



Faculty of Resource Science and Technology

**Characterization of *Fusarium* spp. Associated to *Fusarium* Wilt
Symptom on *Piper nigrum* L. in North-western Region of Sarawak**

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Characterization of *Fusarium* spp. Associated to *Fusarium* Wilt Symptom on
Piper nigrum L. in North-western Region of Sarawak

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DECLARATION

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


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ABSTRACT

Fusarium wilt is one of the major diseases in Sarawak pepper plantation. *Fusarium* spp. had been identified as the causal agent for the disease. Hence, further understanding on the causal agent, *Fusarium* spp. is essential in the initial step to design a sustainable disease control strategies such as development of disease resistance varieties to curb the disease. The objectives for this study were: 1. To isolate and characterise morphologically and molecularly *Fusarium* species associated with *Fusarium* wilt of *Piper nigrum* L.. 2. To study the growth performance of *Fusarium solani* isolates on different pH and nutrient media. 3. To study the genetic relatedness of *Fusarium solani* isolated in Malaysia as well as those across Asia. Ten *F. solani*, three *Fusarium oxysporum* and eleven *Fusarium proliferatum* were isolated from diseased root samples collected from fifteen pepper farms in North-western region of Sarawak and identified based on Internal Transcribed Spacer (ITS) sequence. Variations were found on the colony morphology, growth, sporulation rate and ITS sequence of the *Fusarium* spp. isolates. The isolates also showed morphological variations within each species. Nine isolates of *F. solani* were further characterised for their colony morphology and growth on Potato dextrose agar at pH 5-9. Their morphology and growth were also recorded on five different types of nutrient media. The isolates displayed variation of colony morphology across the pH and showed better growth performance when cultured under pH 7-8 compared to other pH of PDA media. *F. solani* isolates also portrayed variations in colony morphology when cultured on different nutrient media. Isolates cultured on PDA media showed the most colony variations in terms of morphological appearance. Five out of nine isolates were more favourable to grow on Carnation Leaf Agar media. ITS sequence ranged from 517 bp to 525 bp was used for genetic relatedness study. It was found that *F. oxysporum* and *F. proliferatum* isolates are genetically closer with each other as they

shared the same common recent ancestor. Both the phylogenetic analysis of local *Fusarium* isolates and *F. solani* isolates from Asia region highlighted their genetical relationship was not based on the geographical distribution of the isolates. Besides that, the analysis showed similarity of colony appearance of the isolates also does not related with their genetical relationship. In conclusion, the objectives of this study were achieved where three *Fusarium* spp. associated to *Fusarium* wilt of *Piper nigrum* L. were successfully isolated. The colony morphological variations of all the isolates were studied and documented. Different pH and nutrient media were found to affect the colony appearance and growth performance of the *F. solani* isolates. Genetic relatedness of the isolates were found to be not related to their geological distribution.

Keywords: *Fusarium* spp., *Piper nigrum* L, morphological characterisation, growth, genetic relatedness

Pemencilan dan Penyifatan Fusarium Spp. Yang Berkaitan Dengan Simptom Fusarium wilt pada (Piper nigrum L.) dalam kawasan Barat Laut Sarawak

ABSTRAK

Penyakit layu Fusarium merupakan salah satu penyakit utama di ladang lada Sarawak. Fusarium spp. telah dikenal pasti sebagai agen penyebab penyakit tersebut. Oleh sebab itu, pemahaman lanjut mengenai penyebab penyakit adalah penting sebagai langkah awal untuk perancangan strategi kawalan penyakit jangka panjang seperti pembangunan varieti yang mempunyai rintangan penyakit untuk membendung penyakit. Objektif kajian ini adalah: 1. Mengasingkan dan mencirikan spesies Fusarium secara morfologi dan molekul daripada pokok Piper nigrum L. yang dijangkiti penyakit layu Fusarium. 2. Mengkaji pertumbuhan F. solani pada pH dan media nutrien yang berlainan. 3. Untuk mengkaji perkaitan genetik spesies Fusarium di Malaysia dengan F. solani di rantau Asia. Sepuluh F. solani, tiga Fusarium oxysporum dan sebelas Fusarium proliferatum telah dipencilkan daripada sampel akar berpenyakit yang disampel dari lima belas ladang lada di kawasan Barat Laut Sarawak dan dikenal pasti berdasarkan jujukan Internal Transcribed Spacer (ITS). Variasi telah ditemui di kalangan isolat Fusarium yang dipencilkan berdasarkan morfologi koloni, pertumbuhan, kadar sporulasi dan jujukan ITS. Isolat-isolat Fusarium juga menunjukkan variasi morfologi dalam kalangan spesies. Sembilan isolat F. solani telah dicirikan selanjutnya untuk morfologi koloni dan pertumbuhannya pada agar-agar dekstroza kentang dalam keadaan pH 5-9. Morfologi dan pertumbuhan atas lima jenis media nutrien yang berlainan juga direkodkan. F. solani menunjukkan variasi morfologi koloni merentas pH dan menunjukkan pertumbuhan yang baik apabila dikultur dalam pH 7-8. F. solani juga menunjukkan variasi dalam morfologi koloni apabila dikultur pada media nutrien yang berlainan. Isolat yang dikultur pada media PDA telah menunjukkan variasi koloni yang

paling banyak. Majoriti pencilan F. solani tumbuh lebih baik pada media *Carnation Leaf Agar*. Jujukan ITS daripada 517 bp hingga 525 bp telah digunakan untuk kajian hubungan genetik. F. oxysporum dan F. proliferatum didapati lebih seiras secara genetik daripada pencilan Fusarium lain yang dikaji kerana mereka berkongsi asal yang sama. Kedua-dua analisa filogenetik bagi isolat Fusarium tempatan dan isolat F. solani dari rantau Asia menunjukkan hubungan genetik isolat-isolat tidak berdasarkan penaburan geografinya. Selain itu, analisa filogenetik menunjukkan persamaan penampilan koloni tidak berkaitan dengan hubungan genetiknya. Kesimpulannya, objektif kajian ini telah dicapai. Tiga Fusarium spp. yang berkaitan dengan penyakit layu *Fusarium* pada Piper nigrum L. telah berjaya dipencilkan. Variasi morfologi koloni bagi semua isolat telah dikaji dan didokumenkan. pH dan media nutrien didapati akan mempengaruhi penampilan koloni dan pertumbuhan F. solani. Hubungan genetik kulat didapati tidak berkaitan dengan taburan geologinya.

