS) and five Inter Simple Sequence Repeats (ISSR) primers. The UPGMA tree based on the RAMS and ISSR combined fingerprint revealed a close relationship between populations, displaying apparent clustering based on geographical locations. The mean value of the percentage of polymorphic loci, Shannon's Information Index and Nei's genetic diversity were 59.52%, 0.294, and 0.193, respectively. Analysis of molecular variance revealed a higher diversity within populations (73%) than among populations (27%), indicating high gene flow between populations and the possibility of shared origin. This study provides the first baseline information on the genetic diversity and aggressiveness of *P. capsici* in Sarawak, highlighting the potential risk for further genetic diversity if left uncontrolled. As black pepper production in Sarawak faces challenges, this study offers insights into the regional black pepper industry.

Keywords: Aggressiveness, Black pepper, genetic diversity, ISSR, morphology, *Phytophthora capsici*, RAMS

Variasi Genetik dan Keagresifan Phytophthora capsici, Patogen Reput Akar Lada (Piper nigrum L.) di Sarawak

ABSTRAK

Phytophthora capsici, agen penyebab penyakit reput akar Fitoptora lada hitam (Piper nigrum L.), perlu dikaji dengan lebih mendalam kerana penyakit ini dikenali dengan kesannya yang sangat memusnahkan pada tanaman tersebut. Penyakit ini menjejaskan lada hitam pada pelbagai peringkat pertumbuhan, justeru menyebabkan pengurangan hasil tanaman yang ketara. Oleh itu, kajian ini bertujuan (i) untuk memencilkan P. capsici daripada pokok lada hitam yang terjangkit di Sarawak dan mencirikan pencilan tersebut berdasarkan morfologinya; (ii) untuk menguji keagresifan pencilan P. capsici terhadap kultivar lada hitam yang biasa ditanam di Sarawak dan; (iii) untuk menyiasat variasi genetik P. capsici yang dipencil daripada pokok lada hitam yang terjangkit di Sarawak. Oleh yang demikian, projek ini berpotensi untuk menyumbang dalam strategi pengurusan penyakit reput akar yang berkesan. Pertama sekali, pensampelan dilakukan di empat bahagian utama penanaman lada di Sarawak: Sarikei, Betong, Serian, dan Kuching. Sampel telah dikumpul daripada pokok lada yang menunjukkan gejala penyakit yang jelas dan sebanyak 153 sampel telah dikumpul dari 28 ladang berbeza. Untuk pemencilan, dua teknik digunakan: penyemaian tisu dan umpan daun. Secara keseluruhan, 52 pencilan telah diperolehi dari 10 ladang yang berbeza. Melalui jujukan Internal Transcribed Spacers 30 pencilan yang dipilih telah disahkan sebagai P. capsici. Pencilan tersebut juga menunjukkan corak pertumbuhan koloni, morfologi sporangia, dan kadar pertumbuhan radial pada media yang berbeza (Agar Bijian Jagung, Agar Jus V8 dan Agar Dekstrosa Kentang). Semua pencilan menunjukkan struktur hifa torulose secara konsisten, dan terdapat oogonia terbentuk semasa pemasangan pencilan antara satu sama lain. Ini menunjukkan kewujudan jenis

pengawanan yang berlawanan. Keratan batang lada daripada kultivar Kuching dan Semenggok Aman diinokulasi dengan P. capsici untuk menilai keagresifannya. Masa yang diambil untuk pokok mati selepas inokulasi direkodkan dan dianalisa menggunakan penganggar Kaplan-Meier untuk menentukan keterukan penyakit dan melukis lengkungan kelangsungan hidup. Dalam kedua-dua set eksperimen agresif, IJ1 menonjol sebagai pencilan yang paling agresif manakala EBK1 adalah pencilan yang paling kurang agresif secara keseluruhan. Untuk menilai kepelbagaian genetik populasi, 52 pencilan yang diperolehi daripada kajian ini digenotip dengan menggunakan tiga Random Amplified Microsatellites (RAMS) dan lima Simple Sequence Repeats (ISSR). Pokok UPGMA berdasarkan cap jari gabungan RAMS dan ISSR menunjukkan hubungan yang rapat antara populasi, menunjukkan pengelompokan yang jelas berdasarkan lokasi geografi. Nilai purata peratus lokasi polimorfik, Indeks Maklumat Shannon dan kepelbagaian genetik Nei adalah 59.52%, 0.294, dan 0.193, masing-masing. Analisis varians molekul menunjukkan kepelbagaian yang lebih tinggi dalam populasi (73%) daripada antara populasi (27%). Ini menunjukkan aliran gen yang tinggi antara populasi dan berkemungkinan berkongsi asal usul yang sama. Kajian ini menyediakan maklumat asas pertama mengenai kepelbagaian genetik dan keagresifan P. capsici di Sarawak. Ia menunjukkan risiko kepelbagaian genetik yang lebih lanjut jika dibiarkan tanpa kawalan. Memandangkan pengeluaran lada di Sarawak menghadapi cabaran, kajian ini menawarkan pemahaman tentang industri lada serantau pada masa hadapan.

Kata kunci: Keagresifan, Lada hitam, variasi genetik, ISSR, morfologi, Phytophthora capsici, RAMS,

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CaCO ₃	Calcium carbonate
CGS	Centre for Graduate Studies
CMA	Corn Meal Agar
CTAB	Cetyltrimethylammonium bromide
COX	Cytochrome C Oxydase
DNA	Deoxyribonucleic Acid
DnaSP	DNA Sequence Polymorphism
GDNA	Genomic Deoxyribonucleic Acid
GPS	Global Positioning System
ISSR	Inter Simple Sequence Repeats
ITS	Internal Transcribed Spacers
KME	Kaplan–Meier Estimator
MEGA	Molecular Evolutionary Genetics Analysis

NCBI	National Center for Biotechnology Information
<i>P. capsici</i>	<i>Phytophthora capsici</i>
PCR	Polymerase Chain Reaction
<i>P. nigrum</i>	<i>Piper nigrum</i>
PDA	Potato Dextrose Agar
RAMS	Random Amplified Microsatellites
RAPD	Random Amplified Polymorphic DNA
RRNA	Ribosomal Ribonucleic acid
SDW	Sterile Distilled Water
SEM	Standard Error of Mean
SPSS	Statistical Package for the Social Sciences
SSR	Simple Sequence Repeats
UNIMAS	Universiti Malaysia Sarawak
UPMGA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet
V8A	V8 Juice Agar

CHAPTER 1

INTRODUCTION

1.1 Study Background

Piper nigrum L., commonly known as pepper and a member of the Piperaceae family, is a perennial climbing vine that flourishes in warm, humid climates. Pepper is highly cultivated for its berries as it is an important spice that is integral to cuisines around the world (Yogesh & Mokshapathy 2013). Black pepper is thought to have its origin in the tropical regions of Kerala, India, where over 51 different cultivars of *P. nigrum* have been identified (Newerli-Guz & Śmiechowska, 2022; Mathew et al., 2001). In Sarawak, Malaysia, ten *P. nigrum* cultivars have been described (Chen & Tawan, 2020).

The black pepper industry in Sarawak has faced a significant decline since the 1980s, with challenges such as worker shortages, climate effects, and persistent pests and diseases contributing to a drop from 28,667 tonnes in 1992 to 17,233 in 2017 (Sabu et al., 2020; Mazlan et al., 2020). Malaysia, particularly Sarawak, used to be the fourth-largest pepper producer globally, but it has now slipped to fifth (Malaysian Pepper Board, 2020). The production of pepper in Sarawak has been declining since the 1980s, primarily due to the detrimental effects of pests and diseases along with other factors such as shortage of manpower, climate, and a lack of governmental assistance (Paulus, 2007; Mazlan et al., 2020). *Phytophthora* foot rot, known as 'quick wilt,' is one of the primary diseases affecting pepper cultivation, causing significant damage at all growth stages (Kueh, 1990; Bhai et al., 2022).

Phytophthora capsici has been identified as the causal agent of foot rot disease in black pepper vines (Farhana et al., 2013). The disease can result in seedling death, root rot,

crown rot, stem blight, leaf spot, and fruit rot (Babadoost et al., 2015). Severe cases can lead to the death of infected vines within three weeks, with neighbouring vines becoming infected within a few months (Nguyen, 2015). In the mid-1950s, an epidemic of *Phytophthora* foot rot caused nearly 100% yield loss of black pepper in Sarawak (Holliday & Mowat, 1963).

Although no outbreaks have been reported since then, several disease incidences were reported in Sarawak. Between the years 2010 and 2011, Farhana et al. (2013) reported a disease incidence ranging from 5% (Repok, Sarikei) to 75% (Ulu Sarikei, Sarikei), and disease severity ranged from 4% (Repok, Sarikei and Tatau, Bintulu) to 70% (Ulu Sarikei, Sarikei). Kong et al. (2022) reported that *P. capsici* infected two farms in Julau, where the disease incidence was more severe in one farm, destroying 90% of the vines within three months in 2019. As smallholder farmers are Sarawak's main pepper cultivators (Entebang et al., 2020), the disease can cause severe economic hardship.

Hausbeck and Lamour (2004) reported that *P. capsici* could infect up to 16 crops from three families: Cucurbitaceae, Solanaceae and Leguminosae. Since *P. capsici* is a cosmopolitan pathogen with a broad host range, it makes control via crop rotation difficult even with non-host crops, as it might survive on farm weeds (Tian & Babadoost, 2004). *Phytophthora capsici* is also challenging to eradicate due to its ability to form survival structures in the soil. For example, oospores could remain dormant and survive in the soil for several years until they encounter a suitable host (Babadoost & Pavon, 2013). *Phytophthora capsici* can also quickly disperse as it can reproduce asexually through sporangia formation, which can release motile zoospores when in contact with water, allowing the pathogen to disperse via the water column present in soil (Granke et al., 2009).

Latifah et al. (2018) stated that the pathogen's virulence stems from its distinct genetic composition, enabling *Phytophthora* spp. to evolve rapidly. These pathogens

continually acquire new genetic variations through exposure to diverse environmental conditions (Velásquez et al., 2018). Kong et al. (2022) reported that *P. capsici* isolates from Julau, Sarawak possess both A1 and A2 mating types, evident from the presence of oogonia and antheridia during the isolates pairing. The coexistence of these mating types within the same field might facilitate sexual reproduction, further enhancing the pathogen's genetic diversity due to recombination (Li et al., 2012).

Hence, understanding the genetic variation of *P. capsici* is crucial for morphological identification, determining pathogenicity and cross-pathogenicity, and developing effective management strategies against the pathogen (Latifah et al., 2018). Genetic variation studies can be conducted using various techniques such as Random Amplified Polymorphic DNA (RAPD) (Chee & Jee, 2001), Random Amplified Microsatellites (RAMS) (Chee & Jee, 2001; Rai et al., 2020; Truong et al., 2010), Inter Simple Sequence Repeats (ISSR) (Babadoost, 2004; Li & Liu, 2021; Mohammadbagheri et al., 2021), Amplified Fragment Length Polymorphism (AFLP) (Bowers et al., 2007; Hurtado-González et al., 2008; Lamour & Hausbeck, 2001) and Simple Sequence Repeat (SSR) (Chen et al., 2018; Cissin et al., 2015; Meitz et al., 2010).

1.2 Problem Statement

The production of black pepper in Sarawak, Malaysia, has faced significant challenges due to *Phytophthora* foot rot caused by *P. capsici* (Farhana et al., 2013). This destructive disease affects all stages of black pepper plant growth, leading to substantial yield losses and economic hardships for smallholder farmers (Babadoost et al., 2015; Nguyen, 2015). The pathogen's ability to form survival structures in the soil and its pronounced genetic variation creates difficulties in eradicating the disease and developing effective management strategies (Koné et al., 2009; Kumar et al., 2012).

In the context of Malaysia, Latifah et al. (2018) shed light on *Phytophthora* spp. in other crops in Malaysia, indicating extensive genetic diversity and varying levels of virulence. Although Latifah et al. (2018) emphasised the importance of studying the genetic diversity of *Phytophthora* spp. in Malaysia, such analysis has not been extended to *P. capsici* affecting black pepper. Despite the vulnerability faced by Sarawak's black pepper industry, the absence of a focused study on *P. capsici*'s genetic diversity is a significant barrier to the development of effective disease management strategies. This study, therefore, seeks to bridge this knowledge gap, aiming to enhance the understanding of the impact of *P. capsici* on black pepper cultivation in Sarawak.

1.3 Objectives

Hence, the objectives of this project were as follows:

- i. To isolate *P. capsici* from infected black pepper plants in Sarawak and characterise the isolates based on their morphology.
- ii. To examine the aggressiveness of *P. capsici* isolates on common black pepper cultivars planted in Sarawak.
- iii. To investigate the genetic variation of *P. capsici* isolated from infected black pepper plants in Sarawak through molecular analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Black Pepper

The word pepper is linked to Sanskrit, Latin and Old English origins, "*pippali*", "*piper*," and "*pipor*," respectively (Zachariah & Parthasarathy, 2008). Black pepper (*Piper nigrum* L.) is a pungent spice known worldwide as the king of spice. This perennial flowering vine is from the Piperaceae family (Damanhoury & Ahmad, 2014), and it is highly cultivated for its berries as upon dried, it serves as a flavourful spice that compliments many cuisines. Black pepper plants are bisexual, meaning each flower contains a stigma and anthers and are often regarded as self-pollinating species. However, since the anthers are located below the stigma, self-pollination within the same flower without the aid of a pollinator is not possible and hence the geitonogamy mechanism has been adopted by the plant where the pollens are carried downwards, often with water following the gravitational pull to pollinate the stigma of a flower located below within the same plant (Pooja et al., 2022).

The drupe berries grow on a spike (Figure 2.1a) in which they are first observed to be yellowish-green, then their colour slowly evolves to dark green, yellow, and finally red when it has ripened. The leaves are simple, with an obtuse tip and a large base with an even margin. Black peppers exhibit a unique root system, with 10 to 20 primary roots emerging from the base of the mature stem that can penetrate deep into the soil, accompanied by a broad cortex and stele characteristic of dicot roots (Parthasarathy et al., 2007).

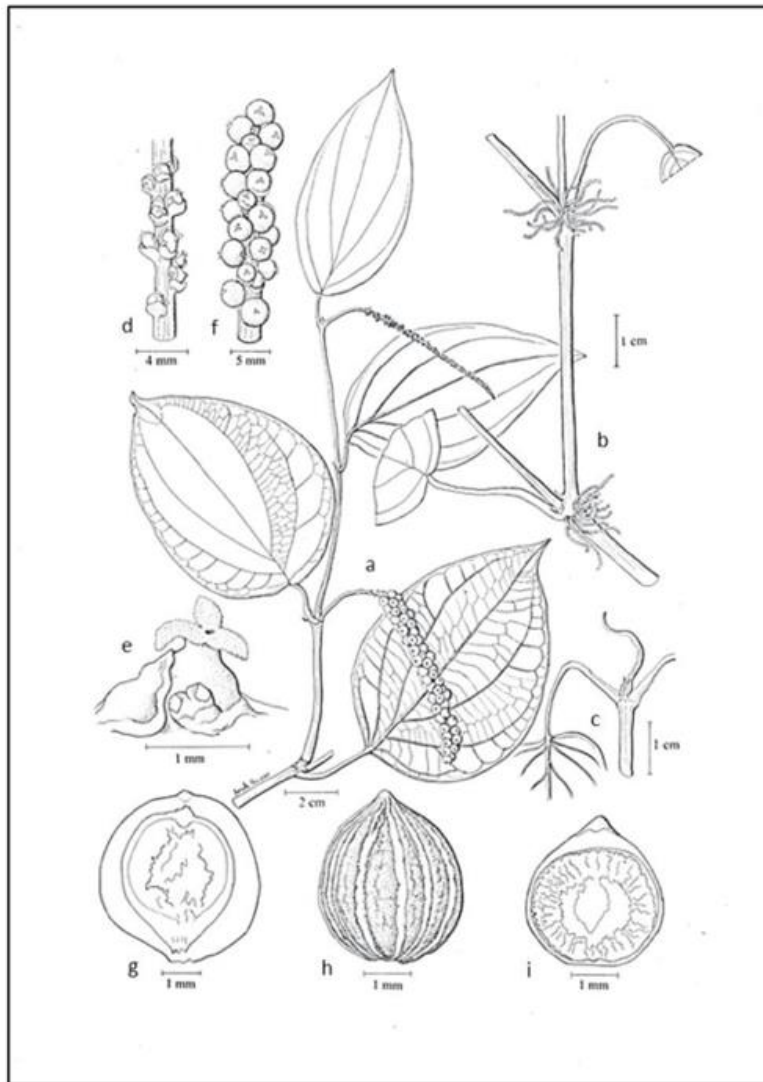


Figure 2.1: Illustration of the black pepper (*Piper nigrum* L.) plant, showcasing the various stages of its drupe berries, from early growth to ripeness, and the distinct features of its leaves and flowers. (a) Lateral branch; (b) Terminal stem node; (c) Shoot tip; (d) Inflorescence/early stage of fruit development; (e) Single flower; (f) Mature stage of fruit spike; (g) Cross section of fresh berry; (h) Outer surface of peppercorn (pericarp removed); (i) Cross section of pepper seed (Chen and Tawan, 2020)

2.1.1 Significance of Black Pepper

The primary use of black pepper remains in the food industry as it is used widely worldwide (Ravindran, 2000). The alkaloid piperine is the primary bioactive compound in

black pepper, giving it its distinct pungent taste and smell (Gorgani et al., 2016). This compound has been reported to show therapeutic properties that can be used as an analgesic for influenza, migraines, and throat infections (Ahmad et al., 2012). The piperine compound present in black pepper also had been related to having health benefits against certain chronic diseases (Derosa et al., 2016). The piperine compound was also linked to better nutrient absorption, and it aids in brain functions (Ashokkumar et al., 2021).

Traditionally, black pepper has been used for many health-related issues. Black pepper has been extensively used in Ayurvedic (traditional medicine from India) medication. It was found in the ancient Indian scripts to be used in many prescriptions for treating numerous ailments between the 6th century A.D. (Atal et al., 1981). Meanwhile, Chinese traditional medicine has employed black pepper to treat epilepsy (Singletary, 2010). Chinese medicine also uses black pepper for various diseases, such as lung-related ailments, infections, and boosting blood circulation (Stojanović-Radić et al., 2019).

Besides that, black pepper has been reported to be used in veterinary medicine in India, where gastrointestinal issues in livestock were treated using pepper (Takooree et al., 2019). Due to pepper's various uses, black pepper holds a significant position in the global spice market, driven by its high demand and widespread consumption.

2.1.2 Introduction of Black Pepper to Malaysia and its Spread

Black pepper has been an export product in Southeast Asia since the 12th century, as Arabic and Chinese traders have been sourcing black pepper from Southeast Asian ports such as Kedah, Pahang, and Java (Bulbeck & Al, 1998). In 1511, Alfonso de Albuquerque arrived in the straits of Malacca, introducing a new region (Southeast Asia) to the west to source black pepper from (Wilkinson, 1912). Chinese settlers later brought black pepper to

East Malaysia (Sarawak) in 1840, and the crop was reported to be extensively cultivated (Izzah & Asrina, 2019). The Borneo Company under the Brooke administration in Sarawak during the 1900s had highly traded black pepper as it was the third highest export of the country after sago and gambier (Kaur, 1995).

Black pepper was deemed a cash crop in West Sarawak, and it was mainly cultivated by Chinese miners who turned to black pepper cultivation after being displaced by the Borneo Company (Kaur, 1995). Throughout the 20th century, sharp price rises led to intensive black pepper planting, notably in 1959 and 1977 (Wadley, 2005). From 1972 in Sarawak, government subsidies and the introduction of commercial fertilisers bolstered the industry, responding to the challenges of the black pepper's three-year maturation and fluctuating prices (Wadley, 2005). By the late 1970s, Sarawak's black pepper industry grew significantly, emphasising its role as an export-oriented crop (Entebang et al., 2020).

2.2 Pepper Cultivation in Sarawak

2.2.1 Black Pepper Cultivation Practices in Sarawak

Cultivating black pepper in Sarawak necessitates regular attention. A significant majority, 90% of Sarawakian smallholder farmers surveyed across 18 farms by Johny et al. (2020), allocate up to two days each month to regular maintenance, encompassing activities such as pruning, weeding, and fertilising.

Selecting the appropriate land and topography is of utmost importance for successful pepper cultivation in Sarawak. The soil should ideally have a pH of 5.0 – 6.0, and almost all soil types, except clay, are suitable. Furthermore, an elevated topography with proper drainage is recommended to prevent waterlogging and other related issues, as stated by Gawang and Lau (2007). Peppers, being climber vines, require sturdy supports. Two primary

types of support are prevalent: living and non-living. The most used non-living support pole is 'Belian' (*Eusideroxylon zwageri*) (Figure 2.2) (Yuen et al., 2018).

The typical propagation technique for black pepper in Sarawak is predominantly via stem cuttings. According to the black pepper planting guide provided by the Malaysian Pepper Board (Gawang & Lau, 2007), cuttings from a healthy disease-free vine below three years old are recommended. Nursery cuttings are sown in a sand bin (Figure 2.3) to promote the production of rootstocks. King et al. (2021) reported an alternative method of producing black pepper rootstock via a deep-water hydroponic system. The hydroponic system is recommended by King et al. (2021) to reduce the susceptibility of the stem and developing roots to soil-borne pathogens, ensuring safer and healthier plant growth. However, the performance of hydroponic cuttings and a comparative study between hydroponic systems and traditional techniques have yet to be reported.

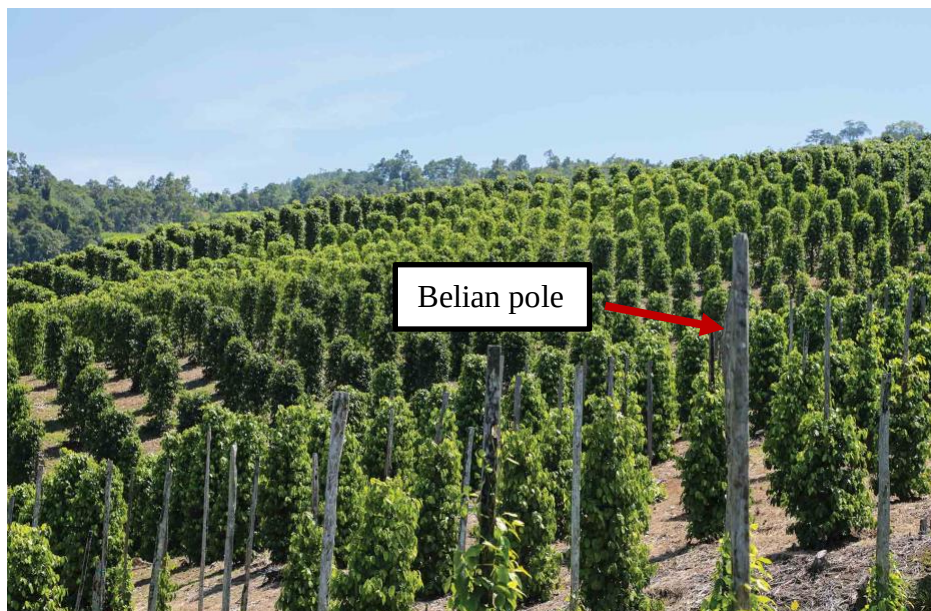


Figure 2.2: Black pepper farms in sarawak showcasing the use of 'Belian' (*Eusideroxylon Zwageri*) poles as non-living support for the climbing vines (Borneo Talk, 2020)



Figure 2.3: Sand bin for sowing black pepper cuttings as recommended by the Malaysian Pepper Board (Gawang & Lau, 2007)

2.2.2 Major Black Pepper Cultivars and Production Division in Sarawak

According to Entebang et al. (2020), in 2017, black pepper was mainly cultivated in three divisions of Sarawak: Sarikei, Betong and Serian, which produced about 61% of black pepper berries. In Sarawak, Kuching and Semongok Emas cultivars are the most abundantly planted, making up 50% and 20% of all cultivated black pepper, respectively (Chen & Tawan, 2020). Other varieties, such as Semongok Aman, India, Nyerigai, Semongok Perak, Yong Petai and Semongok Satu, are also present in Sarawak (Chen & Tawan, 2020; Johny et al., 2020). As of 2007, the Malaysian Pepper Board recommended the varieties Kuching, Semenggok Emas, and Semenggok Aman for planting in Malaysia (Gawang & Lau, 2007). There have been no updates on newer recommended varieties since then.

2.2.3 Trends in Black Pepper Production and Trade in Malaysia

Sarawak contributes about 98 % of Malaysia's black pepper production (Ministry of Plantation and Commodities [MPC], 2023). Over the past four decades, the dynamics of pepper production in Malaysia, have witnessed considerable fluctuations. In 1980, the production of black pepper stood at 31,460 tonnes, but a decline was evident by the end of 1992, with production dropping to 28,667 tonnes (Sabu et al., 2020). This downward trend persisted through the 2000s, with Sarawak recording a production of 16,708 tonnes in 2006 (Amin, 2008). However, a recovery was seen from 2018 onwards. Sarawak's black pepper production in 2018 was 22,056 tonnes, and it slightly increased to 22,477 tonnes in 2022 (MPC 2023). Although a recovery trend is observed, the overall trend in black pepper production in Malaysia is negative (Figure 2.4).

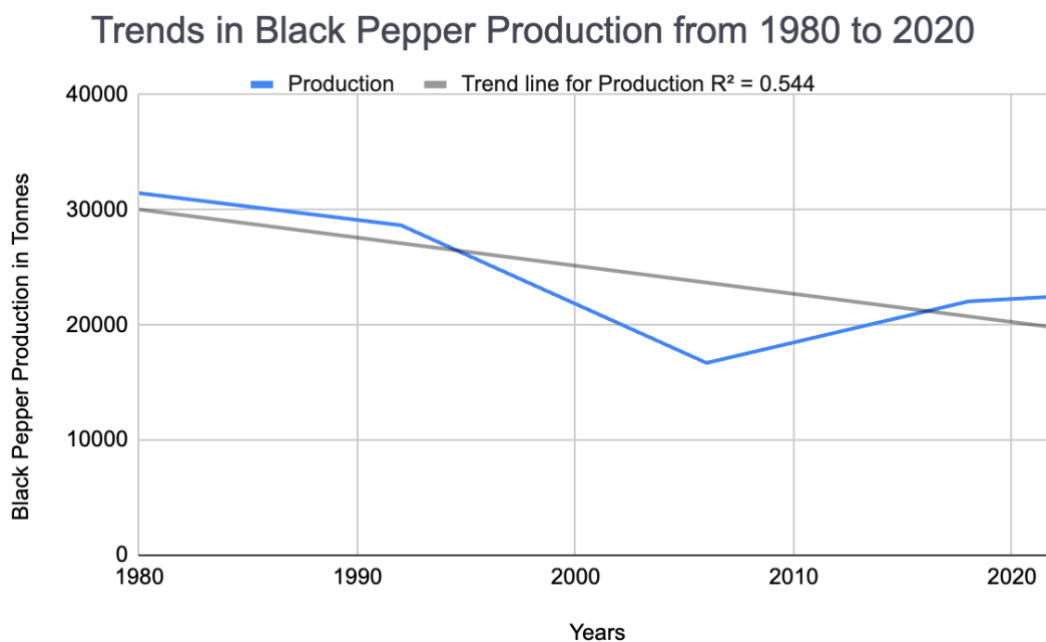


Figure 2.4: Black pepper production in Sarawak (1980 to 2022). Data sourced from Sabu et al. (2020), Amin (2008), and Ministry of Plantation and Commodities (2023).

In terms of economic valuation, in 2006, the export volume from Sarawak earned RM 136 million, and despite a reduced tonnage, the value increased to RM 177 million in 2007 (Amin, 2008). By 2010, the black pepper yield was valued at MYR 190 million (Kamarulzaman et al., 2013). Import values also provided an interesting perspective: RM 47.57 million in 2018, and reaching RM 67.18 million in 2022 (MPC, 2023). Despite fluctuations in production tonnage, the economic value of black pepper exports from Sarawak has consistently increased over the years, with even import values showing a significant rise between 2018 and 2022.