Kata kunci: spesies Fusarium, Piper nigrum L., penyifatan morfologi, tindak balas fisiologi, hubungan genetik

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
ABSTRAK	v
TABLE OF CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement	3
1.3 Objectives	5
CHAPTER 2 LITERATURE REVIEW	6
2.1 Overview	6
2.2 <i>Piper nigrum</i> L. (Black Pepper)	6
2.2.1 General Review for Cultivar of <i>Piper nigrum</i> L.	7
2.2.2 Importance and Constraints of Black Pepper Industry in Malaysia	10

2.3	<i>Fusarium</i> Wilt Disease	12
2.4	Genus <i>Fusarium</i>	13
2.4.1	Life Cycle of <i>Fusarium</i> spp.	14
2.4.2	<i>Fusarium</i> Infection Mechanism	15
2.4.3	Common <i>Fusarium</i> spp. in Malaysia	17
2.5	Nomenclature and Identification of <i>Fusarium</i> spp.	20
2.5.1	Morphological Identification	20
2.5.2	Molecular Identification	22
2.6	Phylogenetic Analysis and Genetic Relatedness Study	25
	CHAPTER 3 MATERIALS AND METHODS	27
3.1	Overview	27
3.2	Sample Collection	27
3.3	Isolation of <i>Fusarium</i> spp. Associated with <i>Fusarium</i> Wilt Disease of <i>Piper nigrum</i> L.	29
3.3.1	Fungal Isolation	29
3.3.2	Pure Culture Establishment and Pre-identification	29
3.4	<i>Fusarium</i> Identification	30
3.4.1	DNA Extraction	31
3.4.2	PCR Amplification	32
3.4.3	DNA Purification and BLAST Analysis	32

3.5	Morphological Characterisation	34
3.5.1	Macromorphology and Micromorphology Study	34
3.5.2	Growth Rate	34
3.5.3	Sporulation Rate	35
3.6	Effect of pH and Different Nutrient Media on the Growth of <i>F. solani</i>	36
3.6.1	Media Preparation	36
3.7	Phylogeny Analysis of <i>Fusarium</i> spp.	38
CHAPTER 4	RESULTS	42
4.1	Sample Collection	42
4.2	Fungal Isolation and Pure Culture Establishment	43
4.3	Species Identification	45
4.3.1	Molecular identification	45
4.3.2	Morphological Characterisation	48
4.3.3	Colony growth rate and sporulation rate	64
4.4	Effect of Different pH on the Growth of <i>F. solani</i> Isolates	67
4.4.1	Colony Growth Rate of <i>F. solani</i> Isolates on Different pH	80
4.5	Effect of Different Nutrient Media on the Growth of <i>F. solani</i> Isolates	82
4.5.1	Colony Growth Rate of <i>F. solani</i> Isolates on Different Nutrient Media	94
4.6	Phylogeny Analysis of <i>Fusarium</i> spp.	96
4.6.1	Phylogeny Study of <i>F. solani</i> within Malaysia	96

4.6.2	Phylogeny Study of <i>F. solani</i> within Asia	98
CHAPTER 5 DISCUSSION		101
5.1	Overview	101
5.2	Isolation of <i>Fusarium</i> spp. Associated with <i>Fusarium</i> Wilt Disease of <i>Piper nigrum</i> L. in North-western Region of Sarawak	101
5.3	Effect of pH and Nutrient Media on the Growth of <i>F. solani</i>	105
5.4	Phylogeny Analysis of <i>Fusarium</i> spp.	109
CHAPTER 6 CONCLUSION AND RECOMMENDATIONS		111
6.1	Conclusion	111
6.2	Recommendations for Further Research	112
REFERENCES		114

LIST OF TABLES

	Page
Table 2.1: Distribution location of <i>P. nigrum</i> L. cultivars	9
Table 2.2: Summary of Previous Studies Done on <i>Fusarium</i> spp. in Malaysia	18
Table 3.1: The Geographical Coordinate of Each Pepper Farm Where Three Diseased Root Samples Were Collected	28
Table 3.2: <i>Fusarium</i> spp. isolates selected for molecular identification included.	30
Table 3.3: Recipe for Five Different Media Used to Observe the Growth of <i>F. solani</i>	37
Table 3.4: List of <i>Fusarium</i> Isolates Used in Phylogenetic Dendrogram Construction	39
Table 4.1: Number of <i>Fusarium</i> spp. Isolated from 15 Pepper Farms in North-western Region of Sarawak	42
Table 4.2: Colony Characteristics Variation Among <i>Fusarium</i> spp. Isolates	44
Table 4.3: Identification of <i>Fusarium</i> spp. Based on ITS Region Sequencing	46
Table 4.4: Summary of Colony Morphological Characteristics of 24 <i>Fusarium</i> spp. Isolates	59
Table 4.5: Number of Variations Observed on the Colony Morphological Characteristics of the Isolated <i>Fusarium</i> Spp.	64
Table 4.6: Growth Rate and Spore Concentration of <i>Fusarium</i> spp. Isolates	65
Table 4.7: Number of Variations Observed on the Colony Morphological Characteristics of the <i>F. solani</i> Isolates Across pH	67
Table 4.8: Number of Variations Observed on the Colony Morphological Characteristics of the <i>F. solani</i> Isolates Across pH	70
Table 4.9: Summary of Colony Morphological Characteristics of 9 <i>F. solani</i> Isolates on PDA with Different pH	71
Table 4.10: Colony Growth Rate of <i>F. solani</i> Isolates on PDA Media with Different pH Value	81
Table 4.11: Number of Variants Between <i>F. solani</i> Isolates Across Nutrient Media	83

Table 4.12: Number of Colony Morphological Characteristics Variation on <i>F. solani</i> Isolates Across Nutrient Media	85
Table 4.13: Summary of Colony Morphological Characteristics of 9 <i>F. solani</i> Isolates on Different Nutrient Media	86
Table 4.14: Colony Growth Rate of <i>F. solani</i> Isolates on Different Media	95

LIST OF FIGURES

	Page
Figure 2.1: <i>P. nigrum</i> L. Plant with <i>Fusarium</i> Wilt Symptom of Canopy Yellowing and Drooping of Leaves Took by Tan Wan Joo During Sampling Session.	13
Figure 2.2: Map of Nuclear Ribosomal RNA Genes and Their ITS Regions (Modified from Toju <i>et al.</i> , 2012). SSU is 18S Small Subunit Gene; whereas LSU is 28S Large Subunit Gene. Positions of Forward (Right-pointing Arrow) and Reverse (Left-pointing Arrow) Primers.	24
Figure 4.1: PCR Products Amplified from 15 <i>Fusarium</i> Isolates Using ITS1-ITS4 Primers. PCR Products of <i>Fusarium</i> Isolates (1)Fs-7SMG, (2)Fs-9T1, (3)Fs-6RN, (4)Fs-8JD, (5)Fs-8JM, (6)Fs-5JG, (7)Fs-JOG1, (8)Fs-JOG2, (9)Fs-JOG3, (10)Fs-6SDM, (11)Fo-JHF, (12) Fo-2BL, (13)Fo-4RN, (14) Fp-1JH, (15)Fp-3JH; Lane 16: 100 bp DNA Ladder.	45
Figure 4.2: Macromorphology and Micromorphology Under Microscope at 40x Magnification of <i>F. solani</i> isolates (a) Upper View of Isolate Fs-6RN (b) Upper View of Isolate Fs-9T1 (c) Upper View of Isolate Fs-7SMG (d) Upper View of Isolate Fs-8JD (e) Upper View of Isolate Fs-8JM (f) Upper View of Isolate Fs-5JG (g) Upper View of Isolate Fs-6SDM (h) Upper View of Isolate Fs-JOG1 (i) Upper View of Isolate Fs-JOG2 (j) Upper View of Isolate Fs-JOG3 (k) Reverse View of Isolate Fs-7SMG (l) Reverse View of Isolate Fs-9T1 (m) Reverse View of Isolate Fs-6RN (n) Reverse View of Isolate Fs-8JD (o) Reverse View of Isolate Fs-8JM (p) Reverse View of Isolate Fs-5JG (q) Reverse View of Isolate Fs-6SDM (r) Reverse View of Isolate Fs-JOG1 (s) Reverse View of Isolate Fs-JOG2 (t) Reverse View of Isolate Fs-JOG3 (u) Macroconidia of <i>F. solani</i> (Bar=20µm) (v) Microconidia of <i>F. solani</i> (Bar=10µm)	51
Figure 4.3: Macromorphology and Micromorphology Under Microscope at 40x Magnification of <i>Fusarium</i> Isolates. (a) Upper View of Isolate Fo-4JH (b) Upper View of Isolate Fo-4RN (c) Upper View of Isolate Fo-2BL (d) Reverse View of Isolate Fo-4JH (e) Reverse View of Isolate Fo-4RN (f) Reverse View of Isolate Fo-2BL (g) Macroconidia of <i>F. oxysporum</i> (Bar=20µm) (h) Microconidia of <i>F. oxysporum</i> (Bar=20µm)	53
Figure 4.4: Macromorphology and Micromorphology Under Microscope at 40x Magnification of <i>F. solani</i> isolates (a) Upper View of Isolate Fp-1JH (b) Upper View of Isolate Fp-3JH (c) Upper View of Isolate Fp-9T2 (d) Upper View of Isolate Fp-3RN (e) Upper View of Isolate Fp-3CX (f) Upper View of Isolate Fp-3JM (g) Upper View of Isolate Fp-2RN	

(h) Upper View of Isolate Fp-5JM (i) Upper View of Isolate Fp-1GM (j) Upper View of Isolate Fp-3PTU (k) Upper View of Isolate Fp-3BTG (l) Reverse View of Isolate Fp-1JH(m) Reverse View of Isolate Fp-3JH (n) Reverse View of Isolate Fp-9T2 (o) Reverse View of Isolate Fp-3RN (p) Reverse View of Isolate Fs-3CX (q) Reverse View of Isolate Fp-3JM(r) Reverse View of Isolate Fp-2RN (s) Reverse View of Isolate Fs-5JM (t) Reverse View of Isolate Fp-1GM (u) Reverse View of Isolate Fp-3PTU (v) Reverse View of Isolate Fp-3BTG (w) Macroconidia of <i>F. proliferatum</i> (Bar=10µm) (x) Microconidia of <i>F. proliferatum</i> (Bar=20µm)	57
Figure 4.5: Colony Morphology of <i>F. solani</i> Isolates on Different pH (a) Upper View of Isolate Fs-8JD (b) Reverse View of Isolate Fs-8JD (c) Upper View of Isolate Fs-6RN (d) Reverse View of Isolate Fs-6RN	79
Figure 4.6: Colony Morphology of <i>F. solani</i> Isolates on Different Nutrient Media (a) Upper View of Isolate Fs-JOG1 on Different Nutrient Media (b) Reverse View of Isolate Fs-JOG1 on Different Nutrient Media (c) Upper View of Isolate Fs-6RN on Different Nutrient Media (d) Reverse View of Isolate Fs-6RN on Different Nutrient Media	93
Figure 4.7: Neighbour Joining (NJ) Phylogenetic Tree of <i>Fusarium</i> Isolates from Malaysia Using Maximum Likelihood Method Supported by Bootstrap Test 1000 Replicates, with 0.02 Unit of Base Substitution per Site. Isolates from Shahnazi <i>et al.</i> (2012) Study Were Marked with *	97
Figure 4.8: Neighbour Joining (NJ) Phylogenetic Tree of <i>Fusarium</i> Isolates from Asia Countries Supported by Bootstrap Test 1000 replicates, with 0.02 Unit of Base Substitution per Site. Isolates from Current Study Were Marked with *	100

LIST OF ABBREVIATIONS

°C	Degree Celsius
ANOVA	One-Way Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
ca.	Circa
cAMP	Cyclic Adenosine Monophosphate
CIA	Chloroform Isoamyl Alcohol
CLA	Carnation Leaf Agar
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
E	East
EtBr	Ethidium Bromide
g	Gram
G-protein	Guanine Nucleotide-Binding Protein
HCL	Hydrogen Chloride
ISSR	Inter Simple Sequence Repeat
ITS	Internal Transcribed Spacer
Kg	Kilogram
L	Litre
LSU	28S Large Subunit
m	Meter
M	Molar

MAPK	Mitogen-Activated Protein Kinase
MgCl ₂	Magnesium Chloride
mL	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	Messenger Ribonucleic Acid
N	North
NaOH	Sodium Hydroxide
NCBI	National Centre For Biotechnology Information
OMA	Oatmeal Agar
PCA	<i>Piper colubrinum</i> Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pmol	Picomolar
PNA	<i>Piper nigrum</i> Agar
Ras protein	Rat Sarcoma Protein
rDNA	Ribosomal Deoxyribonucleic Acid
Rpm	Revolutions Per Minute
spp.	Species
SSU	18S Small Subunit
TAE	Tris Acetate EDTA
U/μL	Unit Per Microliter
v/v	Volume Per Volume
viz.	Videlicet
WA	Water Agar

μL	Microliter
μm	Micrometre

CHAPTER 1

INTRODUCTION

1.1 Background

Spices are plant-derived seasonings that existed in human culinary culture for their flavour enhancement on food since ancient time. Other than that, spices possess medicinal value and have food preservation properties as well, due to their secondary metabolites. In terms of botanical definition, spices are those flavour components stored in root, bark, bud, flower stigma and aromatic seed (Ogbunugafor *et al.*, 2017). Among all the spices, black pepper (*Piper nigrum* L.) is widely known as ‘king of spices’ due to its wide usage.