Land utilisation patterns for black pepper cultivation reflected the production trends as between 2000 and 2010, Malaysia, on average, cultivated black pepper on 13,255 hectares of land (Kamarulzaman et al., 2013) but a significant reduction was noted a decade later. In 2020, the area allocated for black pepper cultivation shrunk to 8,022 hectares, marking a stark decrease of 39% since 2010 (Izmir, 2020; Malaysian Pepper Board, 2020). Overall, the land allocated for black pepper cultivation in Malaysia saw a significant over the years, mirroring the production trends.

Despite the overall declining trends observed, black pepper continues to be an essential agricultural product for Sarawak's farmers. Around 67,000 smallholder farmers are dependent on it as their primary cash crop (Kamarulzaman et al., 2013), encompassing approximately 33,306 families who mostly operate family-owned farms, which limits the hiring of foreign workers (Entebang et al., 2020).

2.2.4 Challenges of the Black Pepper Industry in Sarawak

The decline in production can be traced back to several challenges confronting pepper growers in Sarawak. As identified by Mazlan et al. (2020), these challenges encompass worker shortages, climatic effects, inconsistent profitability, lack of government

support, and the persistent threat of pests and crop diseases. A survey of Sarawak's black pepper smallholders by Johny et al. (2020) found that 29% of farmers pinpointed disease as the principal issue, followed by inconsistent pricing (28%), unsynchronized berry development (21%), fertilization problems (12%), and harvesting challenges (10%). The foot rot disease caused by *Phytophthora capsici* has been especially damaging (Adam et al., 2018; Shashidhara, 2009), posing a threat to production in Malaysia and other significant producers like Vietnam, Indonesia, and India. India, for example, incurs an annual loss of over 1,000 tonnes of black pepper due to this disease (Kumar et al., 2012).

2.3 *Phytophthora* Foot Rot Disease in Black Pepper Plants

The *Phytophthora* foot rot disease is most prevalent during wet seasons in Sarawak, as the oomycete thrives in humid and damp environments. High rainfall, high humidity, lower sun exposure, and high soil moisture are all favourable conditions for the pathogen, *P. capsici* (Truong et al., 2008). The pathogen favours a warm and wet climate and is often more prevalent in poorly drained soils. Johny et al. (2020) reported that 29% of the farmers out of 18 farms in Sarawak surveyed had reported crop loss due to foot rot disease and it was stated that foot rot disease caused by *P. capsici* was a significant issue faced by the black pepper farmers of Sarawak.

2.3.1 Symptoms of *Phytophthora* Foot Rot Disease in Black Pepper Plants

The *Phytophthora* foot rot is also known as black pepper's 'quick wilt' (Truong et al., 2008). The root collar of the infected vines showed symptoms of a dark brown lesion with water-soaked margins (Abebe & Adugna, 2018). The foot rot disease caused by *P. capsici* is considered quick rot because the oomycete penetrates through the roots of the black pepper vines and disrupts the vascular tissues in the roots, thus preventing the transport of water and nutrients to the leaves (Nguyen, 2015). Hence, the infected vines express quick

wilting and dropping of leaves with rotten roots (Truong et al., 2008). Wilting is the first observable above-ground symptom of infected vines. The leaves turn yellow and wilt before completely defoliating (Alconero, 1972; Drenth & Guest, 2004). In a recent study conducted in Julau, Sarawak, the black pepper plants exhibited a characteristic progression of symptoms—from initial yellowing and wilting of leaves to extensive leaf necrosis and finally complete defoliation (Kong et al., 2022) (Figure 2.4).

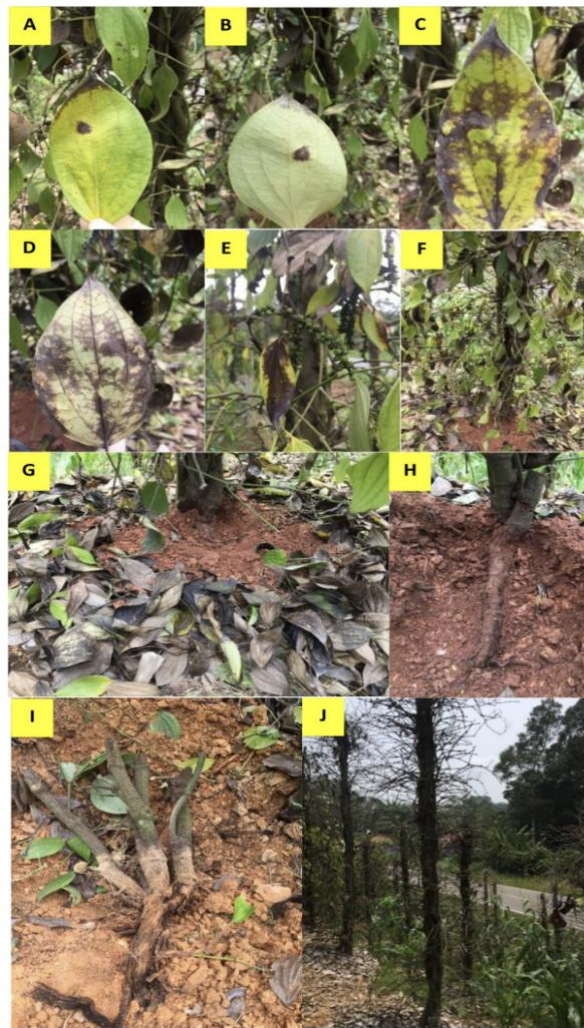


Figure 2.5: Symptoms of *Phytophthora* foot rot disease in black pepper plants observed in Julau, Sarawak (Kong et al., 2022)

2.3.2 Historical Occurrence and Major Outbreaks

Phytophthora capsici has been reported to cause nearly 100% yield loss of black pepper in the mid-1950s in Sarawak (Holliday & Mowat, 1963). Aside from that significant outbreak, no other drastic outbreaks have been reported in Sarawak thus far. However, the prevalence of *Phytophthora* foot rot disease remains in Sarawak. For instance, it was reported by the Malaysian Pepper Board that around 365,000 black pepper plants in Sarawak experienced diseases caused by *Phytophthora* spp. and *Fusarium* spp., between November 2017 and May 2018 (Awit, 2018). This period coincided with persistent heavy rainfall in Sarawak.

2.3.3 Disease Management

Protection of black pepper vines from *P. capsici* based on the emergence of above-ground symptoms is generally inadequate because when the above-ground signs are visible, the collar of the roots would have been severely rotted, making treatment of the disease challenging (Kumar et al., 2012). In many cases, the vines were not curable. A single fungicide had neither been consistent nor effective in controlling *P. capsici* (Kumar et al., 2012).

To manage diseases, the Malaysian Pepper Board recommends phyto-sanitation practices including burning infected plants and treating areas with copper oxychloride (Kannan, 2007). For chemical control, they advise using copper oxychloride, Bordeaux mixture, potassium phosphonate, or metalaxy-mancozeb, especially during monsoon periods. Additionally, biological controls like *Trichoderma* spp. and *Pseudomonas fluorescens* can be applied around trees during monsoonal phases (Kannan, 2007).

Fungicides with mefenoxam as the active ingredient have shown some efficacy against *P. capsici*. However, an increasing concern is the rise of mefenoxam-resistant strains, with studies indicating resistance in up to 58% of strains (Koné et al., 2009). Considering this, alternative strategies have been explored. A study by Bhai et al. (2022) demonstrated that using bacterial antagonists combined with chemicals/bioagents could effectively prevent *Phytophthora* foot rot in black pepper from 2008 to 2013. The combination of *Curtobacterium luteum* and Metalaxyl was mainly effective against *P. capsici*, even though nematode infections increased. Another emerging strategy involves grafting black pepper vines onto resistant rootstocks, which has shown potential to defend against *P. capsici* (An et al., 2019). However, this grafting technique is still in the preliminary research stages.

2.3.4 Climate Change and Disease Dynamics

Climate change alters pathogen evolution, host-pathogen interactions, and pathogen biogeography, increasing the risks of plant disease outbreaks and threatening global food security and ecosystem sustainability (Singh et al., 2023). The rise in global temperatures and increased precipitation associated with climate change can contribute to favourable conditions for *P. capsici* epidemics (Camilo & Quesada-Ocampo, 2022). Sa'adi et al. (2023) reported observing a significant increase in extreme rainfall and temperature events in Sarawak's peatlands due to climate change in the affected peat regions. High rainfall and high soil moisture are highly favourable for *P. capsici*, given that the surface water movements in the field play a crucial role in the pathogen's dispersal mechanism, leading to an increased disease incidence (Sanogo & Ji, 2013).

Studies investigating the survival of *P. capsici* oospores under different temperature regimes have revealed valuable information for understanding the pathogen's response to

climate change. Etxeberria et al. (2011) reported that the oospores of *P. capsici* displayed different survival rates based on the duration of exposure to temperatures ranging from 40 to 53 °C in moistened soil. Additionally, when these oospores were suspended in water, they exhibited potential survival for over a week, even when consistently exposed to a temperature of 40 °C. The oospore's survival capability starts declining for temperatures above 40 °C. These findings indicate that while the overall effects of climate change, such as a rise in temperature and increased precipitation, may provide a more conducive environment for *P. capsici* to thrive, extremely high temperatures could still negatively impact the pathogen's survival.

2.4 Causal agent - *Phytophthora capsici*

The genus *Phytophthora* comprises over 100 species (Kroon et al., 2012), which are known destructive plant pathogens. According to the *Phytophthora* keys developed by Waterhouse (1963), this genus can be divided into six groups based on morphological features such as hyphae and reproductive structures. *Phytophthora capsici* is one of the species under the genus *Phytophthora*. This oomycete plant pathogen infects black peppers, capsicums, and many other commercial crops. For black pepper plants, this species is known to be the causal agent of *Phytophthora* foot rot (Farhana et al., 2013).

2.4.1 Taxonomy, Characteristics and Life Cycle of *Phytophthora capsici*

According to Farhana et al. (2013), *P. capsici* isolated from black pepper farms in Sarawak had shown typical torulose hyphae, lemon-shaped sporangia with a long pedicel and globose oogonia along with perigynous antheridia. However, identifying *P. capsici* based on morphological features has its shortcomings, as the morphological characteristics of these oomycetes are highly influenced by environmental conditions (Drenth & Sendall,

2001). The molecular analysis could be a supplementary tool for more precise identification of *P. capsici* (Li et al., 2007).

Phytophthora capsici, a hemi-biotrophic oomycete, initiates its infection cycle by drawing nutrients from live host cells in a biotrophic phase, while later switching to a necrotrophic phase, consuming the host cells for nutrition (Maillot et al., 2022). This metabolic transition serves as a strategic adaptation for the pathogen to maximize its survival and reproductive success.

Phytophthora capsici reproduces both sexually and asexually (Ristaino & Johnston, 1999) (Figure 2.3). For sexual reproduction, *P. capsici* requires two mating types, A1 and A2, to be present together (Li et al., 2017), and it was reported that type A1 is more pathogenic than type A2 (Truong et al., 2010). Through this sexual reproduction, a thick-walled oospore will be produced (Li et al., 2017).

Smith et al. (2019) found evidence of oogonia when pairing specific isolates, indicating that these isolates possessed the opposite mating type. The occurrence of sexual reproduction in the pathogen contributes to its persistence, as the sexual structure (oogonia) can survive for extended periods, up to 36 months, in the absence of a suitable host (Gobena et al., 2012). Hence, the oospores allow the pathogen to survive between crop cycles (Babadoost & Pavon, 2013). Asexually, upon infecting the host, *P. capsici* can produce sporangium that will release biflagellate-motile zoospores when in contact with water (Gobena et al., 2012; Granke et al., 2009). These zoospores can spread quickly through water particles in the soil, allowing this pathogen to cause epidemics within days (Gobena et al., 2012).

In terms of the growth rate of *P. capsici*, varying data had been previously reported on different growth media. Kong et al. (2022) reported that some isolates from Julau, Sarikei, and Sarawak had higher growth rates on Corn Meal Agar (CMA) and 20% clarified V-8 Juice Agar (V8A) compared to Potato Dextrose Agar (PDA), while others had a higher growth rate on V8A followed by CMA and PDA. Smith et al. (2019) also reported similar growth rate trends where most isolates grew fastest on V8A, CMA and followed by PDA.

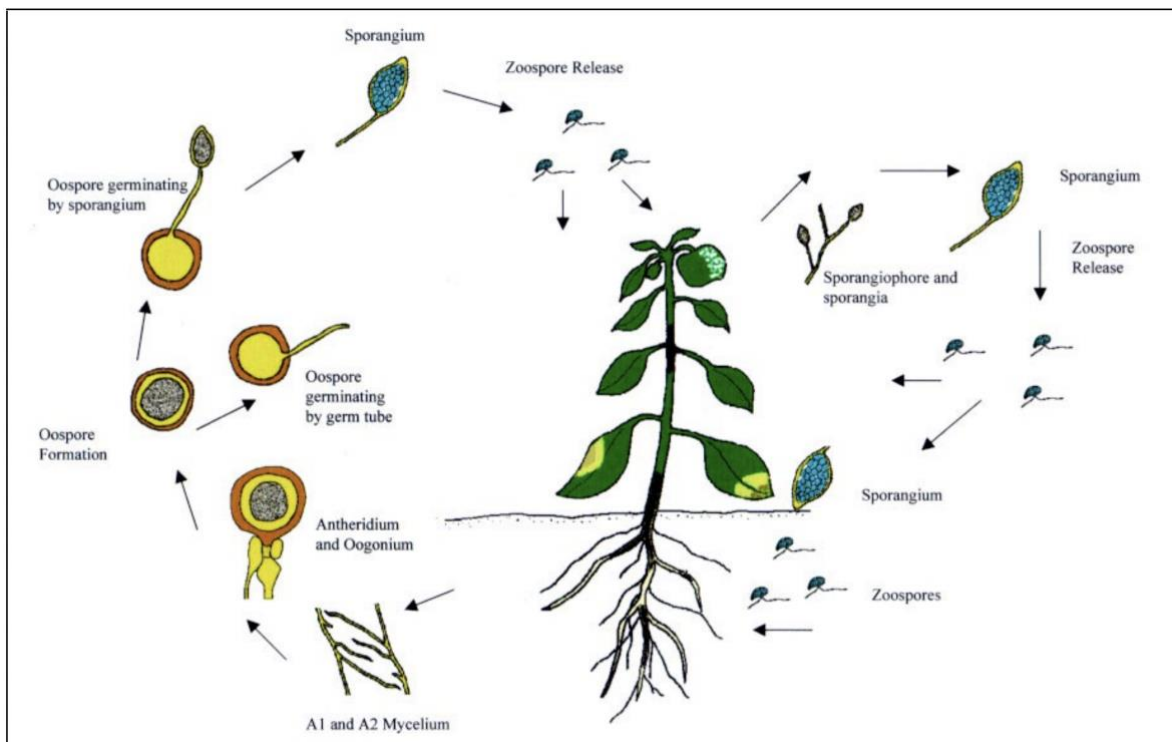


Figure 2.6: Life cycle of *Phytophthora capsici* (Ristaino & Johnston, 1999)

2.4.2 Mode of Infection

The mode of infection of *P. capsici* involves multiple mechanisms that contribute to the devastating impact on black pepper plants. The primary source of inoculum is oospores-contaminated soil, where the oospores can readily germinate and infect plant tissues (Nguyen, 2015). The pathogen primarily enters the plant through collar and root penetration

(Vandana et al., 2019), infecting the root system and spreading to the lower stem and crown regions. Upon infiltration, the pathogen induces necrosis to the root and collar tissues, inhibiting the vascular tissues from transporting water and minerals to the plant, resulting in plant death (Silvar et al., 2005).

While *P. capsici* predominantly targets black pepper through root invasion, findings from Vandana et al. (2019) highlight the plant's inherent biochemical defences, including factors such as membrane conductance, OD phenol, lignin, and peroxidase activity, which fortifies root resistance against this pathogen. However, *P. capsici* has evolved elaborate mechanisms to overcome the multi-layered defences plants possess, such as motility, attachment, and host cell wall degradation to derive nutrients from their hosts. They release an array of effectors that can suppress the immune response of the host plants, further promoting the disease (Chepsergon et al., 2020). Additionally, the presence of root-knot nematodes, specifically *Radopholus similis* and *Meloidogyne incognita*, can complicate this mode of infection as the combined interactions between nematodes and the fungus can intensify the plant's vulnerability, potentially amplifying its susceptibility to the disease (Ravindra et al., 2014).

2.5 Pathogenicity and Aggressiveness of *Phytophthora capsici*

In plant pathology, understanding the impact of pathogens on host plants was significantly shaped by the framework put forth by Vanderplank in 1968 (Sacristán & García-Arenal, 2008). Pathogenicity is defined as the ability of a plant pathogen to cause disease in a host plant, while aggressiveness, is defined as the quantitative negative effect of a pathogen on its host (Andrivon et al., 2010; Michalska et al., 2015; Widmer, 2015). Virulence is defined as the capacity of a pathogen to infect a plant with one or more major genes for resistance (Sacristán & García-Arenal, 2008).). In contrast, virulence is the

capacity of the pathogen to infect its host, following the gene-for-gene model which involves a specific interaction between pathogen avirulence genes and plant resistance genes (Fontyn et al., 2023).

In other words, the concept of aggressiveness refers to the variable impact a pathogen can have on its host, influenced by both the interaction with the host and environmental factors. Virulence, however, is tied to the pathogen's genetic makeup, reflecting its intrinsic ability to infect and overcome the host's genetic defences, regardless of external conditions.

Two types of plant materials have been reported for testing the pathogenicity and virulence of *P. capsici*, namely detached leaves from disease-free vines (Andrison et al., 2010; Michalska et al., 2015; Widmer, 2015) and young black pepper vines (Truong et al., 2008). Truong et al. (2008) tested the pathogenicity of *P. capsici* isolates by inoculating on healthy 3-month-old vines. Through Koch's postulate, the pathogen had been proven to be disease-causing. Lee et al. (2001) explored the aggressiveness of *P. capsici* isolates from pumpkin and pepper hosts on young pumpkin vines, finding that pumpkin isolates were notably more aggressive. This suggests potential host-specific virulence factors.

Muthuswamy et al. (2018) tested using detached black pepper leaves and reported differential expression and temporal regulation of effector proteins during the infection process. They have also reported that the more virulent isolate exhibited a biotrophic stage at 8 hours post-inoculation, transitioning to a necrotrophic stage within 24 hours, whilst the less virulent isolate displayed a similar pattern but reached the necrotrophic stage at 48 hours.

In Sarawak, Kong et al. (2022) performed pathogenicity (detached leaves) and virulence tests on *P. capsici* isolates from Julau. They have reported that one of the isolates (I5) was significantly more virulent than the other (E3), although they originated from the

same district. This suggests the presence of distinct virulence factors or possible genetic variations within the population of *P. capsici* in Sarawak. Understanding such variations is crucial for developing effective management strategies to control the disease and protect black pepper crops in the region.

2.6 Genetic Diversity of *Phytophthora capsici*

2.6.1 Impacts of High Genetic Diversity in the *Phytophthora capsici* Population

Zhang et al. (2006) highlighted the considerable diversity in *P. capsici*'s morphology and genetics, noting the challenges this poses for controlling the pathogen. Both mating types in the pathogen population not only suggest high genetic diversity but are pivotal for sexual reproduction (oogonia) to occur, leading to the emergence of new recombinant races (Mazáková et al., 2010). Meitz et al. (2010) reinforced this observation, reporting a near 1:1 ratio of A1 and A2 mating types across diverse locations, correlating with increased genotypic diversity, especially in their South African study. Similarly, Li et al. (2012) and Castro-Rocha et al. (2017) found both mating types prevalent in their regions, while contrasting studies by Gobena et al. (2012) and Rai et al. (2020) highlighted areas dominated by a single mating type, indicative of reduced genetic diversity.

Sun et al. (2008) underscored the risks in Eastern China, where the pronounced genetic diversity in *P. capsici* isolates from chilli peppers signified potential challenges in combating newer, more resilient pathogen strains. Furthermore, Truong et al. (2008) observed an even greater genetic diversity in *P. capsici* isolates from black pepper than chilli variants. This extensive genetic diversity has far-reaching implications, especially its correlation with the pathogen's prowess in resisting hosts and becoming immune to chemical fungicides, as Chen et al. (2018) support. Such insights accentuate the pressing need to probe

into the genetic variations of the *P. capsici* population influencing black pepper farms in Sarawak.

2.6.2 Molecular Techniques Used for Identification and Assessing Genetic Diversity of *Phytophthora capsici*

Various molecular techniques have been used to identify *P. capsici*, including the nuclear ribosomal Internal Transcribed Spacers (ITS) are described as a universal DNA barcode marker by Schoch et al. (2012). The ITS sequence is located between the 18S and 5.8S rRNA regions and is known to have a high degree of variation (Ghosh et al., 2019). Latifah et al. (2018) used ITS sequence to identify and characterise *Phytophthora* spp., in which ITS 4 and ITS 6 primers were used.

Different molecular markers have been employed to study the diversity of *P. capsici*, such as Random Amplified Polymorphic DNA (Chee & Jee, 2001), Random Amplified Microsatellites (RAMS)(Chee & Jee, 2001; Rai et al., 2020; Truong et al., 2010), Inter Simple Sequence Repeats (ISSR) (Babadoost, 2004; Li & Liu, 2021; Mohammadbagheri et al., 2021), Amplified Fragment Length Polymorphism (Bowers et al., 2007; Hurtado-González et al., 2008; Lamour & Hausbeck, 2001) and Simple Sequence Repeat (Chen et al., 2018; Cissin et al., 2015; Meitz et al., 2010).

The RAMS, a PCR technique introduced by Hantula et al. (1996), which was also used in this study, was specifically designed to characterise genetic variation within fungi. This technique, characterised by its 5' anchored short primers, is adept at detecting interspecific and intraspecific DNA polymorphisms. Utilising this method, Chee and Jee (2001) explored the genetic variation of *P. capsici* in Korea, detecting notable polymorphisms among the isolates. Similarly, Zakaria et al. (2005) employed RAMS to study the genetic relatedness of *Ganoderma* spp. in Malaysia. Their findings showcased

polymorphic banding patterns, with clustering often reflecting geographical origins, suggesting a tight genetic linkage among isolates from varied sources. On the other hand, Truong et al. (2010) used RAMS to investigate the genetic variation of *P. capsici* across Korea and Vietnam, concluding that geographical origin did not significantly influence genetic variation; most isolates belonged to a broad clonal group spanning several provinces and regions.

The ISSR, a PCR-based technique, is widely used to study genetic diversity in plants, animals, and fungi (Pradeep et al., 2002). The technique amplifies a DNA segment located between two identical microsatellites repeat regions oriented in opposite directions (Vijayan, 2005). This technique is used for various purposes, such as fingerprinting, phylogenetic analysis, population structure analysis, line identification, gene mapping, and marker-assisted selection. The ISSR technique was used by Li et al. (2012) to study the genetic diversity of *P. capsici* in Anhui province, China where the authors used 13 primers to produce 158 fragments, that displayed a high polymorphism rate of 90%. This study revealed substantial genetic variation of *P. capsici* population, with genetic similarity coefficients ranging from 0.56 to 0.84 among the studied 51 isolates.

For the interpretation of results from RAMS and ISSR, the DNA fragments from ISSR and RAMS are scored objectively (Chee & Jee, 2001; Truong et al., 2010; Li et al., 2012; Poyraz, 2016) as (1) when a band is produced and (0) when absent. Faint bands are omitted from the data collection (Poyraz, 2016). A similarity matrix and a dendrogram were then constructed upon analysing the score data using an Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) (Nath et al., 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

Sampling was conducted in four divisions of Sarawak Sarikei, Betong, Serian, and Kuching (Figure 3.1) to investigate the prevalence of *Phytophthora* foot rot disease in black pepper vines between December 2019 and June 2021. The samples were collected based on a purposive sampling approach. The sampling locations were determined based on reports from the Department of Agriculture, Sarawak, regarding suspected regions of *Phytophthora* foot rot disease occurrence.

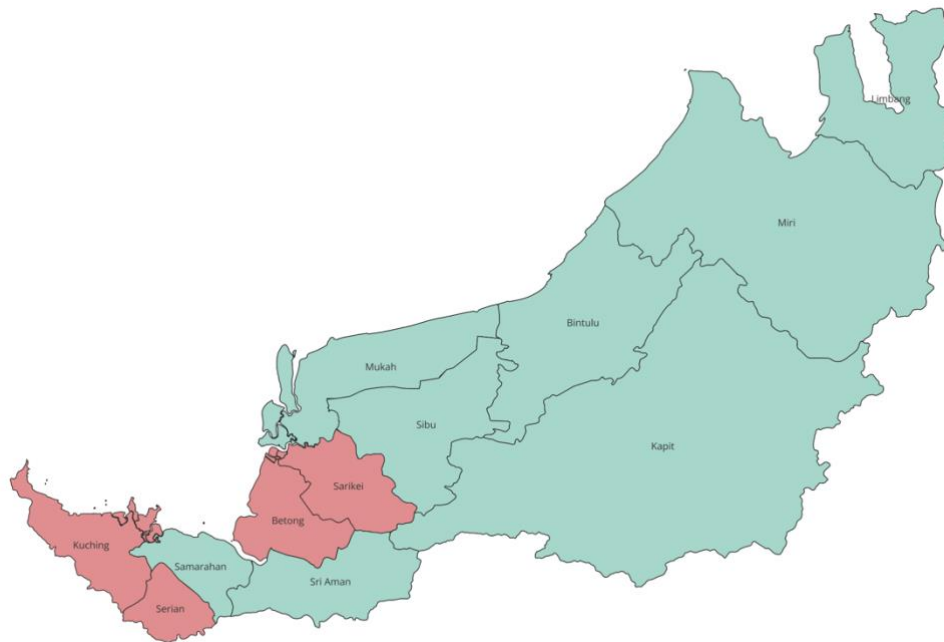


Figure 3.1: Sarikei, Betong, Serian, and Kuching divisions, where suspected regions of *Phytophthora* foot rot disease occurrence were reported by the Department of Agriculture

Within each selected farm, the number of samples collected (Table 3.1) was determined relative to the size of the farm and the severity of the disease, with larger farms and more severe disease cases receiving more samples. To ensure a representative sample, the collection of samples within each farm was carried out purposively by selecting the most visibly affected plants exhibiting typical symptoms of *Phytophthora* foot rot disease, such as round black lesions on the leaves, yellowing of the leaves, severe leaf defoliation, and rotten roots accompanied by a distinct foul smell.

Samples of plant tissues, including infected roots and the collar region, and soil samples surrounding the infected roots were collected from affected black pepper vines exhibiting typical symptoms of *Phytophthora* foot rot disease. Each sample was labelled and kept in a plastic bag to avoid contamination. Upon sample collection, the samples were stored in either a polystyrene box or an ice box and kept in a cool area until transported to the lab. Once brought to the lab, the samples were kept refrigerated and processed as soon as possible, within 14 days after sample collection.