As one of the global pepper producers and exporters, an increase in pepper production for exportation in Malaysia is vital to bring more income to the country. However, the production is limited by several constraints. Crop loss due to diseases is one of the primary constraints faced by pepper production in Malaysia. Diseases caused 2% decrease of the pepper cultivation area annually (Adama *et al.*, 2018). Pepper planting area in Malaysia covered 7,299 hectares, produced 4.65 tonnes per hectare, which worth around RM460.4 millions of pepper in year 2019 (Malaysia Pepper Board, 2019). This means that 2% decrement of pepper cultivation area caused by disease resulted annual loss of approximately 146 hectare pepper cultivation area, estimated to contribute 678.9 tonnes of pepper production which worth about RM10 millions.

One of the major diseases is *Fusarium* wilt, which is also known as slow wilt or yellowing disease (Gordo *et al.*, 2012; Lau *et al.*, 2012). According to (Lau, 2019), *Fusarium* wilt was reported in most of the black pepper farm in some divisions in North-western region of Sarawak namely, Serian, Sri Aman, Betong and Julau. No statistical report on the loss caused by *Fusarium* wilt in Malaysia. However, this disease was reported to cause up to 32% of annual loss in Indonesia (Mustika, 1990). An outbreak of *Fusarium* wilt disease was reported in several black pepper farms around Julau and Betong, Sarawak, (Lau, 2019). The outbreak had resulted severe loss in a short period of time. However, the *Fusarium* species that caused the *Fusarium* wilt outbreak was not specified. Duarte & Albuquerque, (1991) reported that the economic life span of pepper plantation will drop from 20 years to 6–8 years due to the *Fusarium* spp. infestation. The typical symptom of *Fusarium* wilt is the brown to black lesion in the xylem tissue of plant root (Eng, 2011). However, no observable sign of the *Fusarium* wilt was documented on *P. nigrum* L. The infected plant will start yellowing and followed by drooping of leaves of the whole canopy. Due to the malfunctioning roots, flaccid appearance of the plants will be observed, followed by foliar and foot rot. (Eng, 2011; Luz *et al.*, 2017). Severely infected plant will dieback due to the loss of its feeder roots (Eng, 2011).

Most studies reported that *Fusarium* wilt disease is caused by *F. solani* (Shahnazi *et al.*, 2012; Souza *et al.*, 2011; Silva *et al.*, 2014; Luz *et al.*, 2017). However, some studies claimed that *F. oxysporum* is responsible for the disease (Duarte *et al.*, 2001; Edward *et al.*, 2013; Wiratno *et al.*, 2019).

Both *F. solani* and *F. oxysporum* are cosmopolitan and economically important species in agricultural field as they are the causal agent to plant diseases in various crops (Nelson *et al.*, 1981; Encinas Basurto *et al.*, 2020; Bahadur, 2022). Plants that are attacked by the *Fusarium* spp. usually show severe vascular wilts and root rot. This is because the soil borne pathogens attack the plant vascular system, which disrupt the transportation of nutrients and water in plant. Members of *F. solani* species complex (FSSC) and *F. oxysporum* are widely spread in all types of soil worldwide. They have been known as animal, and human pathogens as well (Antonissen *et al.*, 2014; Leslie & Summerell, 2007; Walther *et al.*, 2017). They also caused severe vascular wilts and root rot diseases in various crops (Nelson *et al.*, 1981).

1.2 Problem Statement

Various disease control measures were done in Sarawak pepper plantation in the past, but none of them can control the *Fusarium* thoroughly. *Fusarium* wilt disease does not show disease symptoms at early stage of infection. Farmers usually misinterpreted the yellowing of the plants were caused by malnutrition. Hence, it is too late to perform curing treatment during the final stage of infection (Lau, 2019). Besides that, the high survival period of the spores of *Fusarium* spp. in soil is another factor why the disease is hard to be curb (Thatcher *et al.*, 2016). These are the reasons why the disease would result in significant yield loss to the pepper producing countries such as Malaysia, Indonesia, Brazil, India and Thailand. The massive loss would cause great impact to the economy of the pepper production of the

country. Therefore, extensive studies on the disease are vital in ensuring the sustainability of black pepper production in Malaysia.

Correct and specific identification of the causal agent of *Fusarium* wilt disease are crucial in handling the disease. As both *F. solani* (Shahnazi *et al.*, 2012; Souza *et al.*, 2011; Silva *et al.*, 2014; Luz *et al.*, 2017) and *F. oxysporum* (Duarte *et al.*, 2001; Edward *et al.*, 2013; Wiratno *et al.*, 2019) were reported as the pathogen responsible for the disease, it is necessary to clarify the claim of both pathogens as the causal agent for the disease by isolate and identify *Fusarium* species associated with *Fusarium* wilt of *P. nigrum* L. morphologically and molecularly. Pathogenicity test will follow to determine the disease causal agent (pathogenicity test was not done in this study due to lack of *Piper nigrum* L. plantlet). Besides that, knowledge on the diversity of the pathogen is necessary in developing sustainable disease management.

Different culture media affects the performance of *Fusarium* spp. (Iqbal *et al.*, 2005; Jayashree *et al.*, 2016; Pradeep *et al.*, 2013). Besides that, the difference of pH value of the media also alternate the behaviour of the *Fusarium* isolates (Khilare & Rafi, 2012). It is necessary to understand the type of culture media that are favourable to the pathogen by study the growth performance of the *Fusarium* (in this study *F. solani*) isolates on different pH and different media. This information is lacking for *Fusarium* spp. in Sarawak. It will be helpful in culturing the pathogen efficiently in the lab for *in vitro* research towards sustainable disease management.

Knowledge on the gene flow of the pathogen as well as their genetic relatedness are vital in future development of sustainable disease management. Currently, the study of *Fusarium* species associated with *Fusarium* wilt and their relatedness between the population of different geographical distribution are still poor in Sarawak. Despite the study by (Shahnazi *et al.*, 2014), which reported on the genetic diversity of *F. solani* isolated from black pepper farms situated in Sarikei, Sibu and Johor, the phylogenetic relationship among the *F. solani* from North-western region as well as other regions of Sarawak is still remain unknown.