Table 3.1: Diseased samples collected from 28 farms from four divisions

Farm Code	Division	Location	Coordinates	Year of Collection
IJ	Sarikei	Julau	1°50'47"N 111°55'18"E	2019
NG	Sarikei	Julau	1° 50'47"N 111° 55'18"E	2019
GRF	Sarikei	Julau	1°50'51"N 111°55'16"E	2019
NCY	Sarikei	Julau	1°50'51"N 111°55'16"E	2019
MRI	Sarikei	Julau	1°57'2"N 111°54'14"E	2019
EBK	Sarikei	Julau	1°55'55"N 111°53'34"E	2019
YBK	Sarikei	Pakan	1°52'55"N 111°35'27"E	2019
ED	Sarikei	Ulu Manding, Pakan	1°52'15"N 111°34'12"E	2019
MY	Sarikei	Sg. Petai	1°56'21"N 111°27'22"E	2019

Table 3.1 continued

RWG	Sarikei	Sare	2°55'54"N 111°41'15"E	2019
NLG	Sarikei	Sebangkoi	1°41'43"N 111°41'33"E	2019
PDN	Sarikei	Ulu Pedanum	1°55'55"N 111°53'34"E	2019
IGL	Betong	Melabu Bair	1°35'49"N 111°45'12"E	2020
EG	Betong	Melabu Bair	1°35'35"N 111°45'14 "E	2020
DG	Betong	Melabu Bair	1°36'2"N 111°45'32"E	2020
JG	Betong	Melabu Bair	1°35'35"N 111°45'14"E	2020
GG	Betong	Melabu Bair	1°35'33"N 111°45'26"E	2020
LWN	Betong	Ulu Layar	1°35'51"N 111°43'32"E	2020
GER	Betong	Ulu Layar	1°35'50"N 111°43'31"E	2020
GR	Betong	Ulu Layar	1°35'51"N 111°43'30"E	2020
TYN	Betong	Nanga Penebak	1°37'55"N 111°43'43"E	2020
MBI	Betong	Nanga Penebak	1°37'55"N 111°43'59"E	2020
NGD	Betong	Nanga Penebak	1°37'9"N 111°43'21"E	2020
SRN	Betong	Nanga Penebak	1°35'51"N 111°43'30"E	2020
VC	Kuching	Bau	1°29'4"N 110°0'59"E	2021
MKM	Kuching	Bau	1°29'2" N 110°0'57" E	2021
LC	Serian	Serian	1°6'3"N 110°32'54"E	2021
NRD	Serian	Serian	0°58'45"N 110°39'10"E	2021

3.2 Isolation and Identification of *Phytophthora capsici*

Two isolation techniques were employed to isolate *P. capsici*, namely the tissue plating technique and the baiting technique. The root and collar tissue samples were used for the tissue plating technique, while the soil samples collected were used in the baiting technique.

3.2.1 Tissue Plating Isolation Technique

The isolation technique described by Farhana et al. (2013) was used with slight modification. The samples were processed separately per symptomatic vine. The diseased

tissues were taken from the borderline with internal lesion margin area of collar and root tissue samples. The root tissues were first debarked and placed in a beaker. Then, the root tissues were washed with tap water thoroughly to remove all attached soil and plant debris. The root samples were cut into approximately 1 cm³ cubical shapes. The samples were surface sterilised using 10% commercial bleach (0.5% v/v of sodium hypochlorite; Brand: Clorox) for 1 minute, followed by three rinses in sterile distilled water (SDW). Surface sterilised tissue was then dried on a sterile paper towel. The samples' surface area was then cut and removed. A thin slice of the root tissue (Figure 3.2) of the sample was excised and plated onto Corn Meal Agar (CMA) (Hi-Media, India) media plates amended with antibiotics (Ampicillin (5 mL/L), Rifampicin (0.34 mL/L), Pentachloronitrobenzene (PCNB) (20 mL/L) and Nystatin (1 mL/L) following Gobena et al. (2012) and Meitz et al. (2010).



Figure 3.2: Plating of root tissue onto corn meal agar media plates

The plates were incubated for 48 hours in an incubator at 25 °C in the dark. The plates were then observed under a compound microscope, and pieces containing hyphae with torulose structure, a distinctive characteristic of *P. capsici* (Rai et al., 2020), were sub-cultured onto fresh CMA plates. Subsequently, a single hyphae tip was selected with the help of stereomicroscope and transferred onto 20% clarified V8 Juice agar (V8A) plates to establish a pure culture of *P. capsici*. Three (3) replicates were made, and the plates were incubated in the dark at 25 °C for 48 hours before being observed.

The V8A media was prepared by adding 2g of calcium carbonate (CaCO₃) (UNICHEM, USA) to 200 mL of V8 juice (Campbell) and mixing well. The solution was centrifuged at 4,000 rpm for 20 mins, and the supernatant was filtered through filter paper. The filtered solution was then centrifuged at 7,000 rpm for 10 mins and filtered again. Finally, 800 mL of distilled water and 15 g of agar powder (Hi-Midea, India) were added to the filtrate (200 mL). The solution was shaken thoroughly and autoclaved (121 °C, 103 kPa for 25 mins).

3.2.2 Leaf Baiting Isolation Technique

Soil samples collected during sampling were utilised for isolation, employing the leaf-baiting isolation technique described by French-Monar et al. (2007) with modification in which, instead of lemon leaf, black pepper leaves were used in this study. Five (5) g of soil were measured and transferred onto a surface-sterilised container, and 200 mL of SDW was added into a surface-sterilised transparent plastic container (13 cm by 20 cm) with a lid and mixed thoroughly. A black pepper leaf of Kuching cultivar that was grown from a cutting from a healthy black pepper plant from Real-Living Lab, UNIMAS, was taken and surface sterilised by using 10% of commercial bleach solution (0.5% v/v of sodium hypochlorite; Brand: Clorox) for 1 min, followed by three rinses in SDW. The leaf was added to the

container and submerged in the soil solution for 48 hrs in dark conditions at room temperature (24 ± 2 °C). Symptomatic (black, circular lesion) leaf samples were used for further isolation.

The leaves were surface sterilised using 10 % commercial bleach (0.5% v/v of sodium hypochlorite; Clorox) for 1 min, followed by three rinses in SDW. Surface sterilised leaf tissue was then dried on a sterile paper towel. The margin of the developed lesion was excised and plated onto CMA plates amended with antibiotics as described above. The plates were then incubated at room temperature (24 ± 2 °C) in the dark for 48 hours. Pure cultures were established using the hyphal tip technique described earlier on V8A plates. The leaf baiting technique was employed without the inclusion of a control group.

3.2.3 Pre-Identification Criteria

Preliminary identifications were then conducted based on morphological characteristics such as colony colour, texture and pattern, sporangia morphology, and hyphae characteristics described by Kong et al. (2022) and Farhana et al. (2013).

3.3 DNA Extraction

The fungal mycelia were grown in 20% clarified V8 juice broth for 5 to 6 days to produce a thick mycelia mass. The mycelia were then harvested and dried using filter papers. The gDNA was extracted using the cetyltrimethylammonium bromide (CTAB) method described by Lee et al. (1988) and Wu et al. (2001) with some modifications. The cell walls of *Phytophthora* spp. were physically broken down by grinding with a sterile pestle and mortar without liquid nitrogen. Before grinding, 500 µL of preheated (65 °C) CTAB buffer was added to the mixture. The mixture was subsequently transferred into a 1.5 mL microcentrifuge tube and incubated in a water bath at 65 °C for an hour. Then, 500 µL of

chloroform isoamyl alcohol was added and mixed well, followed by centrifugation at 13,500 rpm for 15 mins. The supernatant was transferred into a new microcentrifuge tube, and an equal amount of cold isopropanol was added and mixed thoroughly by inverting the tube. The mixture was stored at -4°C overnight. The mixture was spun at 13,500 rpm for 15 mins, and the supernatant was discarded. One (1) mL of 70% molecular grade ethanol was then added into the tube containing the DNA pellet, and the mixture was spun again at 13,500 rpm for 15 mins. After which, the ethanol was discarded, and the pellet was air-dried before 50 μL of SDW was added to dissolve the pellet. The DNA concentration was adjusted to approximately 25 ng/ μL using a nanodrop (DeNovix, USA). The extracted DNA was stored at -4°C until used.

3.4 Internal Transcribed Spacers (ITS) Sequencing

For identification, a universal primer Internal Transcribed Spacers (ITS) pair ITS-1: 5'- TCCGTAGGTGAACCTGCGG-3' and ITS-4: 5'- TCCTCCGCTTATTGATATGC-3' was used (Díaz-Nájera et al., 2015; Hyder et al., 2020). Polymerase chain reaction (PCR) was performed in a total volume of 25 μL reaction mixture containing 1X Taq Buffer, one mM MgCl, 0.2 mM dNTPs, 1.25 U of Taq Polymerase (1st BASE Sdn. Bhd.), 0.2 μM forward and reverse primers respectively and template DNA (approximately one ng/ μL). The PCR reaction was carried out by using a thermal cycler according to the following temperature profile: Initial denaturation for 2 mins at 94°C , followed by 35 cycles of amplification denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute and a final extension at 72°C for 5 mins.

The PCR products were electrophoresed (90 V, 200 mA, 35 mins) on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV-transilluminator. The PCR products were purified using a purification kit (FavorPrepTM) according to the given

manufacturer's instructions and then sent for both forward and reverse sequencing (Apical Scientific Sdn. Bhd., Malaysia). The BLAST algorithm from GenBank was used to compare the sequences with the sequences deposited in the GenBank database.

3.5 Morphological Characterisation

3.5.1 Colony Morphology

Colony morphology of ten representative isolates (IJ1, EBK1, MRI1, NG1, NCY1, LWN1, GER1, MBI1, NGD1 and LC1) on three different media: CMA, Potato Dextrose agar (PDA) and V8A. In selecting the ten isolates for assessing radial mycelial growth rates, one representative isolate was chosen randomly from each of ten different farms. A 4 mm mycelial agar plug was harvested from the edge of 5-day-old actively growing cultures on CMA agar. These agar plugs were then carefully transferred onto fresh Petri dishes and placed upside down, where the surface of the mycelium was facing the medium, on the new plates. Colony characteristics such as colour, colony growth pattern, texture, margin, and elevation were recorded. Ten replicates were done.

3.5.2 Hyphae Structure Observation

To observe the hyphae structure of the isolates. The isolates were plated onto CMA media plates with a mycelial agar plug that was harvested from the edge of 5-day-old actively growing cultures on CMA. The plates were observed at different time periods; precisely two days, five days, and 14 days old. Observations were made directly from the Petri dish without the use of any staining techniques. The hyphae structure was observed under a compound microscope (BX51-CCD-DA, Olympus) at 200 times magnifications, and images of the hyphae and sporangia were captured using a camera attached to the microscope.

3.5.3 Sporangia Observation

The 10 representative isolates same as described in section 3.5.1 were used to induce sporangia production, and the method from Demirci and Dolar (2006) was adapted with some modifications. Specifically, ten 4 mm diameter mycelial plugs from each isolate were taken from a 5-day-old culture plate and transferred to sterile Petri dishes filled with SDW. Three replicates were prepared for each isolate. The plates were incubated under fluorescent light at room temperature (24 ± 2 °C) and monitored under a light microscope for sporangia production from 48 hrs onwards.

The characteristics of the sporangia produced in SDW were observed under a compound microscope (BX51-CCD-DA, Olympus) at 200 times magnifications, and images of the hyphae and sporangia were captured using a camera attached to the microscope. The length and width of sporangia were measured using the Cell-D Imaging Software and recorded.

Statistical analysis was conducted on different isolates' sporangia length and width. The data were analysed using one-way ANOVA, and the means of the length or width were compared using the Tukey test at a 5% significance level. The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) Statistics version 26.

3.5.4 Oogonia Production

Following the methods described by Manohara (2007), *P. capsici* isolates were paired on V8A media plates, with two 4 mm mycelial plugs of different isolates placed 5 cm apart. The mycelial agar plug was harvested from the edge of 5-day-old actively growing cultures on CMA. Each pairing was replicated thrice and incubated at 25°C in darkness for two weeks. The appearance of double-walled thick oogonia was examined for the

categorisation into group A or B, using a reference isolate (IJ1 as group A). Pairings producing oogonia with IJ1 were classified as group B and, otherwise, as group A.

Two types of pairing tests were conducted: one involving representative isolates from each farm paired with each other in all possible combinations, and another where isolates within the same farm were paired in all possible combinations.

3.6 Mean Radial Mycelial Growth Rate of *Phytophthora capsici* Isolate on Different Media

The 10 isolates selected from section 3.5.1 were selected to study its growth rate on three different media following methods as described by Kong et al. (2022): CMA, PDA (Merck, Germany), and V8A. A cross was drawn at the back of the Petri dish, and the cross extended the centre of the dish, marking the centre of the plates where a 4 mm mycelial plug was placed. The mycelial plug was taken from a 5-day-old culture grown on CMA media.

The plates were incubated in the dark at $25(\pm 1) ^\circ\text{C}$. The radial growth rate of the isolates was marked from the edge of the mycelial plug and recorded daily on all four drawn cross-sections. The experiment was conducted with ten replicate plates for each media, and this experiment was conducted twice. The experiment was concluded once the radial mycelial growth had reached the edge of the plate. The growth rate between isolates was analysed using SPSS Statistics version 26, in which One-Way ANOVA was used to compare the means between media and isolates of the treatments. The Tukey post-test at a 5% significance level was used to differentiate the means.

3.7 Pathogenicity and Aggressiveness of *Phytophthora capsici*

3.7.1 Pathogenicity of *Phytophthora capsici*

The detached shoot pathogenicity experiment were conducted based on methodology as described by Anith et al. (2003) with modification, where instead of using pinprick technique to inoculate the pathogen, this study applied a mycelial plug directly to the first node from the bottom of each shoot. Fifty-five healthy young black pepper shoots with about four to five leaves were obtained from the same vine of Kuching cultivar, planted in the compounds Real Living Lab, UNIMAS, known to be susceptible to *Phytophthora* foot rot. The shoots were surface sterilised as described in section 3.2.1. Ten isolates, each representing one farm (same isolates tested in section 3.5.1), were tested. Five replicates of shoots were used for each isolate, as well as for the control.

Sterile tissue paper moistened with 2 mL of SDW was lined on the surface-sterilised transparent plastic container (13 cm by 20 cm) with a lid. The surface-sterilised shoots, dried in a laminar flow for an hour, were aseptically placed in the prepared containers, one shoot per container. A single agar plug from a -5-day-old *P. capsici* culture grown on CMA was placed on the first node from the bottom of each shoot. The containers with the inoculated shoots were incubated at 25 °C in an incubator. The length (cm) of the lesions developed was recorded every 48 hours.

On the seventh day post-inoculation, the pathogen was reisolated from the artificially inoculated shoots using similar techniques described above for the pathogen's isolation from the leaf-baiting technique. The cultures were purified, and the colonies' morphology and hyphae structure were recorded.

3.7.2 Preparation of Aggressiveness Test

3.7.2.1 Preparation of Potting Medium

The method described by Truong et al. (2008) was adopted in this study. A mixture of topsoil, sand, and coco peat was mixed in a ratio of 2:1:1. The soil mixture was autoclaved at 121 °C and 103 kPa for 30 mins twice with a 48-hour interval in between. All the autoclaved soil was mixed thoroughly to ensure a uniform mixture, and the soil mixture was autoclaved in separate bags (Figure 3.3). The soil mixture was used to establish black pepper cuttings.



Figure 3.3: Autoclaved potting media

3.7.2.2 Young Black Pepper Vines Establishment

Four hundred fifty (450) black pepper cuttings, of which 250 were Kuching and 200 Semenggok Aman cultivars, were planted into polybags (15 cm by 23 cm) using the sterile

potting medium. To ensure approximately the same amount of soil was in each polybag, the soil was filled compactly to a certain height of the polybag, 18 cm (75% of the polybag).

All Kuching and Semenggok Aman cuttings were from black pepper vines of the same respective mother plant (from a Serian small-holder black pepper farmer, Mr Winty). The cuttings were kept and maintained at the Plant Pathology Lab for one month before being transferred to an outdoor plastic sheet-covered greenhouse under 90% shade (Figure 3.4).



Figure 3.4: Black pepper cuttings in the plant pathology lab, Universiti Malaysia Sarawak (left) and their subsequent relocation to an outdoor shaded greenhouse (right)

Similar to black pepper cuttings, the wild pepper cuttings, are Type 1 (*Piper colubrinum*) and Type 2 (*Piper* spp). were also planted via cuttings (Figure 3.5). They were maintained in a plastic-covered net house outside the Plant Pathology Lab, UNIMAS.



Figure 3.5: Wild pepper cuttings – Type 1: *Piper colubrinum* and type 2: *Piper* spp. in a net house outside the Plant Pathology Lab, Universiti Malaysia Sarawak

3.7.2.3 Inoculum Preparation for Aggressiveness Test

Phytophthora capsici was cultured on sterile millet seeds (Truong et al., 2012). For each isolate, 200 g of millet seeds were thoroughly washed using tap water. The millet seeds were transferred to a flask with 200 mL of distilled water. The millet seeds were autoclaved twice at 121 °C and 103 kPa for 25 mins at a 48-hour interval. Once the millet seeds were cooled to room temperature, ten agar plugs of 4 mm size of selected *P. capsici* isolates were harvested from the edge of the 7-day-old culture and transferred into the flask containing millet seeds (Figure 3.6). The flask was covered with sterile foil and sealed with parafilm. The flask was shaken well to distribute the agar plugs evenly between the millet seeds.

Meanwhile, millet seeds were inoculated with fresh sterile agar plugs (10 agar plugs of 4 mm size) for the control. The flasks were incubated in dark conditions at 25 °C for 14 days. The flask was shaken every five days. Two days before inoculation, a small portion of the prepared inoculum was plated on a fresh CMA plate to examine the culture's viability.

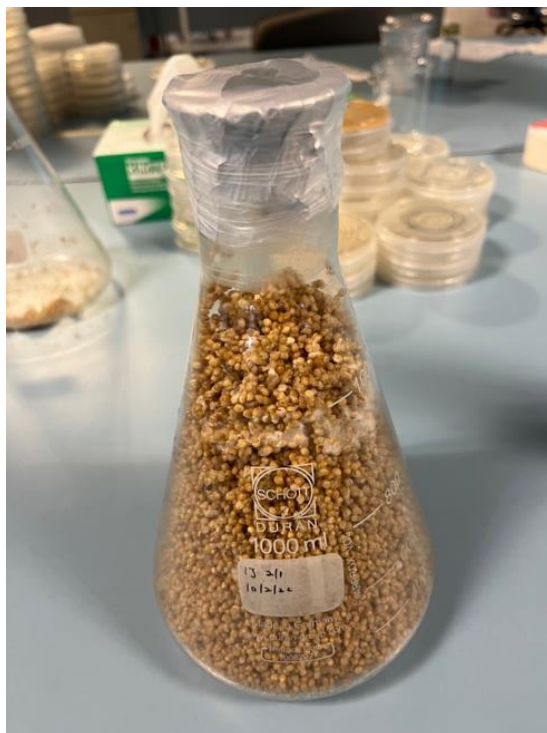


Figure 3.6: Millet seed inoculum

3.7.3 Experimental Set-Up for Aggressiveness Test on Kuching Cultivar

In this experiment, 4-month-old Kuching cultivar cuttings were used. The black pepper cuttings from hereinafter will be referred to as vines. All ten isolates of *P. capsici* used in section 3.5.1 were tested in this experiment with three replicates. The inoculum of 25 g was used for each polybag since 75% of the polybag was filled with soil (Soil Volume $\approx 4000 \text{ cm}^3$), 2% of the soil volume Truong et al. (2012) was 80 cm^3 , which was approximately 25 g of inoculum.

Three (3) cm of surface soil was removed to place the inoculum. Then, the inoculum was covered back with the soil. Each young black pepper vine was placed onto an individual basin (Figure 3.7). The basin was then flooded up to approximately 3 cm of tap water for four consecutive days, and then the water was drained for four consecutive days. The

flooding and draining regimes were maintained until the end of the experiment. The black pepper vines were watered daily with 100 mL of water, where the soil surface was watered.

The black pepper vines were inspected daily to observe the vine's survival or death. The black pepper vine was considered dead when the root collar completely rotted and all its leaves wilted and turned yellow with no possibility of revival. The time taken for each black pepper vine to die was recorded. The observation was for 120 days.



Figure 3.7: Experimental setup of black pepper vines

3.7.4 Experimental Set-Up for Aggressiveness Test on Semenggok Aman Cultivar

In this experiment, the Semenggok Aman cultivar was used and the experimental set-up was similar to that described in section 3.7.3.

3.7.5 Analysis of Aggressiveness Test

Disease severity was assigned with a score based on the following scale: 0 = alive vines and 1 = dead vines. The time taken for the inoculated vines to die was analysed using the Kaplan–Meier Estimator (KME) described by Rich et al. (2010). The survival probability, S_t (Equation 3.1), at any given time was calculated.

$$S_t = \frac{\text{Number of plants living at the start} - \text{Number of dead plants}}{\text{Number of plants living at the start}}$$

Equation 3.1: Survival Probability Equation

A survival curve was plotted based on the probabilities of the occurrence of an event at a certain point in time and by multiplying these successive probabilities by any earlier computed probabilities to get the final estimate. Survival analysis was performed using SPSS Statistics version 26 software where KME was estimated, and the survival curve was plotted. A pairwise comparison was utilized for the survival analysis in SPSS Statistics version 26 to determine significant differences between treatment groups.

3.7.6 Comparison of Degree of Aggressiveness Among Varieties

The comparison of the degree of aggressiveness among the *P. capsici* isolates was based on the data obtained from the aggressiveness tests conducted on both the Kuching and Semenggok Aman cultivars. The percentage of vine death and the average time taken to cause vine death were compared across the different isolates to determine their relative aggressiveness on each cultivar.

In addition, the degree of aggressiveness among *P. capsici* isolates on both the Kuching and Semenggok Aman cultivars was statistically evaluated using the non-parametric Kruskal-Wallis test using SPSS Statistics version 26.

3.7.7 Experimental Set-Up for Virulence Test of IJ1 on Wild Pepper Cuttings

Additionally, two negative controls with two wild-pepper cultivars, *P. colubrinum* and *Piper* spp. were added (Figure 3.5) and the experimental set-up was similar to that described in section 3.7.3. The negative control groups were inoculated with IJ1 isolate. The observation was for 90 days, where the data was collected as described in section 3.7.3.

3.7.8 Reisolating Pathogen (Koch's Postulate)

The collar and root tissues of the dead vines from the aggressiveness tests were re-plated. The root tissues were rinsed with tap water thoroughly and placed into separate beakers according to each replicate. The re-isolation process followed the protocol mentioned in section 3.2.1. The reisolated pathogen was identified based on its colony morphology compared to the original colony.

3.8 Molecular Characterisation of *Phytophthora capsici*

3.8.1 Phylogenetic Analysis and Neutrality Test

The ITS sequences of 30 isolates of *P. capsici* were analysed using MEGA X Software. The sequences were first aligned using the ClustalW alignment. MEGA X was also used to construct a Neighbour-Joining Tree with the Jukes-Cantor Model, the best-fitting model. *Phytophthora citrophthora* was selected as an outgroup. The sequence of *P. citrophthora* was obtained from NCBI (Accession No: KU934210). Bootstrap analysis was set at 1000 replicates. Neutrality tests, Tajima's D, Fu and Li's D and Fu and Li's F, were conducted using DnaSP software v5 to investigate any potential deviation from neutrality.

3.8.2 Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR)

For random amplified microsatellites (RAMS), the method described by Truong et al. (2010) was adopted. The PCR was performed with a 25 µL reaction mixture containing 1X Taq Buffer, 1mM MgCl, 0.2 mM dNTPs, 1.25 unit of Taq Polymerase (1st BASE Sdn. Bhd.), 0.2 µM primer, and template DNA (approximately 1 ng/µL). Six primers reported by Truong et al. (2010) were tested. T100TM Thermal Cycler (Bio-Rad Laboratories, USA) was used to perform this reaction. The initial denaturation was for 10 mins at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at the optimum temperature of the respective RAMS primer (Table 3.2) for 1 minute, and extension at 72 °C for 2 mins, and a final extension at 72 °C for 7 mins.

For inter-simple sequence repeats (ISSR), the procedure described by Li et al. (2012) was adopted. A total of 13 different ISSR primers were tested. The PCR was performed with a 25 µL reaction mixture containing 1X Taq Buffer, 1mM MgCl, 0.2 mM dNTPs, 1.25 U of Taq Polymerase (1st BASE Sdn. Bhd.), 0.2 µM primer and template DNA (approximately 1 ng/µL). The PCR reaction was carried out by using T100TM Thermal Cycler (Bio-Rad Laboratories, USA) according to the following temperature profile: The initial denaturation was for 5 mins at 94 °C, followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at an optimum temperature of the respective ISSR primer (Table 3.2) for 1 minute, and extension at 72 °C for 2 mins, and a final extension 72 °C for 10 mins.

Table 3.2: Primer details

Primer Name	Sequence (5'-3')	Annealing Temperature (°C)
RAMS		
ACA	BDB(ACA) ₅	47
AG	HBH(AG) ₇ A	49
CGA	DHB(CGA) ₅	60
GT	YHY(GT) ₇ G	58
TG	HVH(TG) ₇ T	53
CCA	DD(GA) ₅	61
ISSR		
ILE 120	CAC(GA) ₇	54
P 6	(AG) ₈ YC	54
P 10	(GA) ₈ TG	51
P 15	G(ACAG) ₃ ACAC	54
UBC 807	(AG) ₈ T	52
UBC 808	(AG) ₈ C	54
UBC 809	(AG) ₈ G	50
UBC 811	(GA) ₈ C	50
UBC 816	(CA) ₈ T	52
UBC 821	(GT) ₈ T	48
UBC 835	(AG) ₈ YC	50
UBC 841	(GA) ₈ YC	54
UBC 873	(GACA) ₄	54

3.8.3 DNA Fingerprinting Analysis

The PCR products were electrophoresed on a 1.5% agarose gel at 35 V for 4 hrs. The gel was then stained with ethidium bromide before viewing under a UV transilluminator. The bands produced from the RAMS and ISSR primers were scored conservatively as (1) if the band was present and (0) if the band was absent. GeneAIEx 6.5 was employed to generate a distance matrix based on the pooled ISSR and RAMS scores. MEGA X software was used to construct the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) phylogenetic tree based on the distance matrix. GeneAIEx 6.5 was also used to conduct an Analysis of Molecular Variance (AMOVA) to compute Shannon's Information Index and the percentage of polymorphic loci.

3.9 Quarantine Procedures

All experiments with the isolated pathogen were carried out within the plant pathology lab in the Faculty of Resource Science and Technology. All sampled plant materials were individually stored in plastic bags in the plant pathology lab. After isolation, these samples were autoclaved (121 °C, 103 kPa for 25 mins) twice before disposal. All labware items used in the isolation process were soaked in a bleach solution before washing.

CHAPTER 4

RESULTS

4.1 Sampling

4.1.1 Symptoms of *Phytophthora* Foot Rot Disease

In the sampled black pepper farms across four divisions of Sarawak, namely Sarikei, Betong, Serian, and Kuching, a range of symptoms associated with *Phytophthora* foot rot disease were observed (Figure 4.1). The prominent and easily observable symptoms were the quick wilt of black pepper vines (Figure 4.1 A to B), particularly when more than 50% of the leaves had wilted. Black lesions on leaves (Figure 4.1 C to D) were another common feature of the disease as these lesions, which appeared as irregular dark patches, significantly affected the overall health of the black pepper plants. Rotten root and collar tissue (Figure 4.1 E to G) was another prominent *Phytophthora* foot rot disease symptom. This deterioration was often accompanied by an unpleasant and distinct foul smell, further confirming *Phytophthora* foot rot disease.

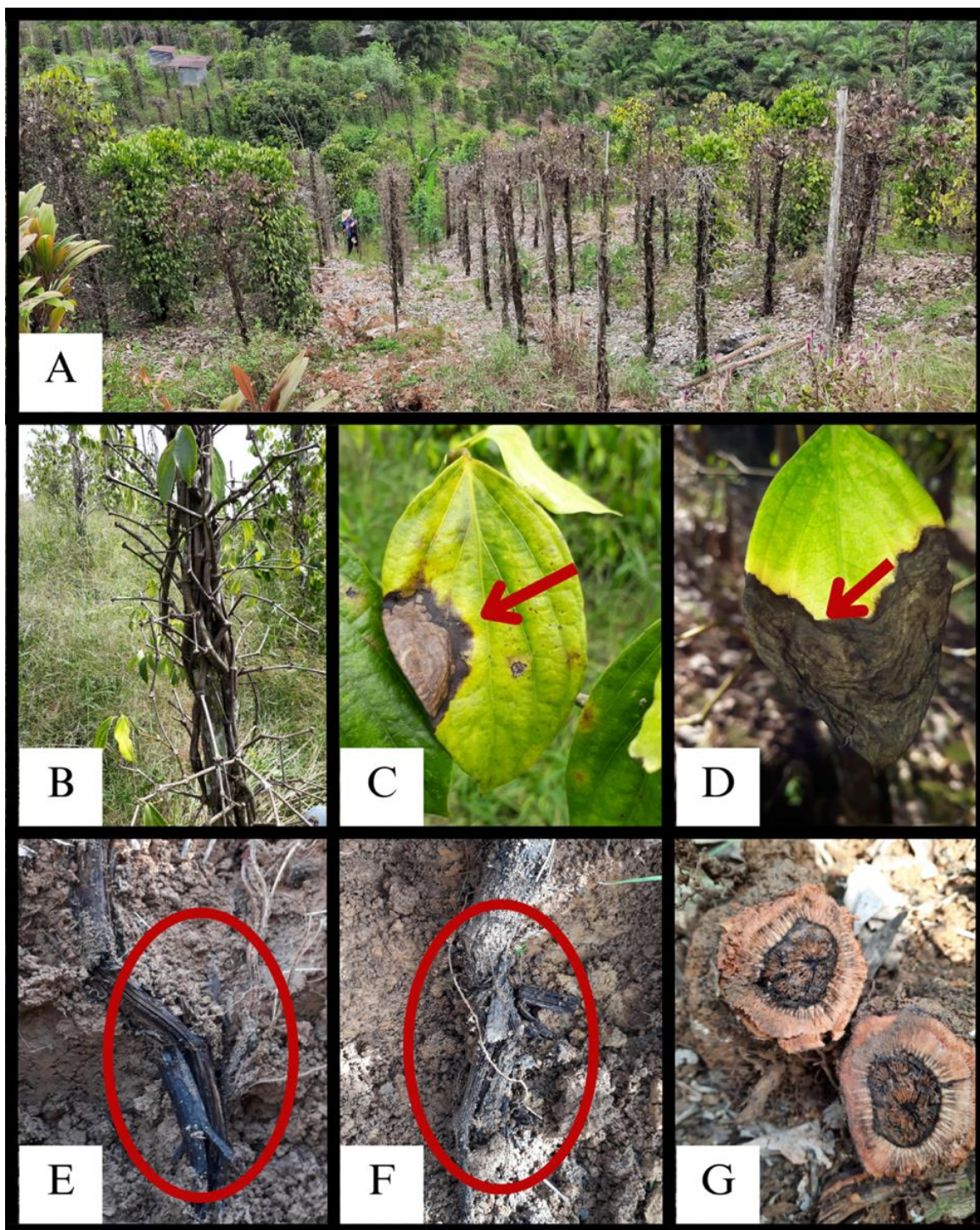


Figure 4.1: Symptoms of *Phytophthora* foot rot disease from sampled farms; (A) infected farm with dead vines; (B) defoliating vine with yellowing of the leaves; (C & D) leaves with a lesion; (E, F & G) infected rotten collar and root tissues

4.1.2 Sample Collection from Infected Black Pepper Farms

In this study, 153 samples from 28 black pepper farms in Sarawak were collected based on exhibited symptoms of *Phytophthora* foot rot disease (Table 4.1).

Table 4.1: Sample collection details

Farm Name	No. of Samples Collected	Division	Location	Year of collection
Ng. Ijit	12	Sarikei	Julau	2019
Rh.Naing	10	Sarikei	Julau	2019
Rh. Griffin	1	Sarikei	Julau	2019
Nancy	5	Sarikei	Julau	2019
Rumah Marai	3	Sarikei	Julau	2019
Embak	6	Sarikei	Julau	2019
Rumah Yosbika	5	Sarikei	Pakan Wuak	2019
Rumah Eddy	11	Sarikei	Ulu Manding, Pakan	2019
Rumah Mary	8	Sarikei	Sg. Petai Sarikei	2019
Rumah Rawing	2	Sarikei	Sare	2019
Rumah Nyalong	7	Sarikei	Sebangkoi	2019
Ulu Pedanum	5	Sarikei	Ulu Pedanum	2019
Inggil Ak. Belaja	3	Betong	Melabu Bair	2020
Entinggi Ak. Giri	3	Betong	Melabu Bair	2020
Dan Ak. Giri	3	Betong	Melabu Bair	2020
Jana Ak. Giri	5	Betong	Melabu Bair	2020
Giang Ak. Guyu	9	Betong	Melabu Bair	2020
Lawan	7	Betong	Rh Tinting, Ulu Layer	2020
Geraman	7	Betong	Rh Tinting, Ulu Layer	2020
Garai	8	Betong	Ulu Layer	2020
Tayan	5	Betong	Rh. Nanga Penembak	2020
Merbai	5	Betong	Rh. Nanga Penembak	2020
Ngadai	5	Betong	Rh. Nanga Penembak	2020
Serani	5	Betong	Rh. Nanga Penembak	2020
Victor	7	Kuching	Bau	2021
Mikim	8	Kuching	Bau	2021
Lucia	2	Serian	Serian	2021
Rh. Ngeradai	2	Serian	Serian	2021

4.2 Isolation and Identification of *Phytophthora capsici*

4.2.1 Isolation Techniques

Of the 28 sampled farms from the four divisions in Sarawak, candidate fungal isolates were isolated from 10 farms (Table 4.2). The primary method for isolating involved the tissue plating technique, which involved directly plating samples of infected root tissues onto corn meal agar (CMA) plates. Most of the isolates from this study except for isolates from MBI and LC farms were retrieved using the tissue plating method. For the isolates from the MBI and LC farms, the leaf baiting method was used for isolation.

4.2.2 Pre-Identification

There were 52 isolates obtained. All 52 isolates were pre-identified as *P. capsici*. Pre-identification was based on the hyphae (torulose) structure, colony morphologies (texture, pattern, and colour) and sporangia morphology. The colony morphologies between isolates varied, but all observed characteristics were typical *P. capsici* characteristics such as smooth and cottony textures, white to creamy colour, and colony growth patterns such as rosette, petaloid and stellate (Bhai et al., 2022; Kong et al., 2022; Moreira-Morrillo et al., 2022). Torulose hyphae were observed on all 52 isolates. Out of the 52 isolates, 30 isolates were selected randomly, in which isolates from all 10 farms were represented in the selected 30 isolates. These selected 30 isolates were then subjected to molecular identification using ITS sequence.

Table 4.2: Isolate and sample information for *Phytophthora capsici* isolates obtained from Sarawak farms

Farm Code	Number of Isolates	Isolate Code	Division	GPS Coordinates
IJ	10	IJ1 IJ2 IJ3 IJ4 IJ5 IJ6 IJ7 IJ8 IJ9 IJ10	Sarikei	1.9633333, 111.905
EBK	2	EBK1 EBK2	Sarikei	1.9319444, 111.8927778
NG	12	NG1 NG2 NG3 NG4 NG5 NG6 NG7 NG8 NG9 NG10 NG11 NG12	Sarikei	1.8457969, 111.9222446
MRI	10	MRI1 MRI2 MRI3 MRI4 MRI5 MRI6 MRI7 MRI8 MRI9 MRI10	Sarikei	1.8958314, 111.9339715
NCY	1	NCY1	Sarikei	1.8475, 111.9211111
GER	3	GER1 GER2 GER3	Betong	1.597222 111.725555
NGD	5	NGD1 NGD2 NGD3	Betong	1.6302292, 111.7299839

Table 4.2 continued

		NGD4		
		NGD5		
MBI	1	MBI1	Betong	1.632106, 111.733093
LWN	6	LWN1	Betong	1.5977195, 111.7256998
		LWN2		
		LWN3		
		LWN4		
		LWN5		
		LWN6		
LC	2	LC1	Serian	1.100885, 110.548525
		LC2		

4.2.3 Internal Transcribed Spacers (ITS)

Thirty isolates were selected from 52 for molecular identification using the internal transcribed spacers (ITS) sequence. The isolate selection criteria for ITS sequencing were first to sequence representatives from each farm. Within the same farm, isolates of different colony morphology (colony pattern) were selected. The ITS region was successfully amplified. The PCR products obtained from this reaction were approximately 800 to 1000 bp (Figure 4.2). While the ITS corrected sequences obtained from both forward and reverse sequences ranged from 797 to 799 bp for all the 30 isolates.

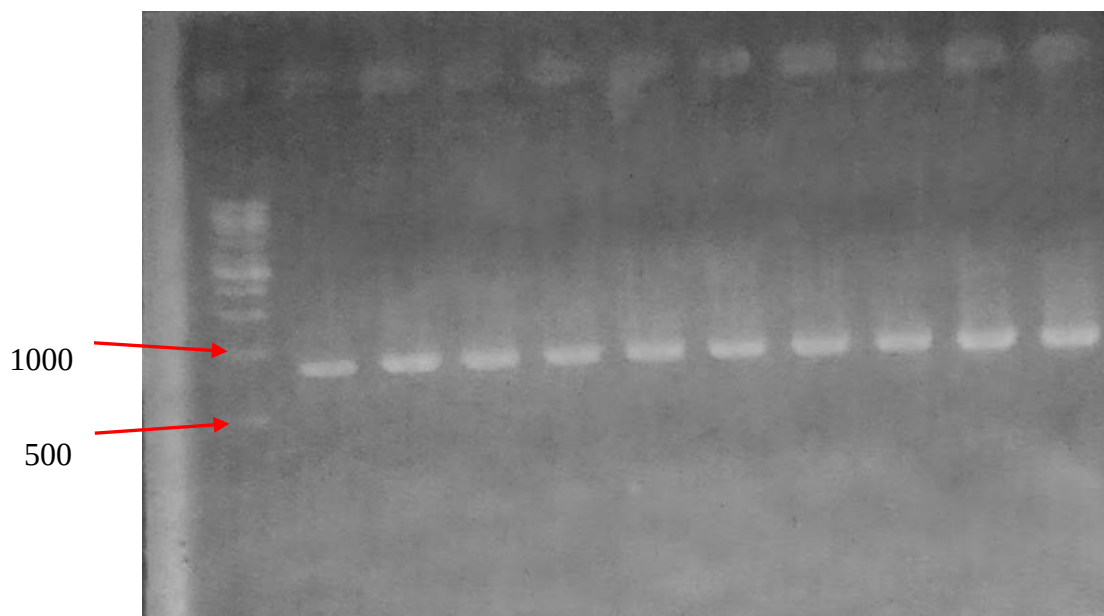


Figure 4.2: Agarose gel electrophoresis (1.5%) with 100 bp DNA ladder (Brand: Vivantis, VC 100 BP Plus DNA Ladder) (Lane 1), and PCR products of different *Phytophthora capsici* isolates (from Lane 2: isolate IJ1, EBK1, NG1, MRI1, NCY1, NGD1, LWN1, GER1, MBI1 and LC1)

Upon blasting the ITS sequences on the NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the BLAST algorithm. It was found that all 30 sequenced isolates were identified as *P. capsici* as they have 99.37% to 100% identity with other *P. capsici* isolates that are found in the NCBI database (Table 4.3). The expectation value (E-value) of 0.0 for all isolates further suggests that the matches are highly significant. Molecular identification complements the initial pre-identification based on morphology, offering a more robust confirmation of the isolates' identity as *P. capsici*. Ten isolates, one selected isolate representing each farm, were further characterised based on their morphology (section 4.3)

Table 4.3: Identification of *Phytophthora capsici* based on the Internal Transcribed Spacers (ITS) sequence

Isolates	Accession number	Sequence Size (Base Pairs)	Division	Best Match Accession in NCBI	Species	Percent Identity (%)	E-Value
IJ1	PP178117	799	Sarikei	MN368092	<i>Phytophthora capsici</i>	99.87	0.0
IJ2	PP178118	799	Sarikei	MN368092	<i>Phytophthora capsici</i>	99.87	0.0
IJ4	PP178119	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	99.87	0.0
IJ5	PP178120	799	Sarikei	FJ887798	<i>Phytophthora capsici</i>	99.87	0.0
IJ7	PP178121	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	99.37	0.0
EBK1	PP178113	798	Sarikei	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
EBK2	PP178114	798	Sarikei	ON680929	<i>Phytophthora capsici</i>	99.87	0.0
NCY1	PP178134	798	Sarikei	KC335550	<i>Phytophthora capsici</i>	99.87	0.0
NG1	PP178135	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	99.87	0.0
NG4	PP178136	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	99.87	0.0
NG5	PP178137	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	100.00	0.0
NG8	PP178138	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	100.00	0.0
NG11	PP178139	799	Sarikei	KC335550	<i>Phytophthora capsici</i>	100.00	0.0
MRI1	PP178130	798	Sarikei	ON680929	<i>Phytophthora capsici</i>	99.87	0.0
MRI4	PP178131	798	Sarikei	ON680929	<i>Phytophthora capsici</i>	99.87	0.0
MRI7	PP178132	798	Sarikei	EU915283	<i>Phytophthora capsici</i>	99.87	0.0
MRI8	PP178133	798	Sarikei	ON680929	<i>Phytophthora capsici</i>	99.87	0.0
GER1	PP178115	798	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
GER3	PP178116	798	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
LWN1	PP178124	797	Betong	ON680929	<i>Phytophthora capsici</i>	99.50	0.0
LWN2	PP178125	797	Betong	ON680929	<i>Phytophthora capsici</i>	99.62	0.0
LWN3	PP178126	797	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
LWN5	PP178127	797	Betong	ON680929	<i>Phytophthora capsici</i>	99.62	0.0
LWN6	PP178128	797	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
MBI1	PP178129	799	Betong	MN368092	<i>Phytophthora capsici</i>	99.87	0.0
NGD1	PP178140	798	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
NGD3	PP178141	798	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
NGD5	PP178142	798	Betong	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
LC1	PP178122	798	Serian	ON680929	<i>Phytophthora capsici</i>	99.75	0.0
LC2	PP178123	798	Serian	ON680929	<i>Phytophthora capsici</i>	99.75	0.0

4.2.4 Prevalence and Potential Transmission of *Phytophthora* Foot Rot

Julau, located in the Sarikei Division, was the only district where samples tested positive for *P. capsici*. Upon observing the distribution of the farms in Julau, which were positive for *P. capsici*. Most farms were situated along the Kanowit River (Figure 4.2), which may serve as the potential medium for transmission of this water-borne oomycetes. Among the farms surveyed in Julau, black pepper vines from Sarikei, IJ farm exhibited the highest susceptibility to *Phytophthora* foot rot disease. Following closely were NG, MRI, EBK and NCY with the latter being in the early stages of disease development at the time of sampling.

Meanwhile, from Betong, most of the positive farms were in the division's North-West region. The terrain of this region was very steep and hilly, and no common water bodies were observed connecting the infected farms based on spatial image observation. As for Serian, upon sampling, only one farm had been identified as *P. capsici* positive. The positive farm LC was in the central region of Serian. Similar to Betong, no open water bodies such as lakes or rivers were observed around the infected farm within a 1 km radius. There is no spatially observable transmission medium between the affected farms in Betong and Serian.



Figure 4.3: Satellite imagery (google earth pro) of Sarikei with *Phytophthora capsici* positive farms along the Kanowit river; A: map of a section of Kanowit river with IJ, MRI and Karms located along the river; B: map of a section of kanowit river with NCY and NG farms located along the Kanowit river

4.3 Colony Morphology

The colony's form (Figure 4.3) of ten selected representative isolates, observed on CMA and V8 Juice agar (V8A) generally had a circular form, except for isolate LC 1 on V8A, which was irregular. On the other hand, the growth pattern observed in all ten isolates on Potato Dextrose agar (PDA) had an irregular form. All the isolates, on V8A and CMA media, had an 'Entire' margin while on PDA had an 'Irregular' margin. Colony elevations

varied, for example, IJ1 and EBK1 were elevated on V8A and CMA, while MRI1 and NCY1 remained flat on V8A but elevated on CMA.

The ten representative isolates showed several distinct colony surface morphologies: petaloid, stellate, rosette, and no pattern (Table 4.4; Figure 4.4). The colony morphology on V8A varied among the isolates, with appearances such as slightly petaloid, concentric rings, and stellate being observed. Patterns such as petaloid, stellate, and concentric rings were prominent on CMA. While on PDA, mostly produced stellate and rosette morphologies. When comparing the morphological characteristics of the same isolate across different media plates, most isolates (7 out of 10) exhibited morphological differences, in which the colony morphology differed in all three media. Other isolates, such as isolate IJ1, LWN1 and NG1 showed two morphologies across three media.

Overall, each of the ten isolates displayed a distinct colony morphology, and no significant grouping or similarities were observed among them based on their morphology. Across all media, petaloid and stellate morphologies were the most and equally prevalent, each accounting for 26.67%, respectively, followed by the 'no pattern' morphology at 23.33%. Rosette and concentric ring characteristics were both present in 13.33% of the observations.

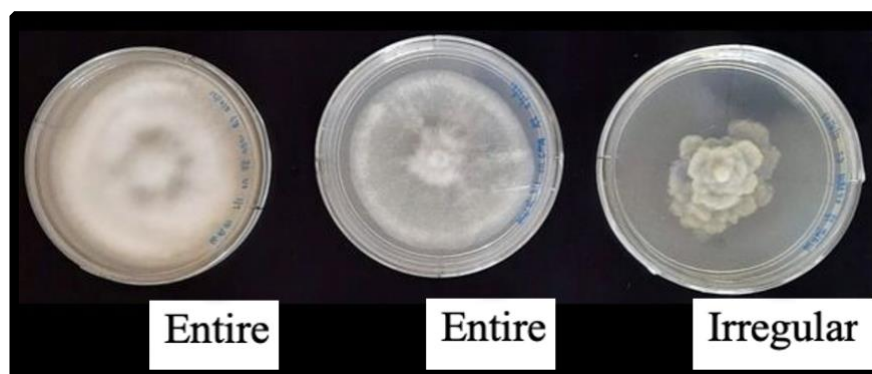


Figure 4.4: From left to right, EBK1 isolate grown on V-8 juice agar (entire margin), corn meal agar (entire margin) and potato dextrose agar (irregular margin)



Figure 4.5: Representative colony morphologies of ten *Phytophthora capsici* isolates observed on different media plates. from left to right: (A) IJ1; (B) EBK1; (C) NG1; (D) MRI1; (E) NCY1; (F) GER1; (G) NGD1; (H) MBI1; (I) LWN1; (J) LC1. The media corresponding to each isolate are labelled as follows: (1) V-8 Juice Agar; (2) Corn Meal Agar ; (3) Potato Dextrose Agar

Table 4.4: Morphological characteristics of *Phytophthora capsici* isolated from infected black pepper vines from Sarawak on V-8 Juice Agar (V8A), Corn Meal Agar (CMA) and Potato Dextrose Agar (PDA) media plates

Isolate Name	V8A				CMA				PDA			
	Colony	Texture	Margin	Elevation	Colony	Texture	Margin	Elevation	Colony	Texture	Margin	Elevation
IJ1	Slightly petaloid	Cottony	Entire	Elevated	Petaloid	No Texture	Entire	Flat	Stellate	No Texture	Irregular	Flat
EBK1	No Pattern	Cottony	Entire	Elevated	Slightly petaloid	No Texture	Entire	Flat	Rosette	No Texture	Irregular	Flat
NG1	No Pattern	Cottony	Entire	Elevated	No Pattern	Cottony	Entire	Elevated	Stellate	No Texture	Irregular	Flat
MRI1	Concentric rings	No Texture	Entire	Flat	No Pattern	Cottony	Entire	Elevated	Rosette	No Texture	Irregular	Flat
NCY1	Petaloid	No Texture	Entire	Flat	Stellate	No Texture	Entire	Flat	Rosette	No Texture	Irregular	Flat
GER1	Stellate	No Texture	Entire	Flat	Petaloid	No Texture	Entire	Flat	Rosette	No Texture	Irregular	Flat
NGD1	Slight petaloid, concentric ring	No Texture	Entire	Flat	No Pattern	Cottony	Entire	Elevated	Stellate	No Texture	Irregular	Flat
MBI1	Slight petaloid, concentric ring	No Texture	Entire	Flat	Petaloid	No Texture	Entire	Flat	Stellate	No Texture	Irregular	Flat
LWN1	Slight petaloid	No Texture	Entire	Flat	Stellate	No Texture	Entire	Flat	Stellate	No Texture	Irregular	Flat
LC1	No Pattern	Cottony	Irregular	Elevated	Concentric rings	Cottony	Entire	Elevated	Slightly rosette	No Texture	Irregular	Flat

4.3.1 Hyphae Structure Observation

The hyphae of the ten representative isolates were observed to be coenocytic. The torulose aseptate hyphae structure was observed on all three media (CMA, PDA and V8A), a distinct characteristic of *P. capsici* hyphae. The hyphae appeared to be swollen and densely packed, coiled, in young cultures (2 days old) (Figure 4.5. A, B) while they appear more elongated in aged cultures (14 days old) (Figure 4.5 E, F).

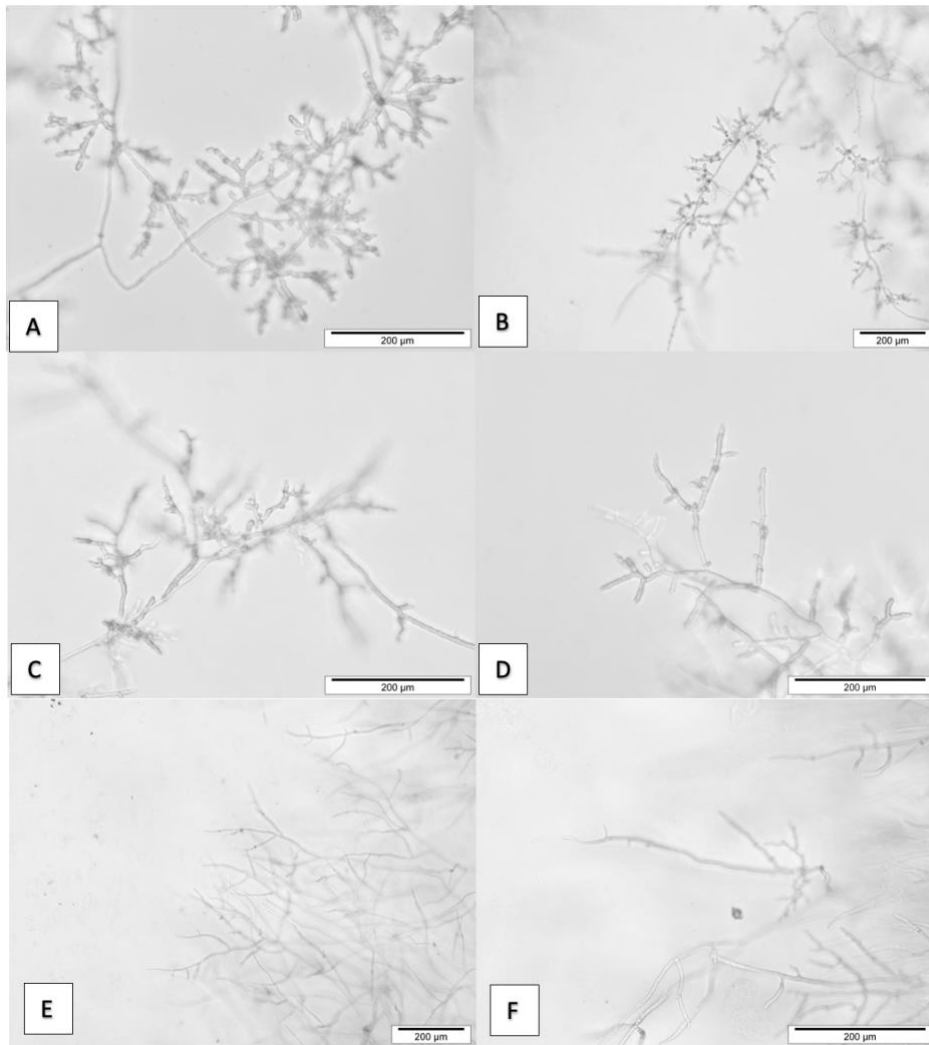


Figure 4.6: (A to B) 2-day old culture with very coiled torulose hyphae of NCY1 and EBK1 isolate at 200x magnification; (C to D) 5-day old culture with torulose hyphae of NCY1 and EBK1 isolate at 200x magnification; (E to F) 14-day old culture with no coiled torulose structure of NCY1 and EBK1 isolate at 200x magnification

4.3.2 Sporangia Observation

All ten selected isolates for characterisation produced sporangia. The sporangia observed had a wide variety of shapes. The sporangia shapes observed included lemon-shaped sporangia, which is one of the identification keys for *P. capsici*. Most of the observed sporangia were obovoid in shape (Figure 4.6).

Both semi-papillated (Figure 4.7 A) and papillated (Figure 4.7 B) sporangia were observed and most sporangia had a long pedicel (Figure 4.7 C). The observed sporangia had a simple sympodium branching (Figure 4.7 D). The spores will be released upon rupturing the sporangia's papillate (Figure 4.7 E to G).

The mean length and width (Table 4.5) of sporangia did not significantly differ from one another at a 95% confidence interval, although variation in sizes can be seen. The length of the sporangia ranged from 21.0 to 28.0 μm , while the width of the sporangia ranged from 15.7 to 22.1 μm .