1.3 Objectives

The objectives of this study were:

- I. To isolate and characterise *Fusarium* species associated with *Fusarium* wilt of *Piper nigrum* L. morphologically and molecularly.
- II. To study the growth performance of the *Fusarium solani* isolates on different pH and different nutrient media.
- III. To study the genetic relatedness of *Fusarium solani* isolated in Malaysia as well as those in across Asia.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Spices play a crucial role in human civilisation since ancient time where they were used for various ways. Spices are usually found in culinary for flavour enhancing, especially for traditional dishes. Besides culinary, ancient society such as China, India and Egypt use spices for medicine, and even mummification, which greatly stimulate the world trade of spices (Katz & Weaver, 2003).

The term “spices” are often being used as an alternative for “herbs”. However, in terms of biological definition, spices are those flavour components stored in their root, bark, bud, flower stigma and aromatic seed; while herbs are those flavour components stored in their leaves (Ogbunugafor *et al.*, 2017). Among all the spices, black pepper is widely known as ‘king of spices’ due to its lucrative value in world trading (Ahmad *et al.*, 2012; Nair, 2011).

2.2 *Piper nigrum* L. (Black Pepper)

Since ancient time, black pepper or *Piper nigrum* L. stands a supreme position among all the spices as most savoury dishes blends well with its flavour and pungency. It is widely used in traditional medicine and culinary purposes. The berries of the black pepper plant, are processed to become peppercorn, or locally known as ‘lada’. *P. nigrum* L. is a perennial woody vine. It belongs to the Piperaceae family, which have the largest number of species (Melo *et al.*, 2016). The genus *Piper* was reported with approximately more than 200 species.

P. nigrum L. originated from the South-western of India, which covers the Kerala forest and Ghats, and north region of Kanara ((Ravindran, 2000). The plants are commercially cultivated in countries such as Malaysia, Brazil, Vietnam, India and Indonesia (Lau *et al.*, 2012). According to Malaysian Pepper Board (2018), the pepper production of Malaysia reached up to 31,073 tonnes with the total export of 11,714 tonnes that worth RM200.95 million in 2018. Among the total coverage area of 17,437 hectares of pepper plantation, 98% of the cultivation is located in Sarawak and operated intensively by the local smallholders, averagely 2000 vines per hectare (Adama *et al.*, 2018).

(Ravindran, 2000) believes that pepper cultivars are developed from wild *Piper* through domestication and selection. According to Sim (1993), the plant was introduced to Sarawak back in the year of 1856. Since then, the plant had become a major agricultural commodity of the state. Even now, the plant still hold an important niche for the economy of the locals, especially the smallholders (Chen, 2011).

2.2.1 General Review for Cultivar of *Piper nigrum* L.

2.2.1.1 Cultivar ‘Kuching’

‘Kuching’ is one of the recommended cultivars for commercial plantation (Det, 2011). This traditional cultivar in Sarawak is mostly grown in Sarawak and Johor due to its vigorous growth and high yielding characteristics. This traditional cultivar is mostly grown in Sarawak and Johor due to its vigorous growth and high yielding characteristics. According to Det (2011), the average annual yield of ‘Kuching’ cultivar ranged from 1.7 - 3.5kg per vine. Chen & Tawan (2020) reported ‘Kuching’ is the most widely distributed cultivar among all the *P. nigrum* cultivars. It was estimated ‘Kuching’ covered approximately 50% of total planting area in Malaysia at location listed in **Table 2.1**.

Growing on disease free and fertile soil, the highest record for annual production of black pepper production from healthy vines of 3 – 3.5m height was 6 – 7kg per vine. However, this cultivar is susceptible to many diseases such as *Fusarium* wilt, *Phytophthora* foot rot, black berries and velvet blight to name a few (Det, 2011). ‘Kuching’ is preferred for the production of creamy white peppercorn, which is the world best grade of white pepper due to its thin pericarp (Det, 2011)

2.2.1.2 Cultivar ‘Semonggok Aman’

In order to overcome the shortcomings of ‘Kuching’, a new variety of *P. nigrum* L., ‘Semonggok Aman’ was developed (Det, 2011). This cultivar was derived from clonal propagation of Germplasm Accession PN106, which was originated from Costa Rica (Det, 2011). Compared to ‘Kuching’, this variety has better fruit setting with longer fruit spikes and bigger berries (Det, 2011). However, ‘Semonggok Aman’ is less popular among the local Malaysian farmers compared to ‘Kuching’. ‘Semonggok Aman’ was estimated to cover around 10% of total pepper planting area in Malaysia at location listed in **Table 2.1** (Chen & Tawan, 2020). Besides that, ‘Semonggok Aman’ showed more tolerant towards diseases such as black berries and *Phytophthora* foot rot as compared to ‘Kuching’ (Det, 2011). However, no report regarding the resistance nor susceptibility of ‘Semonggok Aman’ towards *Fusarium* wilt. ‘Semonggok Aman’ is usually planted for the production of green pepper sauce and premium black pepper, which is world-class black pepper due to its thicker pericarp and higher chemical contents such as *piperine*, oleoresin volatile and non-volatile oils (Det, 2011). These chemicals properties make the product of ‘Semonggok Aman’ to be more pungent as compared to others (Krishnamurthy *et al.*, 2010; Luz *et al.*, 2017).

Table 2.1: Distribution location of *P. nigrum* L. cultivars

Division	‘Kuching’	‘Semonggok Aman’
Kuching	• Bau, • Lundu, • Sematan, • Tanjung Datu, • Padawan, • Mambong, • Tarat, Matang, • Batu Kawa • Bako	• Bau, • Sematan, • Padawan, • Mambong, • Tarat, • Matang
Samarahan	• Muara Tuang, • Asajaya • Simunjan • Batang Sadong	• Muara Tuang • Simunjan • Batang Sadong;
Serian	• Tarat, • Tebakang, • Kedup • Bukit Semuja, • Tedebu • Gedong	• Tarat, • Tebakang • Kedup, • Bukit Semuja • Tedebu • Gedong

Table 2.1 continued

Sri Aman	• Balai Ringin, • Lachau • Lubok Antu • Bukit Begunan • Batang Lupar	• Balai Ringin • Lachau • Lubok Antu • Bukit Begunan • Batang Lupar
Betong	• Saratok • Layar • Saribas • Bukit Saban	• Saratok, • Layar • Saribas • Bukit Saban

Source: E-Pekebun (Sistem Maklumat Pekebun Lada), by Malaysian Pepper Board.

2.2.2 Importance and Constraints of Black Pepper Industry in Malaysia

Black pepper industry supports the socioeconomic of the Sarawakians, especially those living in rural area due to its lucrative value in world commodity trade. According to Malaysian Pepper Board (2018), the pepper production of the nation reached 31,073 tonnes with a total export of 11,714 tonnes that worth RM200.95 million in 2018. Among the total coverage area of 17,437 hectares of pepper plantation, 98% of the black pepper cultivated area is located in Sarawak and operated intensively by the local smallholders with an average 2000 vines per hectare (Adama *et al.*, 2018).

However, the local pepper plantation industry is facing various challenges in expanding and upscaling in both upstream and downstream of the pepper plantation industry. Harry *et al.* (2021) reported that since 2015, the pepper price had been declined more than 70%. This eventually affects the overall community income and indirectly causes the aging population of the pepper farmers as their younger generation urges for better employment opportunities in bigger cities for more stable income. Other than that, Harry *et al.* (2021) also highlighted the problem of low yielding, pest and diseases infestation, soil fertility and fluctuation of pepper global market price as the major reasons of the unstable income of pepper farmers.

Other constraints such as low domestic consumption, farm input cost increment, lack of extension services, low impact of research, development and innovation, intense competition of global market, imbalance of supply and demand of pepper market causing under-pricing of quality pepper, limited subsidy for continuous farming support, lack of new technology, and poor downstream activities investment would also contribute to the limitation of the expenditure of local pepper industry (Harry *et al.*, 2021).

Crop loss due to diseases is one of the primary constraints in increasing the yield of pepper production in Malaysia as highlighted by Harry *et al.* (2021). Adama *et al.* (2018) reported annual crop loss caused by diseases is about 2% of the total pepper area in Sarawak. Diseases such as *Fusarium* wilt often caused high mortality and serious yield loss and damage to the farms within a short period of time (Lau, 2019). For example, (Awit, 2018) reported approximately 365,000 pepper vines in Sarawak were attacked by *Fusarium* spp. and *Phytophthora* related diseases within only 6 months ranging from November 2017 to May 2018.

2.3 *Fusarium* Wilt Disease

One of the major diseases of *P. nigrum* L. is *Fusarium* wilt, which is also known as slow wilt or yellowing disease (Gordo *et al.*, 2012; Lau *et al.*, 2012). The disease may cause as high as 32% of annual loss in Indonesia (Mustika, 1990). Duarte & Albuquerque (1991) also reported that the pepper plantation economic lifespan will drop from 20 years to 6 to 8 years due to *Fusarium* infection.

The typical symptom of the disease is the brown to black lesion in the xylem tissue of root (Eng, 2011). The infected plant will start yellowing and drooping of leaves occurs in whole canopy (**Figure 2.1**). The plant then appeared to be flaccid. Foliar and foot rot will be observed due to the malfunction of the roots (Eng, 2011; Luz *et al.*, 2017). Later, the severely infected plant will dieback due to the loss of its feeder roots.

There are studies reported that *Fusarium* wilt disease is caused by *F. solani* (Shahnazi *et al.*, 2012; Souza *et al.*, 2011; Silva *et al.*, 2014; Luz *et al.*, 2017). Pathogenicity test was done by Shahnazi *et al.* (2012). Briefly, the roots of pepper plantlets (cv. Kuching) of 3 months old were cleaned. Then, the roots inoculation were done in pots (Edel-Hermann *et al.*, 2011) and maintained in glasshouse under optimal conditions. It was found that no symptoms were observed on control plants or plants inoculated with *F. proliferatum*. The symptoms of *F. solani* infection in the glasshouse-grown plants were yellowing of the foliage and root rot, leading to flaccidity. However, some studies claimed that *F. oxysporum* is responsible for the disease (Duarte *et al.*, 2001; Edward *et al.*, 2013; Wiratno *et al.*, 2019). The initial symptom reported by Wiratno *et al.* (2019) was slight to general leaves yellowing. The wilting become severe after infection by *F. oxysporum* causing galls on plant roots to rot, resulting total loss of root system of the host plant. The disease symptom reported were similar with the infection by *F. solani* in Shahnazi *et al.* (2012).

The disease can be controlled through the hygiene cultural practice such as good drainage system, disinfecting land with fungicide such as using dazomet to sterile the soil after removal of infected plant and also disinfecting new cuttings (Zhang *et al.*, 2021). Eng (2011) reported that there is no chemical that can kill the *Fusarium* spp. and keep the *P. nigrum* L. safe at the same time.



Figure 2.1: *P. nigrum* L. Plant with *Fusarium* Wilt Symptom of Canopy Yellowing and Drooping of Leaves Took by Tan Wan Joo During Sampling Session.

2.4 Genus *Fusarium*

Fusarium are cosmopolitan fungi which belongs to the class *Sordariomycetes*, order *Hypocreales* and family *Nectriaceae* (Zubi, 2016). These filamentous fungi are highly diverse genus, which consist of wide range of saprophytic, phytopathogenic and toxigenic filamentous fungi found in different environmental condition. According to (Leslie & Summerell, 2007), the genus consists of more than 200 species. Therefore, *Fusarium* had been one of the popular study subjects among the researchers.

Majority members of the genus are economical important phytopathogen in agriculture sector as they caused destructive loss on various plants such as soybean, guava, black pepper, etc., (Gupta *et al.*, 2010; Shahnazi *et al.*, 2012; Chehri *et al.*, 2015; Li *et al.*, 2018). In previous studies by (Hafizi *et al.*, 2013; Hysing & Wiik, 2014; J. H. Park & Juzwik, 2012; Wu *et al.*, 2011) the pathogen attack vascular system of the plant, which caused various types of diseases such as root rot, stem rot, vascular wilt, seed blights, canker and so on. Brown *et al.* (2013) claimed that plant diseases associated by this genus are not limited by particular plant and geological factor. The disease impact caused by *Fusarium* spp. can be a concern in both temperate and tropical agriculture. Other studies claimed that certain species of *Fusarium* are geographically or climatically confined in their occurrence and diversity (Czembor *et al.*, 2015; Manshor *et al.*, 2012; Mohsen, 2016). Manshor *et al.* (2012) reported *Fusarium* spp. that usually found in the temperate region were also observed in tropical highland, and vice versa. Brown *et al.* (2013) also stated that there are several *Fusarium* spp. which, exhibit the capability to cause multiple diseases on the same plant. However, the host and environment factor can affect their pathogenicity.

2.4.1 Life Cycle of *Fusarium* spp.

Fusarium spp. are grown as a haploid hyphae colony (Ma *et al.*, 2013). According to the description by (Ma *et al.*, 2013), ascospores are produced in a group of eight in a sac known as ascus. The ascus is enclosed in a flask-shaped structure which is known as perithecium. Homothallic species can undergo self-fertilization, where apomixis (clonal ascospore progeny) are produced, while heterothallic species are self-sterile. Asexual spores may be found in three different forms depending on the species, i.e. microconidia, macroconidia and chlamydospores (**Figure 2.2**).