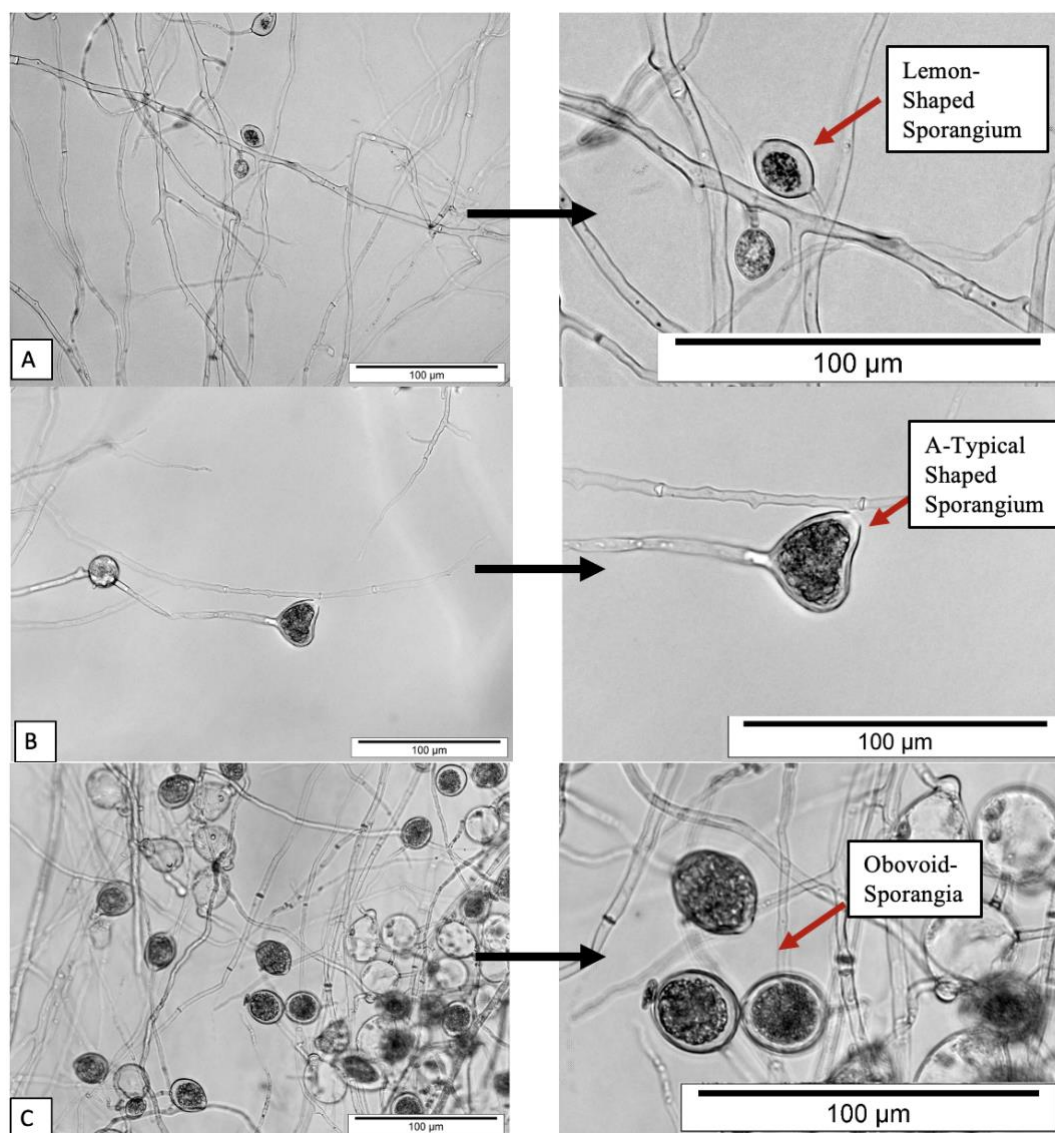


Figure 4.7: (A) Lemon-shaped sporangia produced by isolate LC1, (B) A-typical-shaped sporangia produced by isolate LWN1, & (C) Obovoid-shaped sporangia produced by isolate NG1 in sterile distilled water and viewed under a compound microscope at 200x magnification

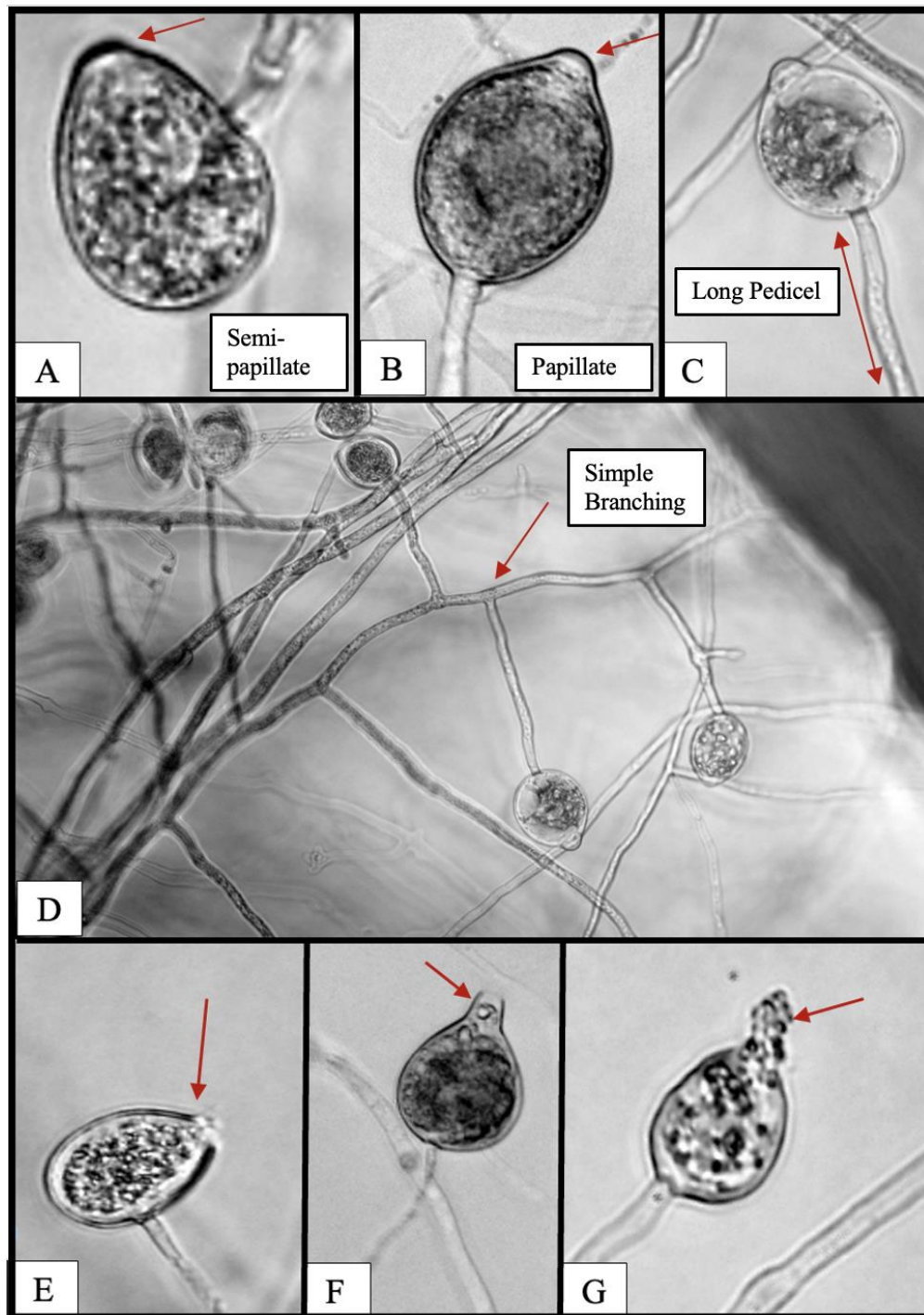


Figure 4.8: (A) Semi-papillate sporangia; (B) Papillate sporangia; (C) Sporangia with long pedicel; (D) Simple sympodium branching; (E, F & G) Germinating sporangium releasing zoospores, viewed under a compound microscope at 200x magnification

Table 4.5: Average sporangia length (µm) and width (µm)

Isolate name	Average Sporangia Length (µm) ± SEM*	Average Sporangia Width (µm) ± SEM*
IJ1	22.5±1.96	14.9±1.14
EBK1	22.8±0.99	16.4±1.13
NG1	24.7±0.44	19.1±1.14
MRI1	24.0±1.32	17.4±0.83
NCY1	21.8±0.81	14.7±1.08
GER1	26.8±2.06	18.1±1.23
NGD1	25.8±1.29	20.2±0.69
MBI1	25.4±2.05	19.1±1.47
LWN1	26.2±1.65	18.3±1.62
LC1	22.4±0.40	16.2±1.55

*SEM represents the standard error of the mean.

4.3.3 Oogonia Production

The ability to produce oogonia (Figure 4.8) on V8A media plates upon pairing the isolates with one other indicated that they are of different mating types, as both Type A1 and A2 are required for sexual reproduction to occur. These double-walled spherical structures and their antheridium were observed in all three replicates of the paired opposite mating type isolates. The isolates that produced oogonia were categorised as group A and group B. Isolates within groups A and B did not produce any oogonia in all three replicate plates when paired with each other.

Two sets of pairing tests were done. The first set was between farms where the representative isolates from each farm were paired with one another (Figure 4.9). Next, pairing was done similarly, but between isolates within the same farm. This process was done for an isolate from all farms, except for isolates from NCY and MBI farms as only one

isolate existed from these two farms. Most of the isolates within the same farm (IJ, EBK, MRI, GER, NGD and LC), when paired together, did not produce oogonia except isolates from NG farm (Figure 4.10) and LWN farm (Figure 4.11).

Isolates NG 4 and NG 7 produced oogonia when paired with other isolates from the farm. While isolates LWN 2 and LWN 3 also produced oogonia when paired with other LWN isolates. Out of the 52 isolates from this study, 28 were grouped in group A, while 24 were grouped in group B (Table 4.6). The ratio of isolates from both groups was 7:6 . For all isolates from this study, chlamydospores were not observed from any of them.

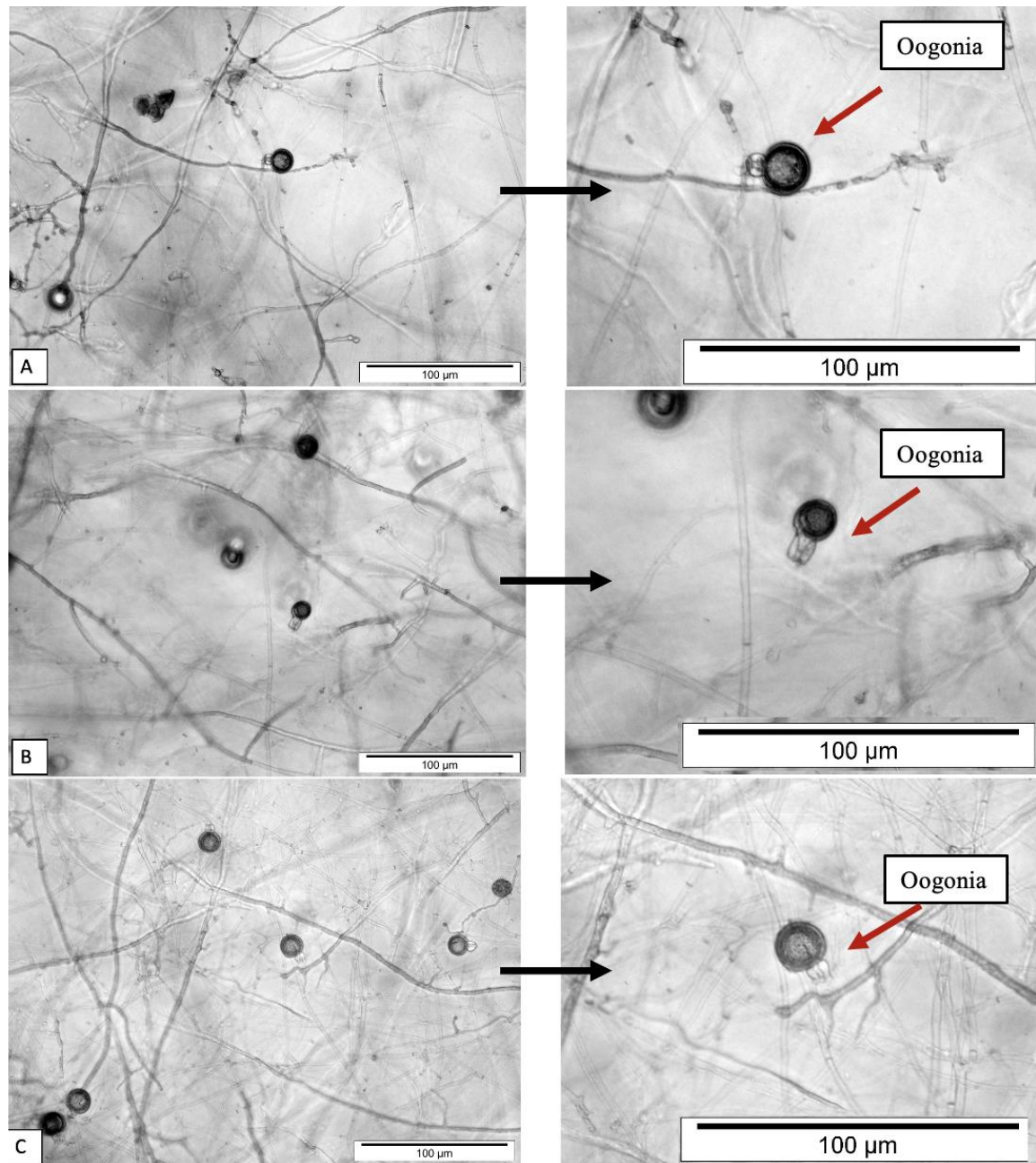


Figure 4.9: Oogonia production upon pairing between (a) IJ1 & EBK1 isolates; (b) GER1 & MBI1 isolates; (c) LC1 & NGD1 isolates

	IJ1	EBK1	NG1	MRI1	NCY1	GER1	NGD1	MBI1	LWN1	LC1
IJ1		Green	Red	Green	Red	Green	Green	Red	Red	Red
EBK1			Green	Red	Green	Red	Red	Green	Green	Green
NG1				Green	Red	Green	Green	Red	Red	Red
MRI1					Green	Red	Red	Green	Green	Green
NCY1						Green	Green	Red	Red	Red
GER1							Red	Green	Green	Green
NGD1								Green	Green	Green
MBI1									Red	Red
LWN1										Red
LC1										

Figure 4.10: Results of pairing of isolates between farms; green boxes indicate the presence of oogonia upon mating, while red boxes indicate the absence of oogonia

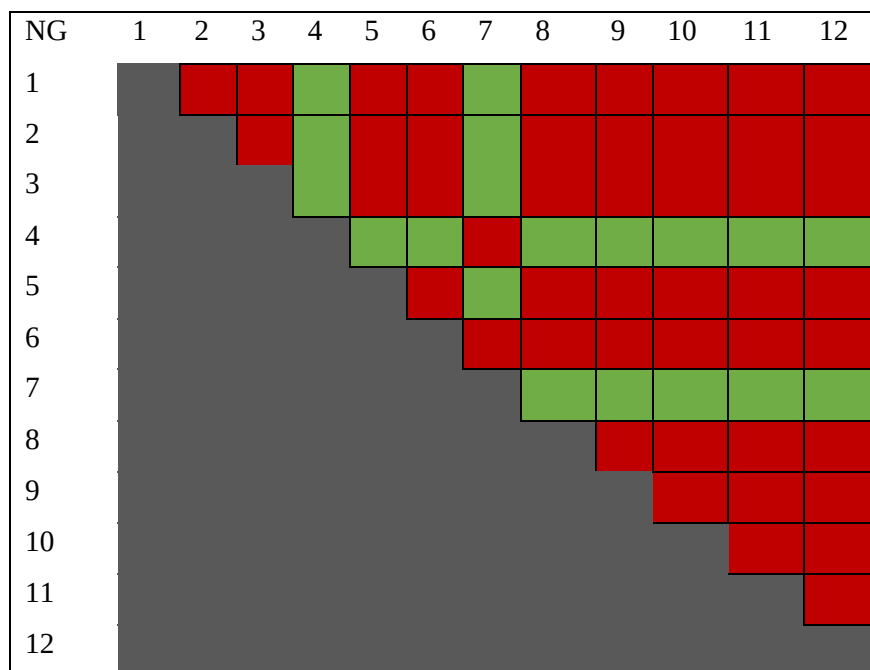


Figure 4.11: Results of pairing of isolates within NG farm; green boxes indicate presence of oogonia upon mating while red boxes indicate the absence of oogonia

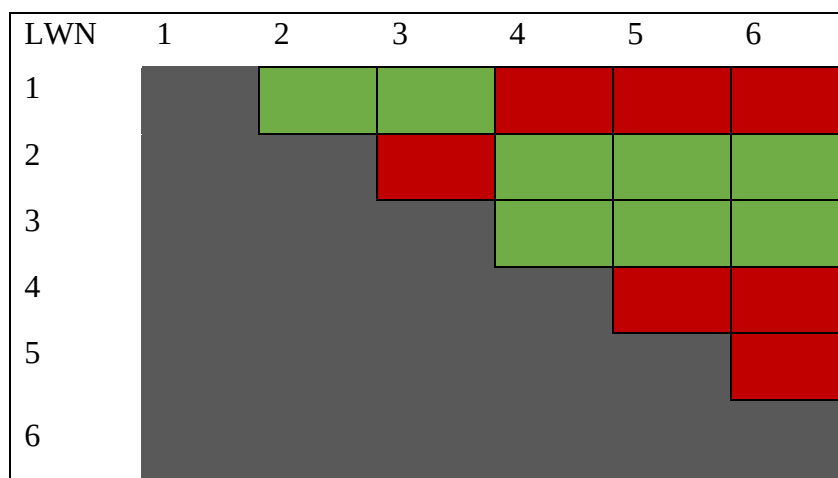


Figure 4.12: Results of pairing of isolates within LWN farm; green boxes indicate presence of oogonia upon mating while red boxes indicate absence of oogonia

Table 4.6: Mating type classification of all isolates

Farm Code	Isolate Code	Division	Mating Type	Farm Code	Isolate Code	Division	Mating Type
IJ	IJ1	Sarikei	A	MRI	MRI1	Sarikei	B
	IJ2	Sarikei	A		MRI2	Sarikei	B
	IJ3	Sarikei	A		MRI3	Sarikei	B
	IJ4	Sarikei	A		MRI4	Sarikei	B
	IJ5	Sarikei	A		MRI5	Sarikei	B
	IJ6	Sarikei	A		MRI6	Sarikei	B
	IJ7	Sarikei	A		MRI7	Sarikei	B
	IJ8	Sarikei	A		MRI8	Sarikei	B
	IJ9	Sarikei	A		MRI9	Sarikei	B
	IJ10	Sarikei	A		MRI10	Sarikei	B
EBK	EBK1	Sarikei	B	GER	GER1	Betong	B
	EBK2	Sarikei	B		GER2	Betong	B
NG	NG1	Sarikei	A		GER3	Betong	B
	NG2	Sarikei	A	NGD	NGD1	Betong	B
	NG3	Sarikei	A		NGD2	Betong	B
	NG4	Sarikei	B		NGD3	Betong	B
	NG5	Sarikei	A		NGD4	Betong	B
	NG6	Sarikei	A		NGD5	Betong	B
	NG7	Sarikei	B	LWN	LWN1	Betong	A
	NG8	Sarikei	A		LWN2	Betong	B
	NG9	Sarikei	A		LWN3	Betong	B
	NG10	Sarikei	A		LWN4	Betong	A
	NG11	Sarikei	A		LWN5	Betong	A
	NG12	Sarikei	A		LWN6	Betong	A
NCY	NCY1	Sarikei	A	LC	LC1	Serian	B
MBI	MBI1	Betong	A		LC2	Serian	B

4.4 Radial Growth Rate

To study the radial growth rate of *P. capsici*, the 10 representative isolates (IJ1, EBK1, NG1, NCY1, MRI1, NGD1, GER1, MBI1, LWN1, and LC1) were plated on CMA, V8A and PDA media plates, in which 10 replicates were made for every isolate on each tested media.

4.4.1 Radial Growth Rate

Analysing all isolates together, the mean growth rates on V8A (0.61 ± 0.01 cm/day), CMA (0.56 ± 0.01 cm/day), and PDA (0.44 ± 0.01 cm/day) indicate an overall trend in growth preferences. This suggests that, on average, isolates tend to perform better on V8A, followed by CMA and PDA (Table 4.7).

The mean growth rates (Table 4.7) (in cm/day) of the ten representative isolates across three different media: CMA, V8A, and PDA. In the context of the V8A media, the isolates exhibited a growth rate ranging from 0.49 cm/day (EBK1) to 0.72 cm/day (NG1). Notably, several isolates (IJ1, NG1, MRI1, NCY1, GER1, LWN1, and MBI1) demonstrated statistically faster growth rates compared to EBK1 and LC1.

For CMA, the colony growth rate ranged from 0.46 cm/day (NGD1) to 0.71 cm/day (MRI1). Isolate NG1, MRI1 and NCY1 demonstrated faster growth on CMA, while isolate IJ1, EBK1, GER1, MBI1, and NGD1 exhibited comparatively slower growth rates. On PDA, the growth rate spanned from 0.29 cm/day (EBK1) to 0.50 cm/day (NG1). Most isolates (IJ1, NG1, MRI1, GER1, NGD1, MBI1, LWN1 and LC1) did not differ statistically from one another in terms of growth rate on PDA except for isolate EBK1.

Table 4.7: Results mean growth rate (cm/day) of isolates on three different media: V-8 Juice Agar (V8A), Corn Meal Agar (CMA) and Potato Dextrose Agar (PDA)

Isolates	Mean Growth Rate (cm) $\pm$ SEM on V8A	Mean Growth Rate (cm) $\pm$ SEM on CMA	Mean Growth Rate (cm) $\pm$ SEM on PDA
IJ1	0.61 $\pm$ 0.023 ^{abc}	0.51 $\pm$ 0.011 ^{cd}	0.43 $\pm$ 0.012 ^{ab}
EBK1	0.49 $\pm$ 0.018 ^{cd}	0.50 $\pm$ 0.017 ^{cd}	0.29 $\pm$ 0.008 ^c
NG1	0.72 $\pm$ 0.038 ^a	0.66 $\pm$ 0.013 ^a	0.50 $\pm$ 0.014 ^a
MRI1	0.71 $\pm$ 0.027 ^{ab}	0.69 $\pm$ 0.011 ^a	0.46 $\pm$ 0.017 ^a
NCY1	0.72 $\pm$ 0.042 ^a	0.71 $\pm$ 0.014 ^a	0.37 $\pm$ 0.010 ^b
GER1	0.64 $\pm$ 0.025 ^{ab}	0.49 $\pm$ 0.014 ^{cd}	0.45 $\pm$ 0.013 ^a
NGD1	0.60 $\pm$ 0.020 ^{bc}	0.46 $\pm$ 0.014 ^d	0.44 $\pm$ 0.018 ^{ab}
MBI1	0.60 $\pm$ 0.025 ^{abc}	0.48 $\pm$ 0.014 ^{cd}	0.49 $\pm$ 0.020 ^a
LWN1	0.62 $\pm$ 0.019 ^{ab}	0.53 $\pm$ 0.015 ^{bc}	0.49 $\pm$ 0.025 ^a
LC1	0.39 $\pm$ 0.027 ^d	0.58 $\pm$ 0.016 ^b	0.44 $\pm$ 0.006 ^{ab}
All Isolates	0.61 $\pm$ 0.01 ^a	0.56 $\pm$ 0.01 ^b	0.44 $\pm$ 0.01 ^c

*SEM represents the standard error of the mean.

** Mean radial mycelial growth on different media for each isolate followed by the same letter indicating no significant difference ($p > 0.05$).

In general, the growth rate observed across V8A, CMA, and PDA media reveals distinct isolate-specific responses. Consistently, isolates NG1 and MRI1 showcased notably higher growth rates across all three tested media – V8A, CMA and PDA – compared to other isolates. Conversely, isolate EBK1 demonstrated comparatively lower growth rates across all three media.

4.5 Pathogenicity and Aggressiveness of *Phytophthora capsici*

4.5.1 Pathogenicity Test for *Phytophthora capsici*

The experiment's primary objective was to evaluate the pathogenicity of the ten selected isolates (IJ1, EBK1, NG1, NCY1, MRI1, NGD1, GER1, MBI1, LWN1, and LC1) over 8 days observation on young Kuching cultivar shoots. No visible lesions emerged within the initial 48 hours after inoculation across all replicates for each of the isolates. Starting from day 3 (72 hours post-inoculation), some isolates (IJ1, NG1, LWN1, GER1, MBI1, and NGD) caused lesions measuring less than 2.00 cm. By day 6, distinct lesions became apparent for most isolates. However, MRI1 displayed only a small lesion, while isolates EBK1, NCY1, and LC1 showed no evident lesions until day 6.

Upon reaching day 8, lesions were observable in all shoots, except for isolate EBK1, where the lesion size remained notably small (less than 1.00 cm). Importantly, all ten tested isolates induced disease in the young Kuching cultivar shoots. Based on the outcomes, it can be inferred that *P. capsici* from Sarikei, Betong, and Serian are pathogenic on the Kuching cultivar. The progression of disease manifestation, as illustrated in Figure 4.12, demonstrates a notable shift in lesion appearance from day 6 to day 8 or 9.

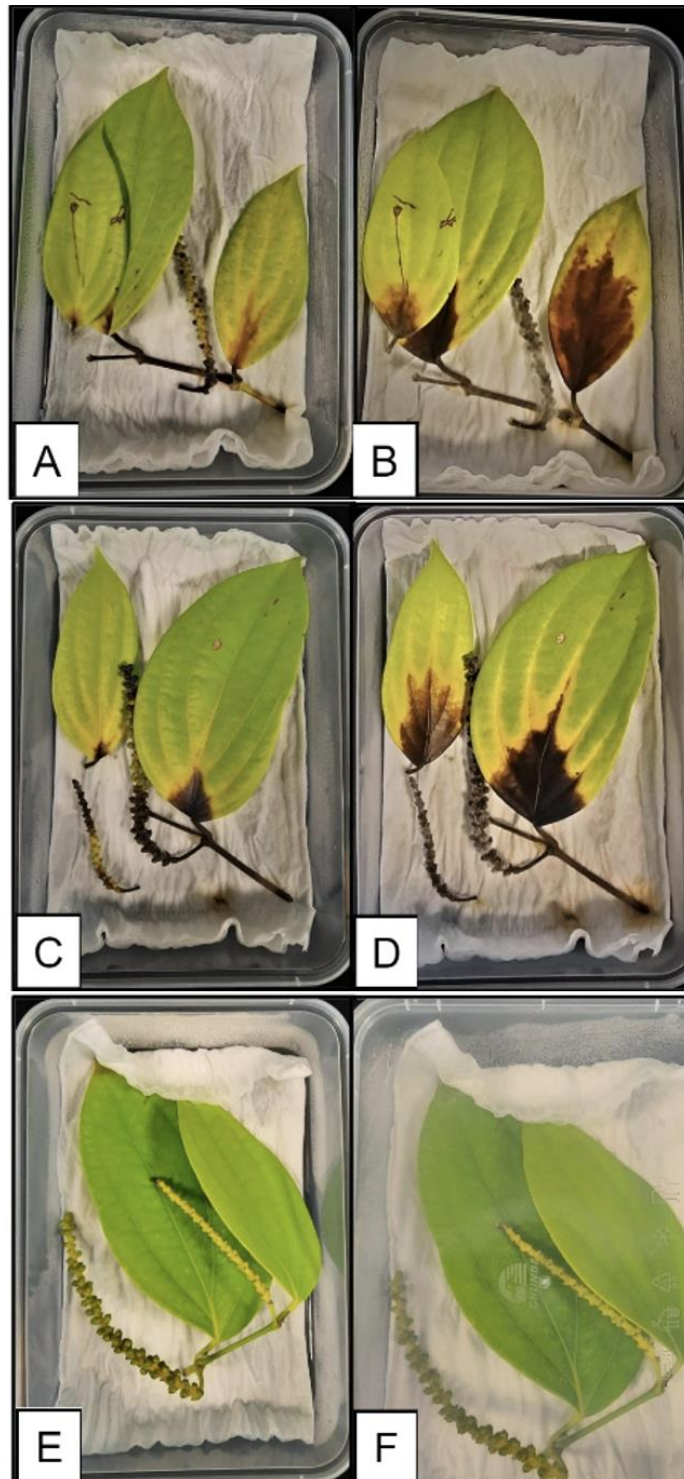


Figure 4.13: Disease progression in black pepper shoots caused by *Phytophthora capsici* isolates. (A & B) show IJ1 infection on shoot on day 6 & 9; (C & D) display GER1 infection on shoot on days 6 & 8; (E & F) represent the control shoot on days 6 & 9, showing no visible lesions

4.5.2 Aggressiveness Test – Kuching Cultivar

In the aggressiveness test of *P. capsici* on the Kuching cultivar which spanned over 120 days of observation, the aggressiveness of 10 representative isolates (IJ1, EBK1, NG1, NCY1, MRI1, NGD1, GER1, MBI1, LWN1, and LC1) from each farm was evaluated on young black pepper vines (4 months old). The primary measure of aggressiveness was the time taken to cause vine death (Figure 4.13).

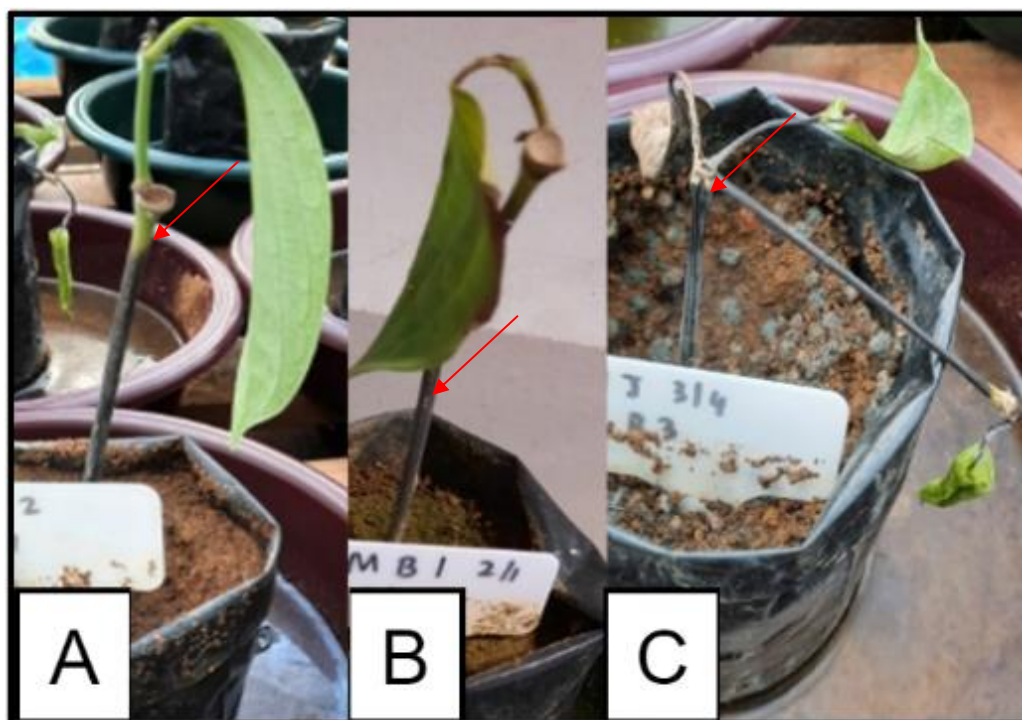


Figure 4.14: Disease progression on black pepper vines caused by *Phytophthora capsici* isolates on the Kuching cultivar during aggressiveness test: (A): lesion on NG1 vine, representing an early stage of infection; (B): lesion on MBI1 vine representing an intermediate stage of infection; and (C): lesion on IJ1 vine representing an advanced stage of infection

Based on the Kaplan Meier Survival analysis (Figure 4.14), there was a significant difference between the aggressiveness of the isolates on the black pepper vines compared to the control vines ($p < 0.05$) except for EBK1, NCY1, MRI1, and NGD1 ($p > 0.05$). This indicates that the remaining isolates are virulent towards black pepper vines. The Log-Rank

(Mantel-Cox) test (refer to Appendix A) was employed for pairwise comparisons to further assess the degree of aggressiveness between the isolates. In this experiment, some isolates stood out as remarkably more aggressive than others in which isolates IJ1, NG1, MRI1, GER1, MBI1, and LWN1 demonstrated a comparatively higher aggressiveness than the other isolates while not significantly differing from one another. Conversely, the isolates EBK1, NCY1, NGD1, and LC1 did not show statistically significant differences in aggressiveness compared to each other or the control group ($p < 0.05$), indicating that the degree of aggressiveness was not distinguishable from the non-inoculated control.