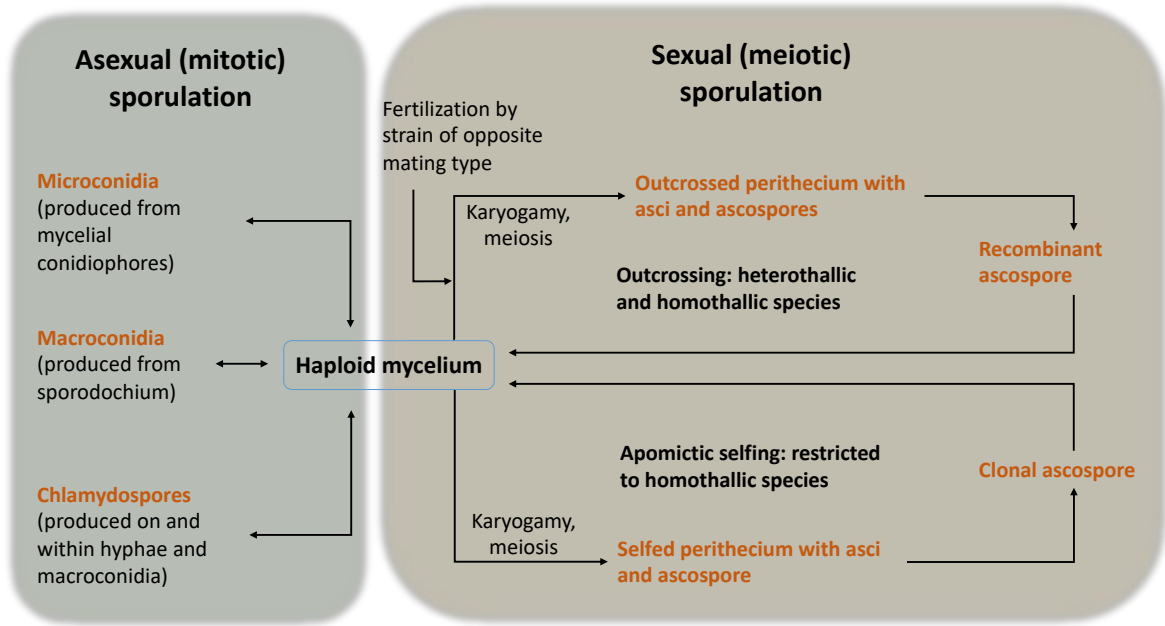


Figure 2.2: Generalised Life Cycle of *Fusarium* Spp. (Modified from (Ma *et al.*, 2013))

2.4.2 *Fusarium* Infection Mechanism

There are astounding diversity of infection strategies among the *Fusarium*, where they are divided into biotrophs or necrotrophs and as either obligate or facultative pathogens (Trail, 2009). However, their polymorphic nature does not restrict by this categorization. According to (Zaini, 2017), most *Fusarium* are categorised as hemibiotrophs. The pathogen displayed biotrophic characteristics where it initially relies on a living host. Eventually, the pathogen transformed to necrotrophic as they continue to feed on the dead host plant due to the failure of plant vascular system (Ma *et al.*, 2013). The trophic divergence from biotroph to necrotrophy of *Fusarium* spp. are affected by fungal effectors and plant complex signalling pathways mediated antagonistically or agonistically. Chemicals such as salicylic acid, jasmonic acid, and ethylene, plant hormones such as auxins, cytokinins, abscisic acid, gibberellins, brassinosteroids, and strigolactones, and nitric oxide regulate the trophic divergence (Huang *et al.*, 2020; Shao *et al.*, 2021).

As most *Fusarium* are soil-borne pathogen, diseases associated with this pathogen usually either start in roots or ground level plant parts (Eng, 2011). The initial phase of *Fusarium* invasion to the host via root penetration is asymptomatic as the pathogen is displaying biotrophic lifestyle. At this phase, the pathogen travels upwards in the plant. At the same time, the pathogen accumulates fungal mycelia and defence related compounds in the plant xylem (biotroph behaviour) (Lyons *et al.*, 2015). As the infection persist, the sign and symptom of *Fusarium* related disease such as yellowing leaves and brown lesion showed as the pathogen switched the way they intake nutrient (necrotrophic behaviour) by killing and feed on the host tissues (Lorang, 2018). The pathogen used both general and pathogen-specific pathogenicity mechanism during the invasion process on their hosts (Zaini, 2017).

General pathogenicity genes are shared by *Fusarium* and other pathogenic fungi. The genes are not specific. General pathogenicity genes are responsible for encoding the essential pathway components that involved in endogenous or exogenous signal recognition. These cellular signalling pathways are usually needed for proper development, and fungal enzymes used for plant cell degradation are shared by *Fusarium* spp. and other pathogenic fungi (Ma *et al.*, 2013). Same general pathogenicity genes such as genes responsible to produce MAPKs, found in *Fusarium graminearum*, can also be found in other pathogenic fungi like *Bipolaris oryzae* (Zhao *et al.*, 2007).

On the other hand, the pathogen-specific pathogenicity genes such as genes producing gibberellin are specific to individual *Fusarium* spp. on specific host (Troncoso *et al.*, 2010; Ma *et al.*, 2013). For example, *F. oxysporum* f. sp. *lycopersici* has established a gene-for-gene interaction with its tomato host (Ma *et al.*, 2013). Virulence factors, such as *SIX* (secreted in xylem) genes played significant roles in determining host specificity. Previous study by Sun *et al.* (2022) showed highly-induced expression of *F. oxysporum* f. sp. *lycopersici* effector during tomato infection. (Ma *et al.*, 2013) stated that the effector secretion and host-specific toxins form the specific pathogenicity factors are the key to overcome the host defence mechanisms. Eventually, the pathogen colonized the vascular tissues of the host plants and triggered symptoms like yellowing and wilting, necrosis, as well as chlorosis of the aerial parts of the plants (Zaini, 2017). The infection area will spread. The toxins released into the host tissue, eventually will kill the plant when colonization is established (Erisléia-Meireles *et al.*, 2016).

2.4.3 Common *Fusarium* spp. in Malaysia

Several studies on *Fusarium* spp. had been conducted in Malaysia on various crops since past decades. Among the previous studies, it was found that both *F. solani* and *F. oxysporum* are the common causal agent of the root rot and wilting diseases in Malaysia. **Table 2.2** summarized the previous studies done on *Fusarium* spp. in Malaysia.