During the aggressiveness test, a rapid onset of vine death was observed within the initial 20 days. Notably, isolates IJ1 and NG1 caused 100% vine death within the first 15 and 17 days, respectively. Similarly, isolates LWN1, MRI1, MBI1, and GER1 resulted in rapid vine death, with all vines in the three replicates succumbing within 30 days.

Isolate NCY1 resulted in the death of two out of three replicates on days 17 and 49, respectively. Notably, re-isolation of the pathogen was successful in both cases. However, for the third replicate of NCY1, the vine died on the 111th day, and pathogen re-isolation of the pathogen was unsuccessful. Isolate EBK1 led to the death of one out of three replicates (46 days), while the other two vines survived until the last day of the experiment. All the control vines remained alive and free from *Phytophthora* foot rot disease symptoms until the end of the experiment (Figure 4.15)

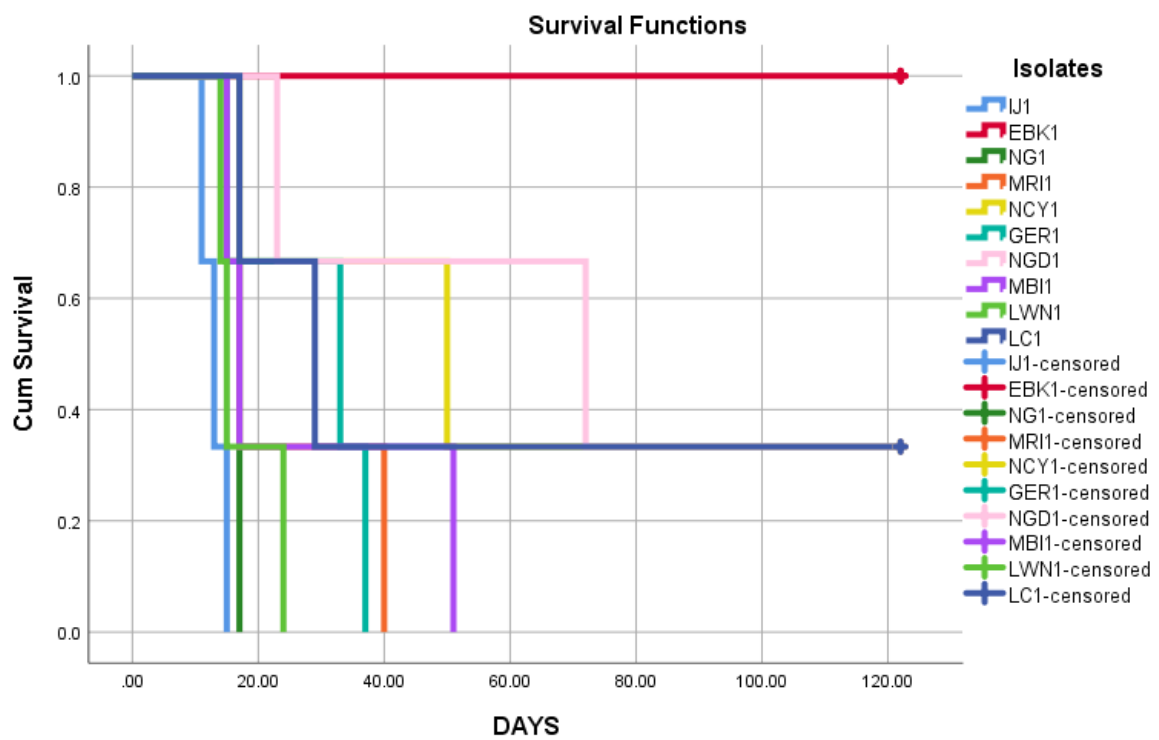


Figure 4.15: Kaplan-Meier survival analysis comparing the aggressiveness of *Phytophthora capsici* isolates on Kuching cultivar black pepper vines against the control vines

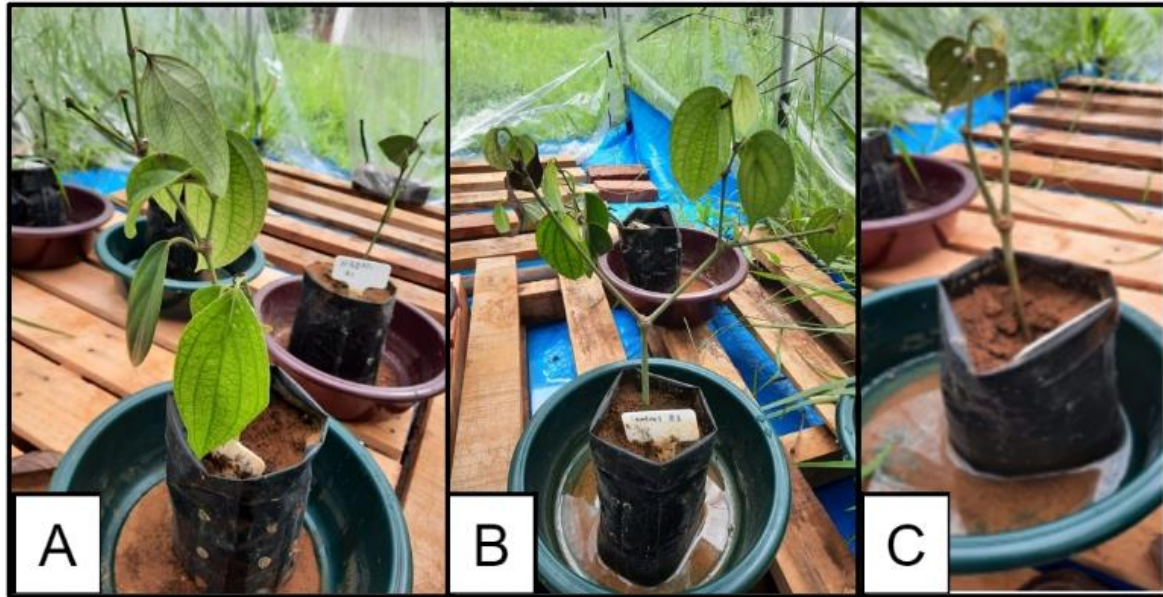


Figure 4.16: Control vines at the end of the experiment (A): control replicate 1; (B): control replicate 2; and (B): control replicate 3.

Pathological observations (Figure 4.16) revealed black lesions and slimy texture on the root tissues, collar, and stem of diseased vines. Successful pathogen re-isolation was predominantly achieved from rapidly killed vines within the initial 30 days. The re-isolated cultures were comparable to the original isolate for its morphological traits such as hyphae structure and colony patterns.

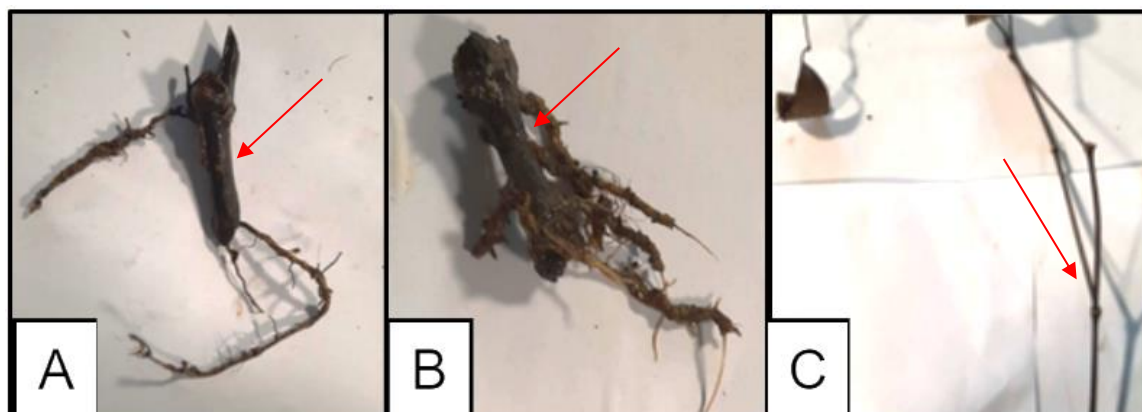


Figure 4.17: (A & B) Black lesions and a slimy texture on the root tissues, collar, and (C) stem of the deceased vines

4.5.3 Aggressiveness Test – Semenggok Aman Cultivar

For the Semenggok Aman cultivar, only five (IJ1, EBK1, GER1, MBI1 and LC1) out of the 10 isolates were selected for the aggressiveness test with seven replicates for each isolate. In this trial, two sets of positive controls were included, with IJ1 isolate being inoculated onto distinct wild pepper *P. colubrinum* and *Piper* spp. plant vines.

All infected black pepper vines exhibited a characteristic set of symptoms (Figure 4.17), including the emergence of black lesions starting from the base. These lesions could extend to all plant components, encompassing roots, collar tissues, stems, leaves, and black pepper berries. Root tissues, collar tissues, and stems transitioned from light brown to black. Despite these changes, the distinct foul smell noted in the field was absent in black pepper vines. Control vines (7 replicates) remained alive until the experiment ended on day 90 (Figure 4.18).

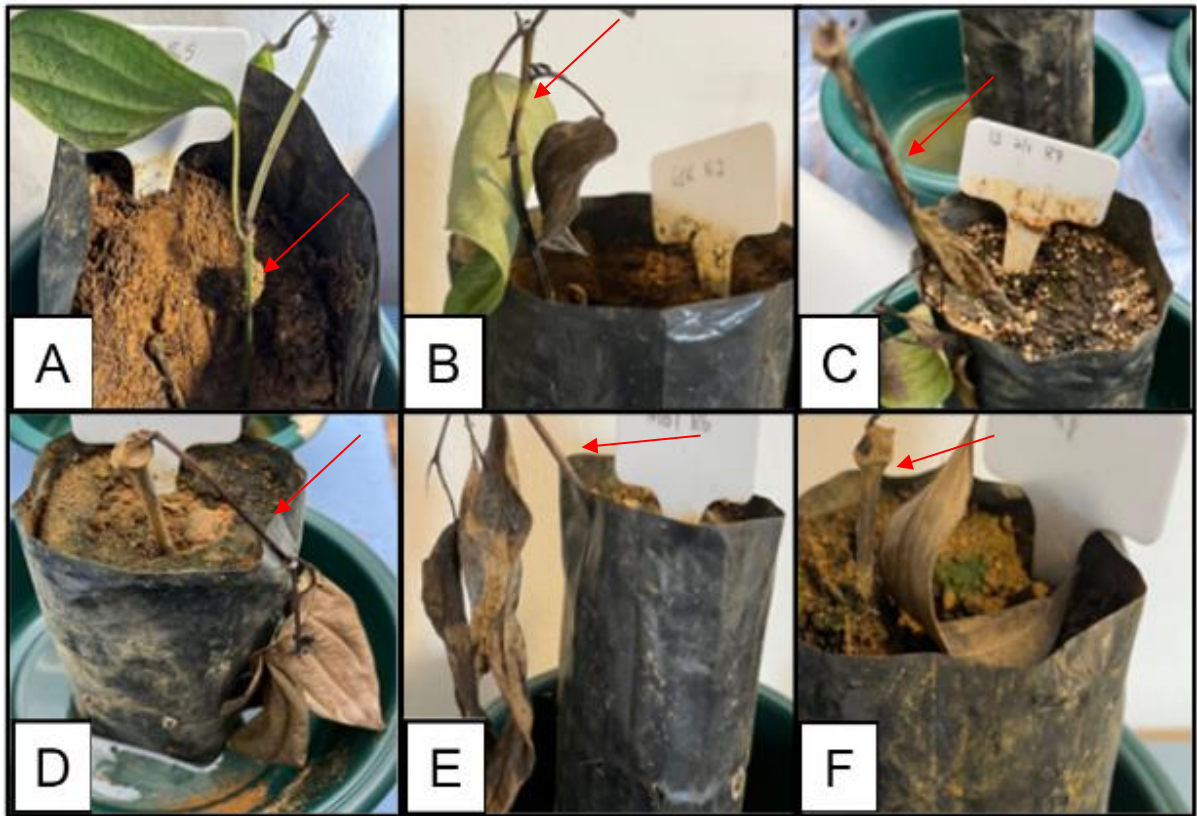


Figure 4.18: Infected black pepper semenggok aman vines with (A): EBK1 ; (B): GER1 ; (C): IJ1; (D): IJ1; (E): MBI1; and (F) LC1 isolates.

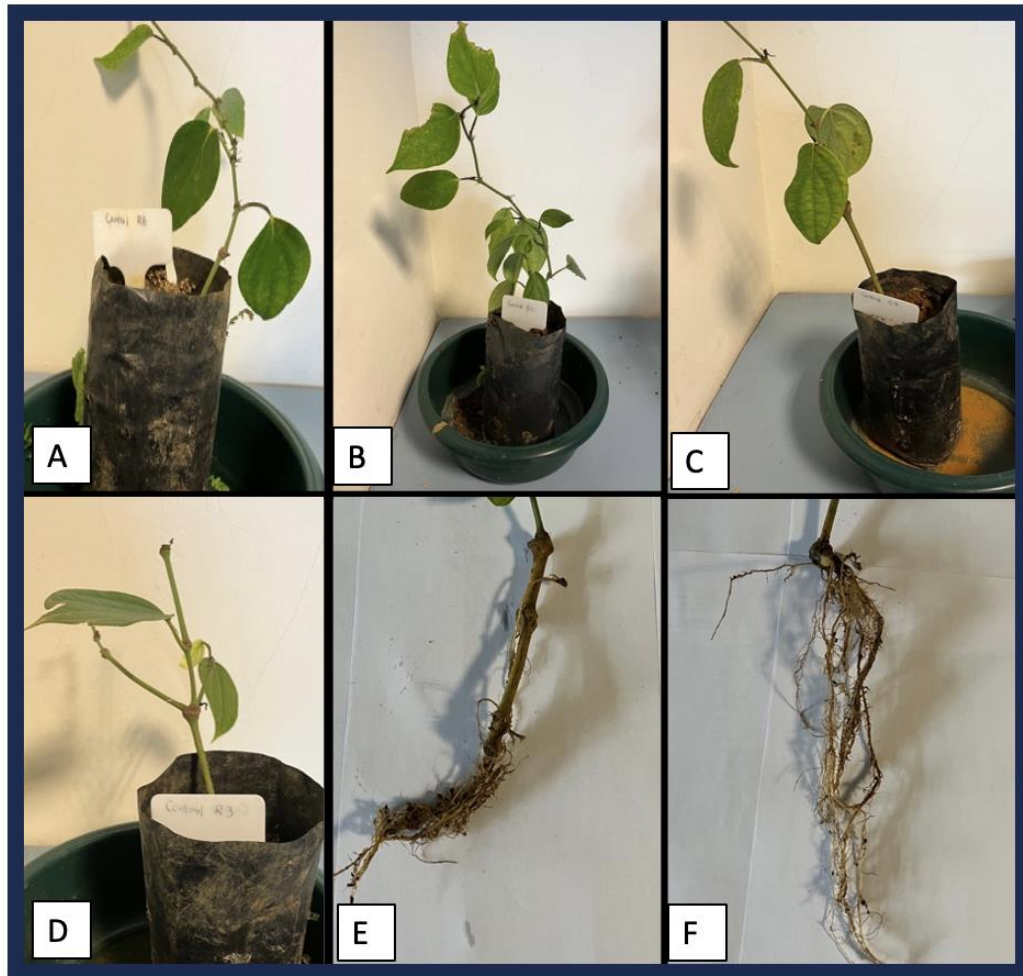


Figure 4.19: Control vines at the end of the experiment and their disease-free roots (A): Control replicate 1; (B): control replicate 2 ; (C): Control replicate 3; (D): Control replicate 4; (E): Control replicate 1 healthy root tissues; and (F) cControl replicate 2 healthy root tissues

The Kaplan-Meier survival plot (Figure 4.19) illustrates the survival plot of black pepper vines inoculated with *P. capsici* isolates, providing a comprehensive view of their relative aggressiveness. The plot shows a clear divergence in survival rates, indicating significant differences in the ability of the isolates to induce vine death. Isolate IJ1 exhibited the most rapid decline in survival, followed by GER1 and MBI1. Conversely, isolates EBK1 and LC1 displayed a comparatively slower decline. The control group, lacking the pathogen, demonstrated the highest survival rate throughout the observation period.

The pairwise comparison (Appendix B) performed further highlighted significant differences in survival patterns among *P. capsici* isolates, providing key insights into their relative aggressiveness. All tested isolates were significantly different ($p < 0.05$) from the control group indicating that all tested isolates from this experiment were disease-causing. Notably, isolate IJ1 exhibited significantly higher aggressiveness ($p < 0.05$), inducing vine death more rapidly than EBK1, GER1, MBI1 and LC1,

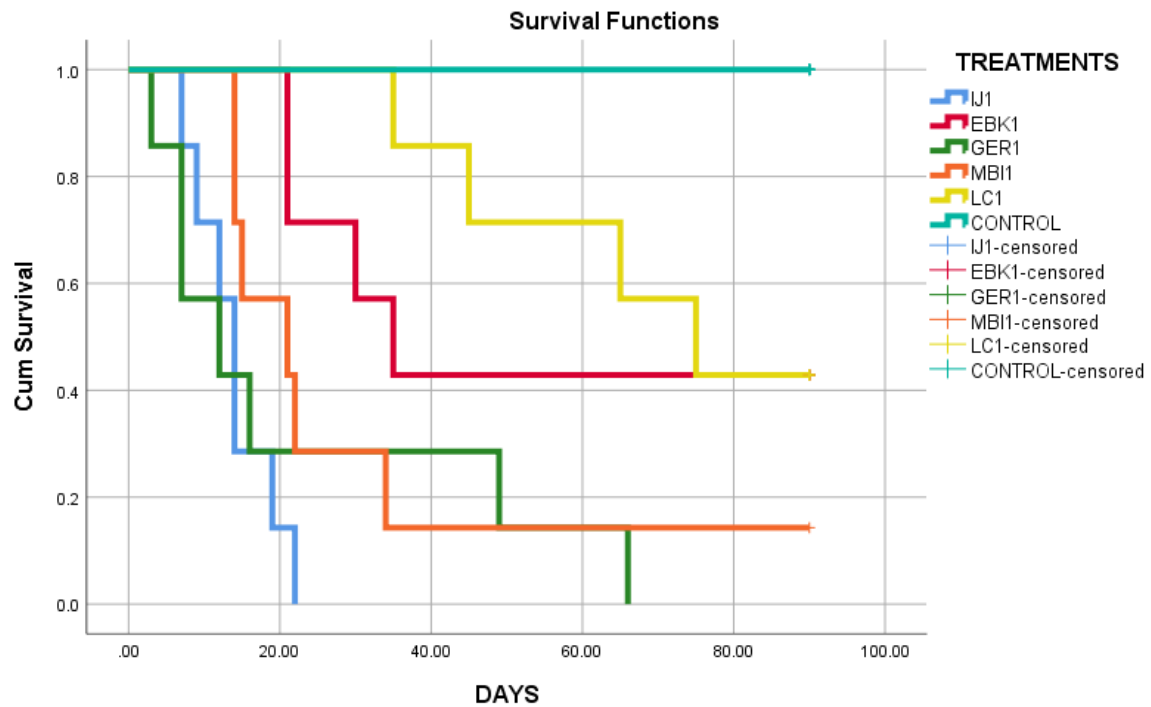


Figure 4.20: Kaplan-Meier survival analysis comparing the aggressiveness of *Phytophthora capsici* isolates on Semenggok Aman cultivar black pepper vines against the control vines

GER1 was significantly more aggressive ($p < 0.05$) compared to EBK1 and LC1 indicating variations in their ability to induce vine death. Similarly, MBI1 was also more

virulent compared to EBK1 and LC1 ($p < 0.05$). The aggressiveness between GER1 and MBI1 was not significantly different. It was also true between EBK1 and LC1.

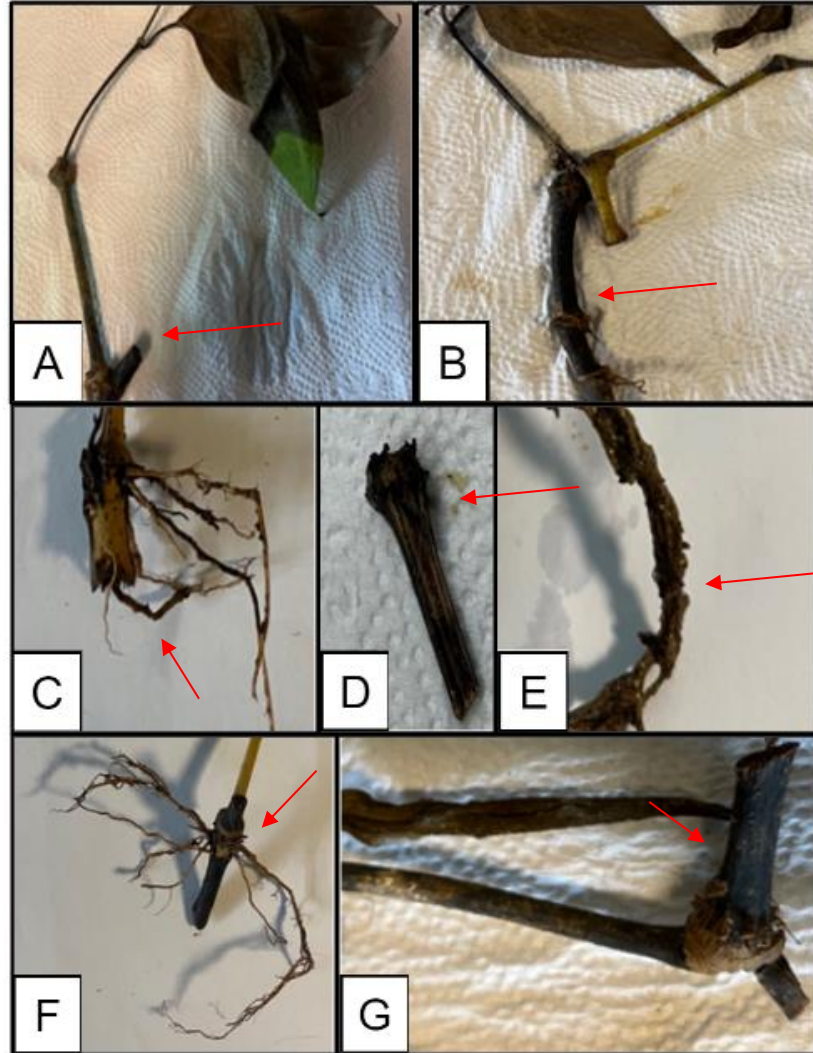


Figure 4.21: Symptoms of foot rot disease observed in inoculated black pepper shoots (A to B): stem and leaf with lesion; (C to G): infected roots with black lesion.

4.5.4 Aggressiveness Comparison of *Phytophthora capsici* isolates between Cultivars

To assess the varying levels of aggressiveness among different *P. capsici* isolates on two black pepper cultivars, namely Kuching and Semenggok Aman, five *P. capsici* isolates (IJ1, EBK1, GER1, MBI1, and LC1) which were inoculated onto the two cultivars (section

4.5.2 and section 4.5.2) were compared based on the time it took for the isolates to cause plant death.

The median time to vine death across both cultivars was 27 days. The nonparametric Kruskal-Wallis test revealed no statistically significant difference in the time to vine death between the two cultivars ($H(1) = 0.090$, $p = 0.764$), suggesting that the cultivar type does not have a significant impact on the aggressiveness of the isolates as measured by time to plant death.

4.5.5 Virulence of IJ1 against Wild Pepper Species

Wild Cutting, Type 1 (*P. colubrinum*) displayed no symptoms when inoculated with IJ1 isolate (Figure 4.21: A and B); all seven replicates remained healthy, and no pathogen was isolated from their root tissues.

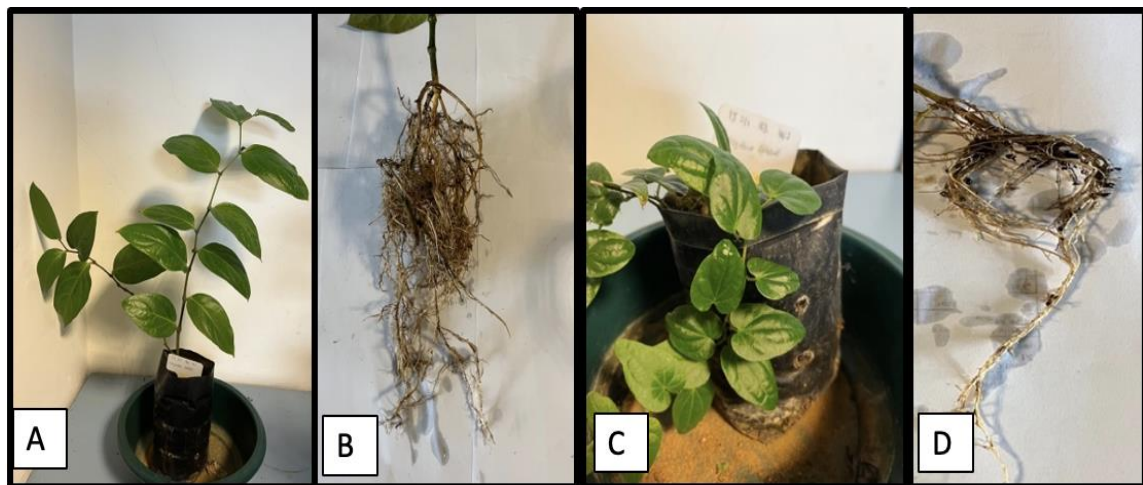


Figure 4.22: (A & B) Wild cutting Type 1 (*Piper colubrinum*); (C & D) wild cutting Type 2 (*Piper* spp.); at the end of the experiment

In contrast, wild cuttings Type 2 (*Piper* spp.) were susceptible to IJ1 isolate, resulting in disease development and vine death for 4 replicates (Figure 4.22); 2 replicates remained healthy at the end of the experiment (Figure 4.21: C and D).

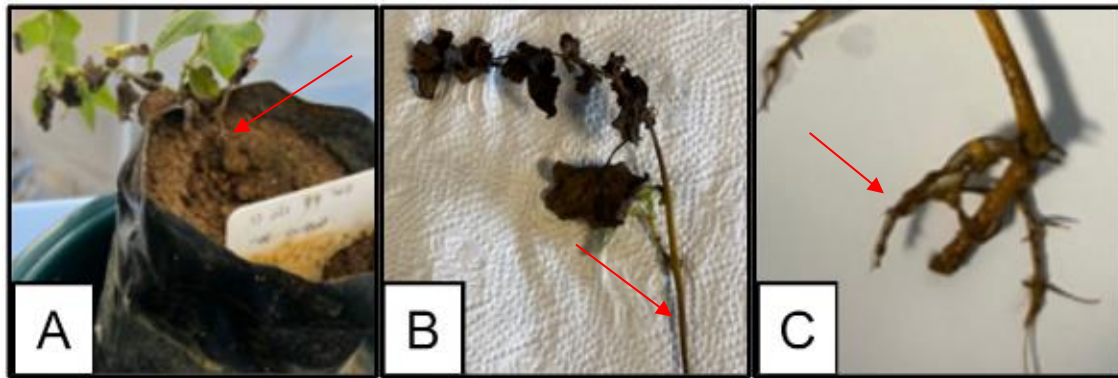


Figure 4.23: Infected wild Type 2 (*Piper* spp.) (A): wild Type 2 vine infection; (B) leaf and shoots with black lesion; and (C) rotten wild Type 2 root tissues

To compare the degree of virulence of IJ1 isolate between Semenggok Aman cultivar black pepper cuttings and wild cuttings Type 1 and Type 2 another Kaplan Meier survival analysis was conducted (Figure 4.23). The pairwise comparison (Appendix C) revealed that there is a significant difference ($p < 0.05$) among all three plant groups. IJ1 isolate was the most virulent on the Semenggok Aman cultivar. Isolate IJ1 also had a certain degree of virulence towards Type 2 wild cutting although not as virulent as it was on the Semenggok Aman cultivar. Meanwhile, isolate IJ1 did not have any virulence towards Type 1 wild cutting. This result may be indicative that this black pepper wild cutting may be resistant to IJ1 isolate.

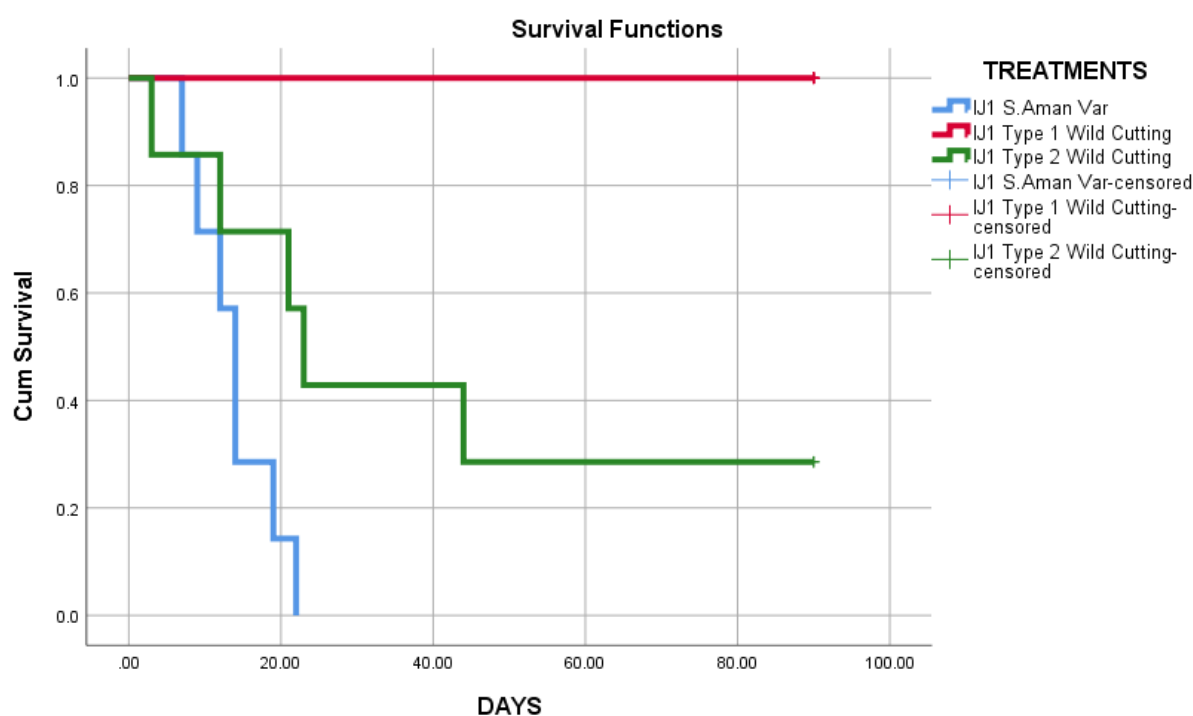


Figure 4.24: Kaplan-Meier survival analysis comparing the virulence of *Phytophthora capsici* IJ1 isolate on Type 1(*Piper colubrinum*) wild pepper and Type 2 (*Piper* spp.) wild pepper cuttings against the Semenggok Aman cultivar black pepper vines

4.6 Molecular Characterisation of *Phytophthora capsici* Isolates

4.6.1 ITS Sequence Analysis and Neutrality Test

The 30 sequences were aligned using Clustal W (Mega X) which resulted an aligned sequence of 799 bp. Out of the 799 bp, 16 sites were variable (7, 10, 11, 12, 48, 118, 122, 153, 449, 726, 733, 742, 755, 757, 765, and 790) (Appendix D) Most of the polymorphisms can be seen located towards the terminals of the sequence (5' – site 7 to 153 and site 726 to 765 – 3'), only one site in the middle, site 449, is polymorphic. Overall, the region in the middle of the alignments was observed to be highly conserved.

From the Neighbour-Joining Tree (Figure 4.24) with the Jukes-Cantor Model, the 30 sequenced isolates formed two clusters, but no clear clustering based on location, morphology, or mating type was observed. Isolates from Betong, Sarikei and Serian can be seen to be closely related as they clustered except for isolate IJ5. IJ5 was observed to form its own clade.

The analysis of polymorphism and neutrality tests of the ITS sequence for *P. capsici* isolates from Sarawak is provided in Table 4.8. The 30 *P. capsici* ITS sequences revealed a total of 11 segregating sites (S) and 9 haplotypes (H!). The observed haplotype diversity (Hd) was 0.559, and the nucleotide diversity (π) was calculated to be 0.00123. Theta (θ), another measure of nucleotide polymorphism, was estimated to be 0.00350. Neutrality tests, including Tajima's D, Fu and Li's D, and Fu and Li's F, were negative values and were significantly different.

Table 4.8: Result summary DNA Sequence polymorphism (DNASP) of polymorphism and neutrality test of ITS region

Population	N	S	H!	Hd	π	θ	Tajima's D	Fu Li's D	and Fu Li's F
Sarawak	30	11	9	0.559	0.00123	0.00350	- 2.08590*	- 2.56970*	- 2.83297*

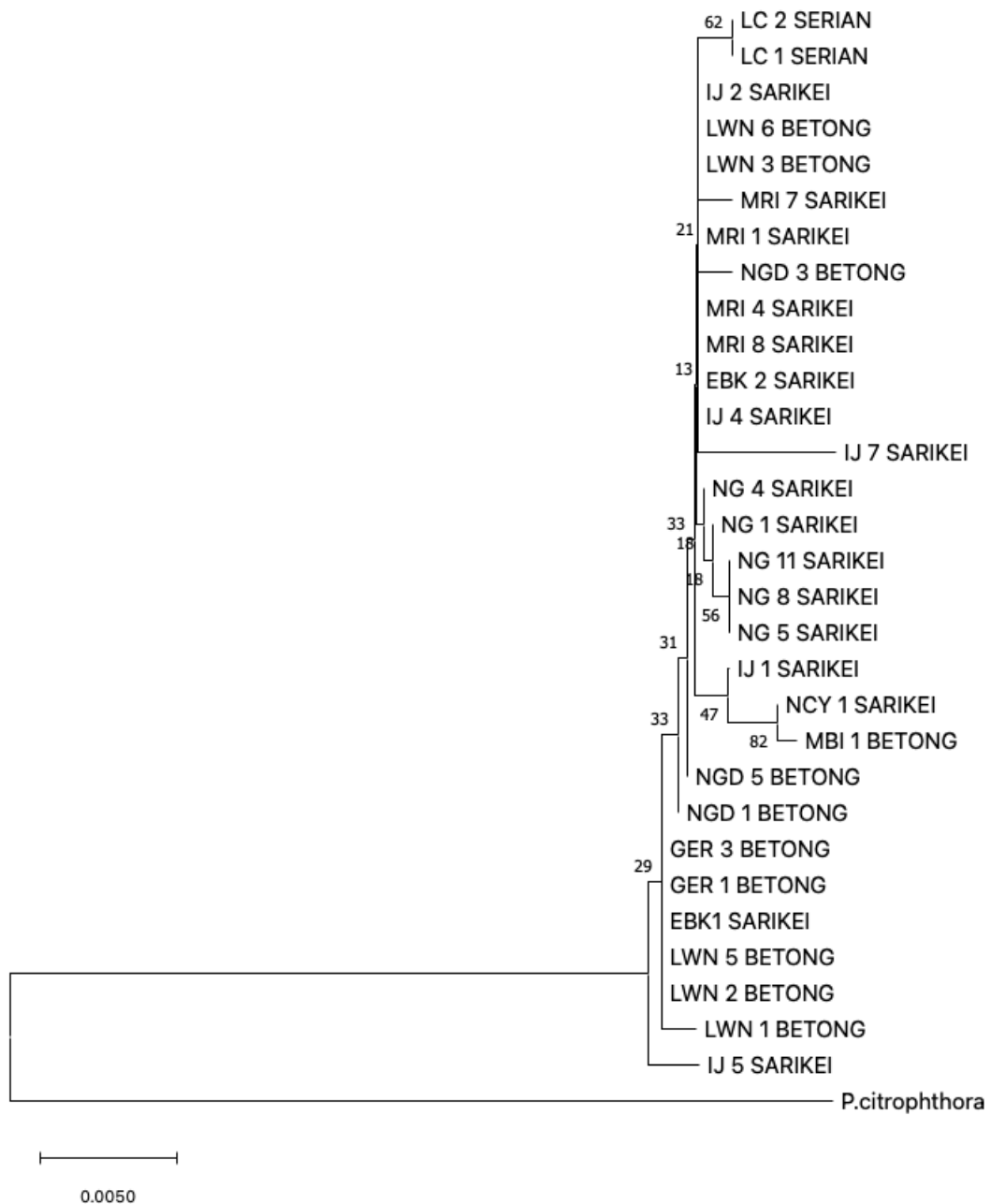


Figure 4.25: Neighbour-Joining dendrogram of 30 isolates of *Phytophthora capsici* from Sarawak, constructed using Jukes-Cantor model and with *Phytophthora citrophthora* as outgroup

4.6.2 Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR)

In the genetic characterization of *P. capsici* isolates, both Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR) were employed. For RAMS, six primers described by Truong et al. (2010) were tested. However, after optimisation, three primers—CGA, CCA, and GT (Table 4.9 and Figure 4.25; F to H) were selected due to their consistent production of clear and reproducible bands. While, for ISSR, a set of thirteen primers reported by Li et al. (2012) were initially evaluated. After optimization, five primers—UBC835, UBC873, UBC807, UBC808, and UBC841 (Table 4.9 and Figure 4.25; A to E) were chosen. These eight selected primers were then employed to fingerprint the 52 *P. capsici* isolates (30 molecularly identified isolates and 22 morphologically identified isolates). In this case, a total of 63 bands were scored.

Table 4.9: Selected primer name, sequence and annealing temperature upon optimisation

Primer Name	Sequence (5'-3')	Annealing Temperature (°C)
RAMS		
CGA	DHB(CGA) ₅	57
CCA	DD(GA) ₅	55
GT	YHY(GT) ₇ G	59
ISSR		
UBC 835	(AG) ₈ YC	49
UBC 808	(AG) ₈ C	51
UBC 873	(GACA) ₄	51
UBC 841	(GA) ₈ YC	49
UBC 807	(AG) ₈ T	49

The ISSR and RAMS primers exhibited different numbers of bands scored and the number of polymorphic bands detected (Table 4.10 and Table 4.11). Notably, the RAMS primer GT produced the highest number of polymorphic bands (8 polymorphic bands), while

UBC 841 and UBC 853 from the ISSR primers yielded the highest number of polymorphic bands (9 polymorphic bands).

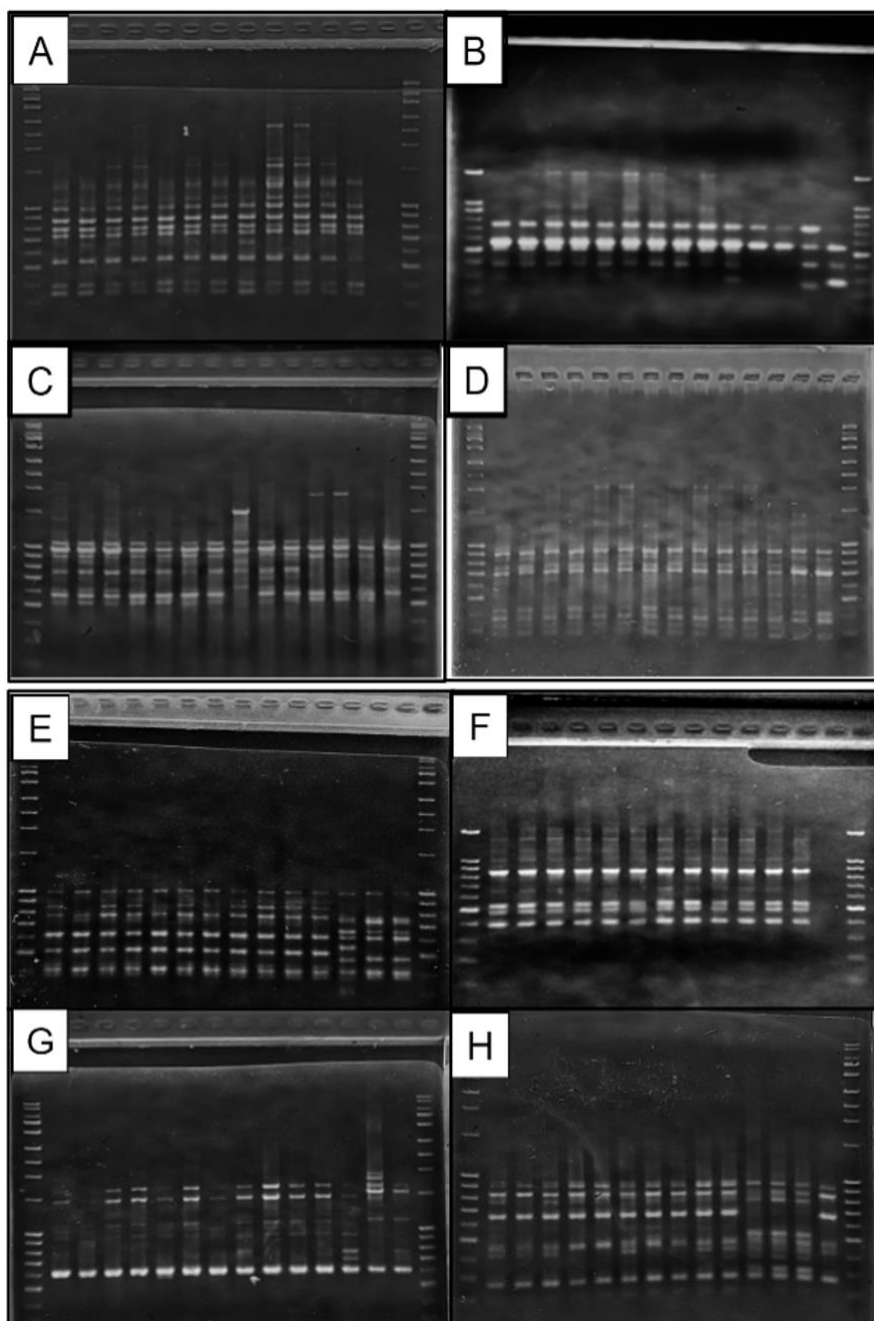


Figure 4.26: Gel electrophoresis images (A) UBC 808; (B) UBC 835; (C) UBC 873; (D); UBC 841(E) UBC 807; (F) CGA; (G) CCA; (H) GT

Table 4.10: Performance of each Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR) primer tested on *Phytophthora capsici*

Primer	Band Scored	Polymorphic Bands
RAMS		
CGA	7	6
CCA	6	6
GT	9	8
ISSR		
UBC 853	11	9
UBC 808	6	5
UBC 873	6	6
UBC 841	10	9
UBC 807	8	8

Table 4.11: Summary statistics of Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR) analysis

No.	Particulars	Molecular Marker System		
		RAMS	ISSR	ISSR + RAMS
1	Number of individuals	52	52	52
2	Total Number of Primer Combination	3	5	8
3	Total Number of Scorable Bands	22	41	63
4	Average Number of bands per primer	7	8	8
5	Total Number of Polymorphic Bands	20	37	57
6	Total Number of Monomorphic Bands	2	4	6
7	Percent of Polymorphism (%)	91	90	90

4.6.3 DNA Fingerprinting Analysis

RAMS and ISSR scoring matrices were combined to study the population structure of *P. capsici*, resulting in a total of 63 polymorphic bands scored (Table 4.11). UPGMA dendrogram was constructed and three main clades were observed (Figure 4.26).

Clade 1 comprised isolates from all three studied divisions: Sarikei, Betong, and Serian. Subsequently, Clade 1 can be further divided into two sub-clades: Sub-clade A,

consisting of all Betong isolates and two isolates from EBK in Sarikei, and Sub-clade B, comprising all IJ isolates from Sarikei and LC isolates from Serian. It is worth noting that Clade 1 primarily consisted of isolates from Betong and Serian, except for isolates from IJ and EBK which were from Sarikei.

Clade 2 was composed of isolates exclusively from Sarikei. Within this clade, isolates from Sarikei farms (NG, MRI, and NCY) clustered together, except for isolate MRI 8 (Sarikei), which formed its own distinct clade, referred to as Clade 3. Additionally, Clade 2 can be further divided into two sub-clades: Subclade C, consisting of isolates from MRI and NCY, and Subclade D, solely composed of all the isolates from the NG farm. Overall, the population structure analysis revealed that most isolates clustered based on their geographic location of origin.

The population genetic analysis was performed based on isolates of different divisions. The two isolates from Serian were excluded due to the low sample size. Only two populations, Sarikei and Betong were studied. Table 4.12 summarises key genetic parameters, indicating a higher genetic diversity in Sarikei with an observed number of alleles (N_a) at 1.587 and a polymorphic loci percentage (P) of 69.84%. In contrast, Betong shows lower diversity ($N_a = 1.365$, $P = 49.21\%$). Shannon's Information Index (I), Diversity (h), and Unbiased Diversity (u_h) all follow this trend, with Sarikei displaying higher values, suggesting a greater genetic variability among its isolates.

The analysis of molecular variance (AMOVA) based on geographical location revealed that the genetic variation among the *P. capsici* population was 27% (Table 4.13). The variation within the population accounts for a larger portion (73%) of the total genetic variation.

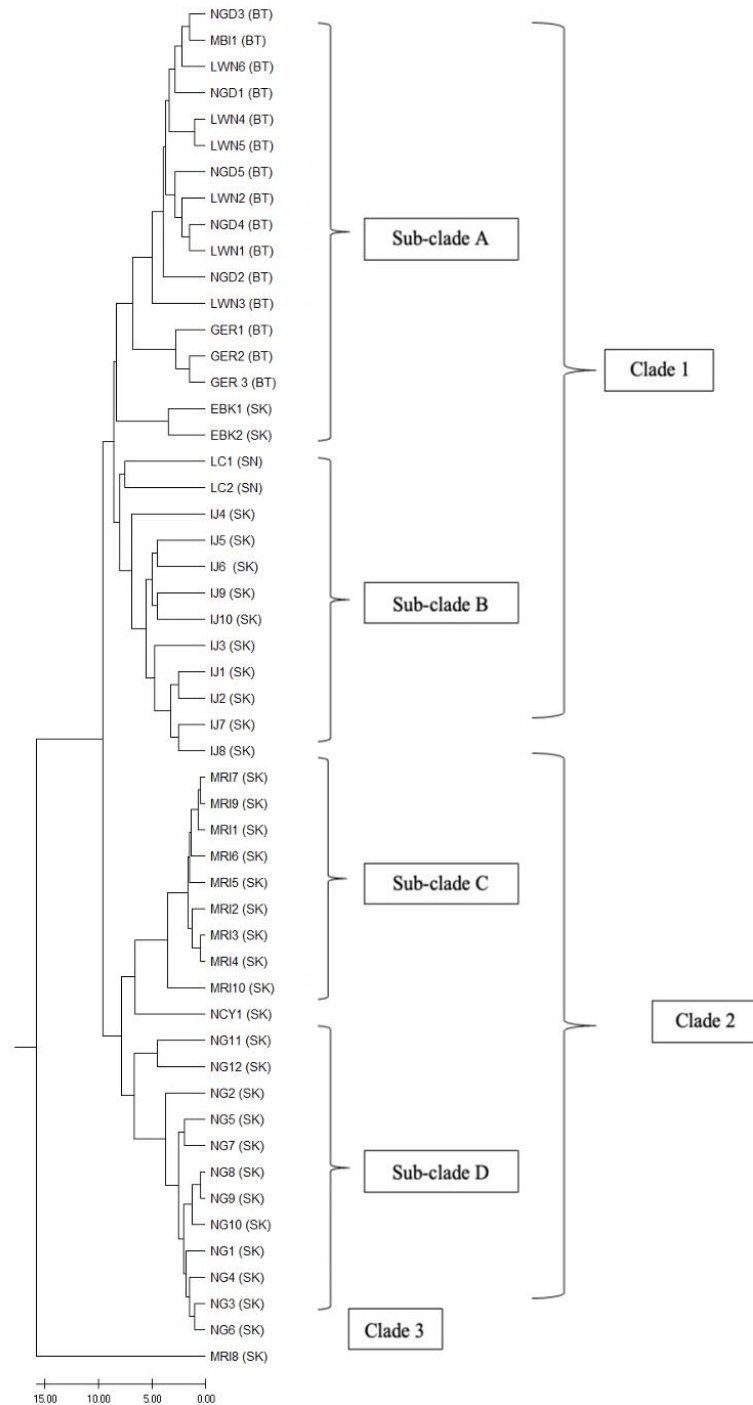


Figure 4.27: Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram of 52 *Phytophthora capsici* isolates based on combined Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR) data; (SN) Serian; (SK) Sarikei; (BT) Betong

Table 4.12: Genetic variation and polymorphic metrics amongst *Phytophthora capsici* isolates from Sarawak using Random Amplified Microsatellites (RAMS) and Inter Simple Sequence Repeats (ISSR) markers analysed with GenAlex

Parameters	N	Na	Ne	P (%)	I	h	uh
Location							
Sarikei	34	1.587± 0.087	1.398± 0.047	69.84	0.353± 0.034	0.234± 0.025	0.241± 0.025
Betong	15	1.365± 0.089	1.248± 0.042	49.21	0.234± 0.033	0.152± 0.023	0.162± 0.025
Grand mean	24.5	1.476± 0.062	1.323± 0.032	59.52± 10.32	0.294± 0.024	0.193± 0.017	0.202± 0.018

N = Number of isolates; Na = Observed number of alleles; Ne = Effective number of alleles; P = Percentage of polymorphic loci; I = Shannon's Information Index, as a measure of genetic diversity; h = Diversity; uh = Unbiased Diversity

Table 4.13: Summary of results of Analysis of Molecular Variance (AMOVA)

Parameters	df	SS	MS	Est. Var.	%
Location					
Among Population	1	63.740	63.740	2.692	27
Within Population	48	346.000	7.208	7.208	73
Total	49	409.740	-	9.900	100

Df = Degree of Freedom; SS = Sum of Squares; MS = Mean Squares; Est. Var. = Estimated Variance; % = Percentage of Genetic Variation

CHAPTER 5

DISCUSSION

5.1 Sampling

5.1.1 Symptoms of *Phytophthora* Foot Rot Disease in Sarawak Pepper Farms

The symptoms observed in the field from this study, such as rotten roots with foul smell, yellowing leaves, and quick wilt, are consistent with those described in previous reports for *Phytophthora capsici* from black pepper crops by Kong et al. (2022) and Farhana et al. (2013) in Sarawak and by other authors from various countries (Jibat and Alo, 2021; Nguyen, 2015; Truong et al., 2008). The damage caused by the pathogen to the root tissue is likely to inhibit the vascular tissues from transporting water to the leaves and other parts of the vine (Nguyen, 2015), causing commonly observed symptoms such as yellowing leaves and quick wilt.

5.2 Isolation of *Phytophthora capsici*

5.2.1 Isolation Techniques

The tailored strategy of employing two distinct techniques based on sample conditions was pivotal for successfully isolating *P. capsici*. For the samples from Betong and Sarikei, tissue plating was the method of choice, primarily due to its compatibility with newly infected samples and the intact nature of root tissues.

In contrast, leaf baiting was deemed necessary for isolates from LC (Serian) and MBI (Betong), mainly when root tissues were extensively decomposed. A significant finding in this study was the effectiveness of using black pepper leaves in the leaf baiting technique,

deviating from the conventional method of *Capsicum annuum* leaves described by French-Monar et al. (2007).

5.2.2 Internal Transcribed Spacers (ITS)

This study effectively identified the selected isolates by applying Internal Transcribed Spacers (ITS) sequencing. Recognised for its high reliability, the ITS regions serve as DNA barcode markers for fungal identification, as Schoch et al. (2012) reported. The 30 examined isolates from this study displayed identity percentages between 99.37% and 100% compared to sequences deposited in NCBI, reconfirming their morphological-based pre-identification as *P. capsici*.

This study's outcomes align with prior research, affirming ITS sequence's validity for fungal pathogen identification (Ghosh et al., 2019) and specifically with findings from Latifah et al. (2018) and Kong et al. (2022) which highlights the technique's reliability for distinguishing *P. capsici* from other *Phytophthora* spp. In this study, ITS sequencing alone proved effective for identifying isolates at the species level. Although integrating it with other additional molecular methods, such as mitochondrial cytochrome c oxidase gene (COX) sequences, could enhance accuracy and identification—as demonstrated by Smith et al. (2019), who confirmed the presence of *P. capsici* in Trinidad's hot peppers using both ITS and COX sequences.

5.2.3 Prevalence and Potential Transmission of *Phytophthora* Foot Rot

Notably, the disease's prevalence was substantial in specific farms in Sarikei, like IJ and MRI, where nearly 90% of vines were dead. Furthermore, a spatial analysis revealed a concentration of affected farms along the Kanowit River, hinting at a potential waterborne transmission of the pathogen, given *P. capsici*'s characterisation as a “water mould” (El

Meleigi et al., 2019) and is substantiated by several documented instances of *Phytophthora* spp. isolation from rivers and canals (Gevens et al., 2007; Brazee et al., 2016; Stamler et al., 2016). Although there are observations related to potential waterborne transmission in the current study, this remains speculative without direct sampling and testing from the river. Future studies could incorporate water sampling and analyse the presence and concentration of the pathogen in river water to draw a definitive conclusion.

Black pepper farms in Betong and Serian presented a contrasting scenario with steep terrains and no observed interconnecting water bodies, suggesting a lower likelihood of waterborne transmission. However, the presence of the disease implies alternative transmission routes, possibly via contaminated tools, planting materials, or lack of sanitisation. In Serian, LC's pepper garden experienced a 100% total yield loss. Nonetheless, no other infected pepper farms were identified in Serian, highlighting the isolated nature of the outbreak in this region.

5.3 Morphological Characteristics

5.3.1 Colony Morphology

The morphologies were media-dependent. On V8A, we observed variations such as slightly petaloid, concentric rings, and stellate. In contrast, CMA primarily showed patterns like petaloid, stellate, and concentric rings. On PDA, however, predominantly exhibited stellate and rosette morphologies. Notably, a significant portion of the isolates (7 out of 10) displayed morphological differences across the three media. This highlights the adaptability of *P. capsici* and its response to varying growth conditions. Each of the ten representative isolates from this study exhibited unique morphological responses across the three tested media types: CMA, V8A, and PDA. For instance, the isolate IJ1 displayed a petaloid

morphology on V8A and CMA while displaying a stellate pattern on PDA; these morphologies were unique to IJ1, and no two isolates behaved identically across the three tested media.

In a breakdown of morphological prevalence across all media, petaloid and stellate morphologies emerged as dominant, each accounting for 26.67%. The 'no pattern' morphology followed at 23.33%, and rosette and concentric ring characteristics were observed in 13.33% of cases. The cottony, petaloid and stellate colony patterns are also commonly observed (Bhai et al., 2022; Kong et al., 2022; Moreira-Morrillo et al., 2022; Smith et al., 2019). The colony patterns observed from the ten representative isolates from different farms were all unique in terms of colony patterns. Tian & Babadoost (2004) reported that from their morphological study on PDA involving six different *P. capsici* isolates, they have observed various growth patterns such as stellate, rosaceous and petaloid that also did not depend on the geographical origin of the isolate.

It is to note that, upon long term culturing of the isolates, it was observed that the colony morphology of the isolates have faced slight changes. Hence, depending on colony morphology for variant identification did not seem appropriate due to the changing nature of colony morphological characteristics as the isolate ages upon re-culturing from stock over a certain period of time. Similar observations have been reported by Kong (2023) who noted that long term stock culture incubation could influence the colony morphology of *P. capsici* isolates.

5.3.2 Hyphae Structure Observation

In contrast to the diversity observed in the growth patterns across different media, the hyphae structures exhibited a striking uniformity among all isolates. Every isolate

consistently displayed swollen torulose hyphae (Figure 4.6). Interestingly, hyphae showed a pronounced coiling during their younger stages, which became progressively less dense as the cultures matured. The swollen, torulose hyphae is consistent with the *P. capsici* morphological structure reported by Leonian (1922), Granke et al. (2009), Farhana et al. (2013), Shivakumar and Somasekhara (2018), and Kong et al. (2022).

5.3.3 Sporangia Production

All isolates from this study could produce sporangia, with the most common sporangia shape being globose in shape, contrasting the typically reported lemon-shaped sporangia, a characteristic feature of *P. capsici* as described by Mchau and Coffey (1995) and Farhana et al. (2013). Nevertheless, the deviation from commonly observed lemon-shaped sporangia was consistent with that reported by Kong et al. (2022) and Moreira-Morrillo et al. (2022), in which spherical to ovoid shapes were commonly observed.

The isolates yielded diverse sporangia shapes, including the key identification feature for *P. capsici*, the lemon-shaped sporangia, albeit less frequent. The morphological observation encompassed both semi-papillate and papillate sporangia, with a prevalent appearance of a long pedicel and simple sympodium branching. Spores are released when the sporangia's papillate ruptures. These morphological observations are consistent with the findings of Kong et al. (2022), Moreira-Morrillo et al. (2022), and Farhana et al. (2013),

The statistical analysis showed no significant variations in the dimensions of sporangia, with the lengths and widths observed ranging from 21.0 to 28.0 μm and 15.7 to 22.1 μm , respectively. These results mirror the dimensions reported by Kong et al. (2022), where the author found the sporangia dimensions of *P. capsici* isolated from black pepper vines of Sarawak to be between 24.15 to 25.66 μm in length and 15.28 to 21.89 μm in width.

Conversely, the findings of Smith et al. (2019) from Trinidad presented larger sporangia dimensions, with lengths ranging from 29.6 to 55.4 μm and widths from 23.3 to 37.1 μm . This highlights the potential influence of geographical and environmental factors on sporangia morphology, as Li et al. (2007) reported.

5.3.4 Oogonia Production

Similar to the findings of Smith et al. (2019), upon pairing the isolates, the presence of oogonia could indicate that the pairing isolates are of the opposite mating type. The observation of oogonia upon pairing specific isolates together may suggest that both mating types (A1 and A2) might be present in the farms of the Sarikei and Betong divisions. In Sarikei, the ratio between the opposite mating type (A1:A2) could be 3:2. At the same time, in Betong, it is likely to be 1:1. As there were limited isolates (2 isolates) from Serian, only one mating type was detected. The detection of both A1 and A2 mating types within the studied regions signifies the potential for sexual reproduction among the isolates, which is a critical driver of genetic diversity due to the possibility of gene recombination.

5.4 Mycelial Radial Growth Rate

This study examined the growth rate of *P. capsici* on three media: CMA, V8A and PDA. Overall, a pronounced growth rate was observed on V8A (mean = 0.61 ± 0.01^a cm/day; ranging from 0.49 to 0.72 cm/day), which aligns with the trends documented by Jung et al. (2011) and Decloquement et al. (2021). The growth rate on CMA (mean = 0.56 ± 0.01^b cm/day; ranging from 0.46 to 0.71 cm/day) and PDA (mean = 0.44 ± 0.01^c cm/day; ranging from 0.29 to 0.50 cm/day) displayed significant differences when compared to V8A (Table 4.7).

Drawing comparisons with prior research, the findings from this study align with the observations made by Kong et al. (2022) and Smith et al. (2019), both of whom documented a similar preference in media-based growth rates for *P. capsici* isolates. However, it is to be noted that the observed growth rate of V8A media from this study is marginally slower than the growth rate of *P. capsici* isolates documented in Trinidad, which hovered between 0.7 to 0.8 cm/day (Smith et al., 2019).

Isolates NG1 and MRI were part of the group that consistently showed higher growth rates on all three tested media (V8A, CMA, and PDA) in comparison to other isolates. Conversely, isolate EBK1 belonged to the group with lower growth rates on these media. NCY1, GER1, and IJ1 were also grouped with isolates showing higher growth on V8A and CMA but had moderate to lower rates on PDA. This pattern aligns with the observations of Kong et al. (2022) and Smith et al. (2019), who noted elevated growth rates of some *P. capsici* isolates on CMA and V8A compared to PDA.

While the predominant trend of isolates preferring V8A isolate LC from Serian was notable for its uniquely slow growth on V8A, setting it apart from the others. The observed trends in radial mycelial growth across different media highlight that specific isolates demonstrate clear media preferences. The overall preference of *P. capsici* for V8A and CMA media over PDA provides insights when choosing a medium for the best cultivation efficiency of this pathogen.

5.5 Pathogenicity and Aggressiveness of *Phytophthora capsici*

In the pathogenicity test, all tested isolates (10 representative isolates from different farms) are pathogenic. The results of this study align with multiple studies conducted on

Phytophthora foot rot disease, where *P. capsici* is identified as the primary invader of black pepper (Kong et al., 2022; Truong et al., 2008; Vinitha et al., 2016).

The same 10 representative isolates (IJ1, EBK1, NG1, NCY1, MRI1, NGD1, GER1, MBI1, LWN1, and LC1) were tested for the aggressiveness test on the Kuching cultivar. However, in the aggressiveness test conducted on the Kuching cultivar, the number of tested isolates was reduced from ten to five compared to the previous experiment conducted with Kuching cultivar. This reduction was strategically implemented to allow for an increased number of replicates per treatment—seven instead of the previous three. The five isolates selected for this experiment, namely IJ1, EBK1, GER1, MBI1, and LC1, were chosen based on their location where isolate IJ1 and EBK1 were from Sarikei, isolates GER1 and MBI1 were from Betong while isolate LC1 was from Serian. Furthermore, these isolates also represent a mix of opposite mating types.

From the series of experiments conducted in this study, it was clear that varying degree of aggressiveness was observed between isolates (Figure 4.14 and Figure 4.19). In the aggressiveness test performed on the Kuching cultivar, the four isolates named EBK1, NCY1, NGD1, and LC1 exhibited a level of aggressiveness that was not significantly different from the non-infected control group. While these isolates are pathogenic, meaning they could cause disease in the Kuching cultivar, they did not lead to a significant amount of death of the pepper vines within 120 days. Compared to the other six isolates tested, these four isolates (EBK1, NCY1, NGD1, and LC1) were significantly less aggressive ($p < 0.05$).

Conversely, when tested on the Semenggok Aman cultivar, isolates EBK1 and LC1 did not show significant differences in aggressiveness compared to the control group ($p > 0.05$). However, isolates IJ1, GER1, and MBI1 demonstrated significantly higher

aggressiveness than both EBK1, LC1, and the control group ($p < 0.05$), indicating that these isolates are consistently more aggressive.

Furthermore, the lack of correlation between aggressiveness and geographical location, as observed by Vinitha et al. (2016), resonates with the findings of this study, where aggressiveness varied significantly even among isolates from the same divisions, reinforcing the notion that different isolates can exhibit significantly diverse behaviours, possibly due to genetic heterogeneity, environmental interactions, or host-specific factors. This can be seen as isolates IJ1 and EBK1 are both from Sarikei with a close proximity of 5 km distance, but, both of these isolates displayed varying levels of aggressiveness.

The sample size in the aggressiveness test, especially for the Kuching cultivar (3 replicates for each treatment), might have introduced potential biases. Hence, a larger sample size could provide more precise insights into the pathogenicity and aggressiveness variability of *P. capsici* isolates.

Given the diverse nature of *P. capsici*, the limited source of resistant cultivars and complex plant-host dynamics, Barchenger et al. (2018) reported that breeding resistant varieties (chilli peppers) against *P. capsici* is challenging. The findings from this study, where resistance was observed in wild cuttings Type 1 (*Piper colubrinum*), could indicate a potential measure of crop protection and genetic source for resistance breeding. In this context, resistant wild pepper cuttings could be used as rootstocks onto which commercially valuable, susceptible black pepper varieties are grafted as scions (An et al., 2019). This approach could confer the resistant rootstock's protective traits against *P. capsici* to mitigate infection of *P. capsici* through the soil. Grafting has been successfully employed in various crops to manage soil-borne diseases, improve abiotic stress tolerance, and enhance nutrient

uptake therefore the wild species can be considered in a breeding program to transfer the resistance from wild species into the existing black pepper varieties (An et al., 2019).

5.6 Molecular Characterisation of *Phytophthora capsici* Isolates

5.6.1 Relationship Between Isolates

Initially, a Maximum Likelihood (with Jukes-Cantor Model) tree was constructed, however, the Neighbour-Joining tree was favoured for the clarity it brought to the phylogenetic relationships, which was crucial for the interpretative aspect of this study. The construction of the Neighbour-Joining Tree using the Jukes-Cantor Model based on the ITS sequence of the 30 selected isolates although it did not display any distinct clustering based on geographical location, morphology, or mating type, it is noteworthy that isolates from Betong and Sarikei appeared to be closely related, suggesting a potential regional genetic similarity. Interestingly, isolate IJ5 (Sarikei) formed its distinct clade, indicating its genetic distinctiveness within the dataset.

While the Neighbour-Joining Tree provided some insights on the relatedness based on the ITS sequence, to further elucidate the genetic relationships among the isolates, the UPGMA cluster analysis was done. The UPGMA cluster analysis (Figure 4.28) based on the microsatellite (ISSR and RAMS) scoring offers valuable insights into the genetic relationship among *P. capsici* isolates in this study. The selection and optimisation of specific primers for both methods ensured the reproducibility and clarity of the results, as evident in the gel electrophoresis images and polymorphic index values. Truong et al. (2010) reported employing 6 RAMS markers on 118 *P. capsici* isolates from black pepper which resulted in 83.3% polymorphism. While Li et al. (2012) reported a 100% polymorphism rate using 13 ISSR primers. From this study, the overall high percentages of polymorphism

(Table 4.10 and Table 4.11) observed in both RAMS (91%) and ISSR (90%) emphasises the efficiency of RAMS and ISSR markers in detecting genetic polymorphism within the *P. capsici* populations.

Based on the UPGMA dendrogram constructed using the combined RAMS and ISSR markers, the isolates are primarily clustered based on geographic origin, with Clade 1 containing 27 isolates from Betong, Serian, and Sarikei. Clade 2 is exclusive to Sarikei isolates (22 isolates), indicating a close genetic relationship among the isolates from different farms within this location. Meanwhile, Clade 3, defined solely by isolate MRI 8 from Sarikei, suggests the presence of unique genetic variants.

Unlike the distinct clustering observed based on the geographic location of isolates, the relationship between an isolate's morphological characteristics and its genetic diversity appears unclear. The morphological diversity among the isolates varies across different media plates, and furthermore, isolate morphology also changes with long-term culturing. These factors likely contribute to the absence of a direct correlation between morphological characteristics and genetic diversity.

Comparing the phylogenetic tree constructed based on the ITS sequence and based on the ISSR and RAMS markers reveals very different clustering patterns of isolates. This may be because the tree based on ISSR and RAMS may be attributed to the differing degrees of polymorphic alleles present. The ISSR and RAMS loci typically originate from non-coding genome regions, exhibiting higher variability rates (Wang et al., 2022). Unlike the ITS region, known for its conserved and low mutation rates (Dung et al., 2013), these non-coding areas, represented by ISSR and RAMS, tend to accumulate mutations faster, offering a more detailed picture of the genetic landscape (Oliveira et al., 2006).

5.6.2 Genetic Variability Analysis

The ITS sequence analysis identified 16 polymorphic sites among the 799 aligned positions. Notably, these sites predominantly reside at the sequence ends, between sites 7-153 and 726-765, except at site 449 in the sequence's central region. This central site emphasises the conservation of the central alignment area, consistent with the known higher polymorphism in ITS 1 and ITS 2 (terminal sites) compared to the well-conserved 5.8S region (central site) (Seifert, 2009). The analysis further revealed nine distinct haplotypes across 11 segregating sites. With a haplotype diversity (Hd) of 0.559, the data suggests a moderate genetic diversity (Cárdenas et al., 2011).

Meanwhile, both the nucleotide diversity (π) and theta (θ) were observed to be low, at 0.00123 and 0.00350, respectively, suggesting limited nucleotide variations in the dataset. The individual nucleotide positions within those sequences are not that varied. These findings could suggest that some of these haplotypes may share a similar origin.

However, studying several different genes from these samples would provide more substantial evidence as reported by Wang et al. (2022) in their study demonstrated that multiple DNA barcode regions exhibited greater genetic diversity compared to the ITS region alone. For instance, the comprehensive study by Cárdenas et al. (2011) demonstrated that multiple DNA barcode regions in the study of genetic diversity of *Phytophthora infestans* in the Northern Andean region, suggesting that the inclusion of additional genomic regions such as Cox1, Ras, b-tubulin, and Avr3a can enhance the resolution of phylogenetic and diversity assessments.

From the neutrality test, a significant negative value for Tajima's D (-2.08590*), Fu and Li's D (-2.56970*), and Fu and Li's F (-2.83297*) indicate deviation from neutrality.

These negative values suggest two primary evolutionary scenarios: population expansion and selective sweep, each of which has different implications (Villarejo et al., 2020). The favourable environment in Sarawak, for *P. capsici*, might have driven a swift population expansion or on the other hand, a selective sweep arises when a beneficial mutation spreads rapidly in a population, leading to reduced genetic variation due to "hitchhiking" (Yang et al., 2018). In *P. capsici*, such a sweep could be driven by mutations that increase virulence, stress resistance, or host adaptability (Villarejo et al., 2020). The quick dominance of these traits diminishes surrounding genetic diversity.

On the other hand, in this study, the Shannon's Information index for *P. capsici*, based on combined RAMS and ISSR markers, was found to be 0.294. This figure falls within a moderate range, as evidenced by comparisons with indices from similar studies in Bhutan, China, and Vietnam, which reported values of 0.42, 0.20, and 0.33, respectively (Rai et al., 2020; Li et al., 2012; Truong et al., 2010). Similarly, studies focusing on hosts in Malaysia have demonstrated Shannon's Information Index ranging from 0.24 to 0.33 for *Phytophthora* spp. (Alsultan et al., 2021). Notably, the Betong population exhibits a lower diversity index (0.234), whereas the Sarikei population shows a slightly higher diversity (0.353).

From the analysis of molecular variance (AMOVA), most of the genetic variation is observed from within populations (73%) compared to among populations (27%). The higher genetic variation observed within the population may reflect a high incidence of either sexual reproduction amongst isolates or mutations derived asexually from the same parental source, contributing to the genetic diversity within populations (Samen et al., 2003). Meanwhile, the low genetic variation among populations may indicate ongoing gene flow between

populations (Sousa & Hey, 2013) or the possibility of a shared single origin (Truong et al., 2010)

The moderate genetic diversity indicated by the Shannon's index, coupled with signs of a selective sweep or population expansion from the neutrality test, suggests that while there is some genetic variation within the *P. capsici* populations, recent evolutionary events may have led to a proliferation of certain haplotypes over others. This proliferation, possibly due to advantageous traits, may have contributed to the moderate level of diversity detected by the RAMS and ISSR markers.

5.6.3 Relationship of Genetic Diversity with Geographical Origin of Isolates

The AMOVA results with a higher genetic variation observed within populations as opposed to among them could be reflective of these selective pressures or reproductive events occurring in localised areas, with the potential for gene flow (Sousa & Hey, 2013). The phylogenetic tree supports this, clustering isolates from both populations (Sarikei and Betong) within Clade 1, implying either historical or ongoing exchange of genetic material.

The localised clustering observed in Clade 2 suggests a potential for gene flow between farms in Sarikei such as the Kanowit River, which could facilitate gene flow within the farms of Sarikei. The observed genetic similarities between the two regions may result from shared agricultural practices or during the transfer of plant material, emphasising the need for a deeper investigation into the pathogen's transmission routes (McDonald & Stukenbrock, 2016).

5.6.4 Relationship of Genetic Diversity with Isolate's Mating Type

Understanding the relationship between genetic diversity and mating type distribution in *P. capsici* isolates is crucial, as the presence of both A1 and A2 mating types suggests the potential for sexual reproduction, a key factor in genetic diversity due to gene recombination. A balanced representation of both mating types with a ratio close to 1:1 is generally associated with higher genetic diversity, as reported by Castro-Rocha et al. (2017), Li et al. (2012), and Meitz et al. (2010). In this study, while Betong's mating type ratio is closer to the equitable 1:1 than Sarikei's, suggesting a potential for greater genetic diversity, the Shannon's Information index and AMOVA results do not reflect this. The lower diversity in Betong, contrary to expectations, could be influenced by factors such as environmental selection pressures, historical population dynamics, or even sampling bias.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study represents the first intensive study of *Phytophthora capsici* isolates from black pepper plants from three major black pepper-producing divisions of Sarawak. The findings contribute to a broader understanding of the morphological and population genetic structure of *P. capsici*, the causal agent of *Phytophthora* foot rot disease in Sarawak. From 10 of the 28 sampled black pepper farms, 52 *P. capsici* isolates were obtained, indicating the disease's prevalence in the region.

Ten representative isolates from each farm were thoroughly studied for their morphological characteristics. The morphological characterisation proved invaluable in understanding *P. capsici*'s adaptability, with noticeable variations in its growth patterns across different media. This observed variability in colony morphologies and growth rates highlights the diversity within the *P. capsici* population. The presence of both A1 and A2 mating types is particularly concerning, suggesting the potential for sexual reproduction in *P. capsici*. This capability can enhance the pathogen's genetic diversity, making it even more challenging to control and manage.

All tested isolates of *P. capsici* were found to be pathogenic on black pepper shoot cuttings. In aggressiveness tests, while all isolates were pathogenic on the Kuching and Semenggok Aman cultivar, varying degrees of aggressiveness were observed between isolates. Isolate IJ1, GER1 and MBI1 consistently demonstrated high aggressiveness in both Kuching and Semenggok Aman aggressiveness tests, whereas EBK1 and LC1 were the least virulent. Finally, the study's findings suggest that grafting commercial varieties onto

resistant wild pepper cuttings, *Piper colubrinum*, might offer protection against *P. capsici* infection.

Furthermore, the molecular characterisations, primarily ITS sequence analysis and DNA fingerprinting using RAMS and ISSR, revealed significant genetic diversity within the *P. capsici* population in Sarawak. The relatively higher genetic variation observed within populations than among populations emphasises the need for continuous monitoring and the implementation of effective cultural practices to prevent further genetic recombination. These findings contribute valuable insights for devising comprehensive strategies to manage *Phytophthora* foot rot disease and protect pepper crops in the region. This study not only highlights the genetic and morphological diversity of *Phytophthora capsici* affecting black pepper crops in Sarawak but also paves the way for targeted management strategies to combat *Phytophthora* foot rot, ultimately contributing to sustainable agricultural practices

6.2 Recommendations

Future studies could expand sampling beyond Sarawak to other pepper-growing regions in Malaysia, providing a more comprehensive view of *P. capsici*'s distribution, diversity, and aggressiveness patterns. This broader sampling would offer a comprehensive perspective on *P. capsici*'s distribution and reveal more about its diversity and aggressiveness patterns. Further investigations into environmental factors—like soil pH, temperature, and moisture—might shed light on their role in the prevalence and spread of *Phytophthora* foot rot disease.

Waterborne transmission potential, especially in areas like Sarikei with interconnected water bodies (Kanowit River), warrants deeper investigation, such as pathogen testing and examining how water quality and flow dynamics may affect pathogen

spread. With the observed variability in aggressiveness among different isolates, there is a need to investigate the underlying genetic and physiological reasons. Furthermore, an aggressiveness assay with a more extensive set of replicates would further reduce bias and better understand the varying degrees of aggressiveness amongst *P. capsici* isolates. Given the potential sexual reproduction hinted at by the presence of both A1 and A2 mating types, future research should also focus on the life cycle of *P. capsici* and how sexual reproduction might influence its evolution and adaptability. Finally, building on this study's molecular characterisation insights, advanced genomic tools like whole-genome sequencing can further clarify *P. capsici*'s genetic structure, evolutionary history, and vulnerabilities.

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APPENDICES

Appendix A: Log-Rank (Mantel-Cox) Pairwise Comparison of *Phytophthora capsici* Isolates on Kuching Cultivar Black Pepper Vines

Isolates	IJ1		EBK1		NG1		MRI1		NCY1		GER1		NGD1		MBI1		LWN1		LC1		CONTROL	
	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.	Chi - Squ are	Sig.
IJ1			5.05 2	0.02 5	3.45 9	0.06 3	3.45 9	0.06 3	5.05 2	0.02 5	3.45 9	0.06 3	5.05 2	0.02 5	3.45 9	0.06 3	1.55 6	0.21 2	5.05 2	0.02 5	5.05 2	0.02 5
EBK1	5.05 2	0.02 5			4.73 8	0.03 0	5.05 2	0.02 5	2.46 9	0.11 6	5.05 2	0.02 5	2.46 9	0.11 6	5.05 2	0.02 5	5.05 2	0.02 5	2.46 9	0.11 6		
NG1	3.45 9	0.06 3	4.73 8	0.03 0			0.38 5	0.53 5	2.77 0	0.09 6	1.36 4	0.24 3	4.73 8	0.03 0	0.38 5	0.53 5	0.00 1	0.97 1	2.77 0	0.09 6	4.73 8	0.03 0
MRI1	3.45 9	0.06 3	5.05 2	0.02 5	0.38 5	0.53 5			2.24 4	0.13 4	0.09 9	0.75 3	2.55 7	0.11 0	0.25 4	0.61 4	0.69 4	0.40 5	0.69 4	0.40 5	5.05 2	0.02 5
NCY1	5.05 2	0.02 5	2.46 9	0.11 6	2.77 0	0.09 6	2.24 4	0.13 4			1.66 7	0.19 7	0.07 4	0.78 6	0.69 4	0.40 5	2.55 7	0.11 0	0.03 2	0.85 8	2.46 9	0.11 6
GER1	3.45 9	0.06 3	5.05 2	0.02 5	1.36 4	0.24 3	0.09 9	0.75 3	1.66 7	0.19 7			1.66 7	0.19 7	0.09 9	0.75 3	2.24 4	0.13 4	0.15 5	0.69 4	5.05 2	0.02 5
NGD1	5.05 2	0.02 5	2.46 9	0.11 6	4.73 8	0.03 0	2.55 7	0.11 0	0.07 4	0.78 6	1.66 7	0.19 7			2.55 7	0.11 0	2.55 7	0.11 0	0.07 4	0.78 6	2.46 9	0.11 6
MBI1	3.45 9	0.06 3	5.05 2	0.02 5	0.38 5	0.53 5	0.25 4	0.61 4	0.69 4	0.40 5	0.09 9	0.75 3	2.55 7	0.11 0			0.69 4	0.40 5	0.69 4	0.40 5	5.05 2	0.02 5
LWN1	1.55 6	0.21 2	5.05 2	0.02 5	0.00 1	0.97 1	0.69 4	0.40 5	2.55 7	0.11 0	2.24 4	0.13 4	2.55 7	0.11 0	0.69 4	0.40 5			2.55 7	0.11 0	5.05 2	0.02 5
LC1	5.05 2	0.02 5	2.46 9	0.11 6	2.77 0	0.09 6	0.69 4	0.40 5	0.03 2	0.85 8	0.15 5	0.69 4	0.07 4	0.78 6	0.69 4	0.40 5	2.55 7	0.11 0			2.46 9	0.11 6
CTRL	5.05 2	0.02 5			4.73 8	0.03 0	5.05 2	0.02 5	2.46 9	0.11 6	5.05 2	0.02 5	2.46 9	0.11 6	5.05 2	0.02 5	5.05 2	0.02 5	2.46 9	0.11 6		

Appendix B: Log-Rank (Mantel-Cox) Pairwise Comparison of *Phytophthora capsici* Isolates on Semenggok Aman Cultivar Black Pepper

Vines

	IJ1		EBK1		GER1		MBI1		LC1		CONTROL	
	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
IJ1			10.927	0.001	0.239	0.625	3.645	0.056	14.356	0.000	14.356	0.000
EBK1	10.927	0.001			4.445	0.035	2.539	0.111	0.284	0.594	5.264	0.022
GER1	0.239	0.625	4.445	0.035			0.668	0.414	7.140	0.008	14.587	0.000
MBI1	3.645	0.056	2.539	0.111	0.668	0.414			5.072	0.024	10.366	0.001
LC1	14.356	0.000	0.284	0.594	7.140	0.008	5.072	0.024			5.241	0.022
CONTROL	14.356	0.000	5.264	0.022	14.587	0.000	10.366	0.001	5.241	0.022		

Appendix C: Log-Rank (Mantel-Cox) Pairwise Comparison of *Phytophthora capsici*, IJ1,
Isolates on Wild Pepper Vines

Treatments	IJ1 S.Aman Var		IJ1 Type 1 Wild Cutting		IJ1 Type 2 Wild Cutting	
	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
IJ1 S.Aman Var			14.356	.000	4.257	.039
IJ1 Type 1 Wild Cutting	14.356	.000			7.397	.007
IJ1 Type 2 Wild Cutting	4.257	.039	7.397	.007		

Appendix D: 16 polymorphic sites from ITS sequence

Sites/ Isolates	7	10	11	12	48	118	122	153	449	726	733	742	755	757	765	790
IJ1	A	G	G	A	T	G	C	T	G	G	G	T	C	T	A	C
IJ2	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	C
IJ4	A	T	G	A	T	G	C	T	G	G	G	T	C	T	A	C
IJ5	A	T	G	A	C	G	T	T	A	G	G	T	C	T	A	C
IJ7	A	T	G	A	T	G	C	T	G	C	C	A	C	T	C	C
EBK1	-	T	G	A	T	G	T	T	G	G	G	T	C	T	A	C
EBK2	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	C
NCY1	A	G	-	G	T	G	C	T	N	G	G	T	C	T	A	C
NG1	A	T	G	A	T	G	C	T	N	G	G	T	C	T	A	C
NG4	A	T	G	A	T	G	C	T	N	G	G	T	C	T	A	C
NG5	A	T	G	A	T	G	C	T	A	G	G	T	C	T	A	C
NG8	A	T	G	A	T	G	C	T	A	G	G	T	C	T	A	C
NG11	A	T	G	A	T	G	C	T	A	G	G	T	C	T	A	C
MRI1	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	C
MRI4	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	C
MRI7	-	T	G	A	T	G	C	T	G	G	G	T	T	T	A	C
MRI8	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	C
GER1	-	T	G	A	T	G	T	T	G	G	G	T	C	T	A	C
GER3	-	T	G	A	T	G	T	T	G	G	G	T	C	T	A	C
LWN1	-	T	G	A	T	G	T	T	G	G	G	T	C	C	A	-
LWN2	-	T	G	A	T	G	T	T	G	G	G	T	C	T	A	-
LWN3	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	-
LWN5	-	T	G	A	T	G	T	T	G	G	G	T	C	T	A	-
LWN6	-	T	G	A	T	G	C	T	G	G	G	T	C	T	A	-
MBI1	A	G	T	G	T	G	C	T	G	G	G	T	C	T	A	C
NGD1	-	T	G	A	T	G	N	T	G	G	G	T	C	T	A	C
NGD3	-	T	G	A	T	G	C	C	G	G	G	T	C	T	A	C
NGD5	-	T	G	A	T	G	N	T	G	G	G	T	C	T	A	C
LC1	-	T	G	A	T	A	C	T	G	G	G	T	C	T	A	C
LC2	-	T	G	A	T	A	C	T	G	G	G	T	C	T	A	C