Table 2.2: Summary of Previous Studies Done on *Fusarium* spp. in Malaysia

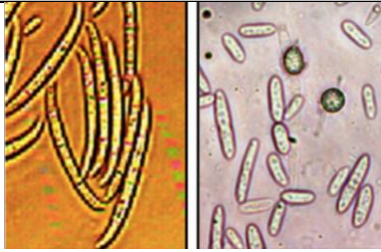
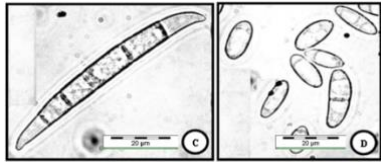
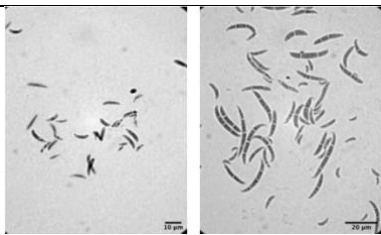
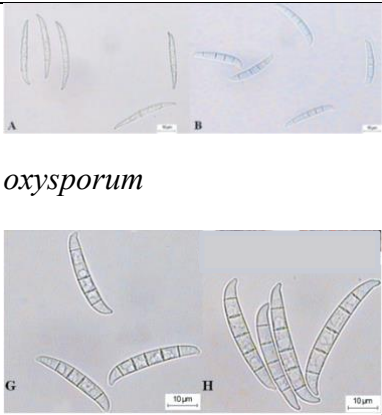
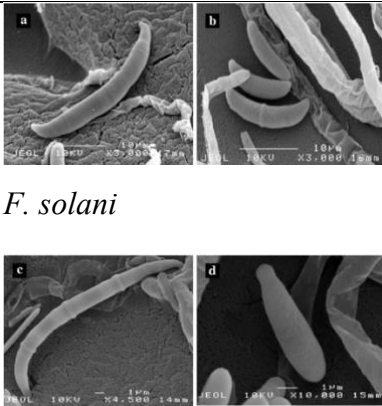
Host	<i>Fusarium</i> spp. isolated	Infection part of the host	Disease symptoms
Potato tuber (Chehri <i>et al.</i> , 2011)	 <i>F. oxysporum</i>  <i>F. solani</i>	• Tuber	• Brownish lesion • Decolouration • Necrosis
Banana (Ujat <i>et al.</i> , 2021)	 <i>F. oxysporum</i>	• Stems • Corms • Roots	• Yellowing of leaves • Pseudostem splitting • Corm rot and discolouration
Tobacco (Norhafeez <i>et al.</i> , 2013)	<i>F. solani</i> *No picture of conidia found*	• Lower stem part • Roots	• Roots discoloration

Table 2.2 continued

Oil palm (Hafizi <i>et al.</i> , 2013)	 <i>F. oxysporum</i> <i>F. solani</i>	• Fronds and leaves • Rachis bending • Breaking and rotting of some spears and growing leaves
Pineapple (Ibrahim <i>et al.</i> , 2015)	<i>F. oxysporum</i> and <i>F. solani</i> *No picture of conidia found*	• Fruits • Sunken fruit skin • Brown lesions • Presence of mycelia in the lesions of some infected fruits
Black pepper (Gordo <i>et al.</i> , 2012; Lau <i>et al.</i> , 2012)	 <i>F. solani</i> <i>F. proliferatum</i>	• Roots • Brown to black lesion in the xylem tissue of the plant root • Yellowing and drooping of leaves occurs in whole canopy • Plant appeared to be flaccid • Foliar • Foot rot • Severely infected plant will dieback due to the loss of its feeder roots

2.5 Nomenclature and Identification of *Fusarium* spp.

According to (Leslie & Summerell, 2007), Link was the first to describe the genus *Fusarium* based on the shape of conidia in the year of 1809. Gradually, Wollenweber & Reinking, (1935) organised the genus from about 1000 species into 65 species in the book of Die Fusarien. Eventually, the classification system for *Fusarium* was improvised by Snyder & Hansen (1954), followed by Booth (1971).

Later, Nelson *et al.* (1983) came out with a classification system of *Fusarium* which is a combination of several previous classification system. Microscopic features of macroconidia, microconidia, conidiophores and chlamydo spores were used for the species identification of the fungi. Finally, Leslie & Summerell, (2007) published a comprehensive laboratory manual for *Fusarium* identification where the manual includes compilation of species descriptions by several *Fusarium* taxonomists. Other than identification and isolation method of *Fusarium*, molecular identification method and mating studies of the species in the genus were recorded in detail in the manual published by (Leslie & Summerell, 2007).

2.5.1 Morphological Identification

Morphological identification is the observation and the classification of the fungus colony based on the macro-morphology and micromorphology. Macro-morphology includes the colony characteristics such as texture, margin, colony elevation, growth rate and pigmentation and micromorphology involve characteristics such as the shape, size and other microscopic features of macroconidia and microconidia, conidiophores and chlamydo spores (Leslie & Summerell, 2007; Nelson *et al.*, 1983).

Macro-morphology such as colony appearance, growth rate and pigmentation aided in the species identification of the *Fusarium*. However, all the colony characteristics differ depends on the media used to cultivate the fungi (Leslie & Summerell, 2007). The morphological variations of a colony may be due to the different nutrient preference by the fungi (Robles-Carrión *et al.*, 2016).

According to Leslie and Summerell (2007), a crucial feature in differentiating the *Fusarium* spp. is the shape of macroconidia of the fungi. These spores with long, septate and canoe-shaped are produced in conidiophores in the aerial mycelium or sporodochia, which is cushion-shaped aggregations of conidiophores (Ma *et al.*, 2013). Different shape of the macroconidia may indicate different species of *Fusarium* (Leslie & Summerell, 2007). The shapes of macroconidia vary from slender, falcate, straight, fusiform to needle like. Besides that, the laboratory manual by (Leslie & Summerell, 2007) also states that the size, number of septa as well as the shape of both apical and basal cells of the macroconidia also being assessed during the species identification of *Fusarium*. In the laboratory manual, the shape of the apical cell of the *Fusarium* ranges from blunt, papillate, hooked to tapering, while basal cell can be foot-shaped, elongated foot-shaped or distinctly-elongated.

The shape of microconidia range from oval, two or three-cell, obovoid with a flattened base, reniform, pyriform or globose, with nil to three septa observed (Leslie & Summerell, 2007; Nelson *et al.*, 1983). The size of microconidia is smaller than macroconidia and produced in the mycelium from conidiophores (Ma *et al.*, 2013). (Leslie & Summerell, 2007) recorded microconidia are the most distinguishing feature for certain species of *Fusarium*.

Chlamydospores, which is the survival structures of the fungi exhibit thick-walled and rough structures. These structures are produced within or on either hyphae or macroconidia (Ma *et al.*, 2013), and can be found singly, in pairs or in short chains on somatic hyphae (Ibrahim *et al.*, 2015; Nelson *et al.*, 1983). However, not all *Fusarium* spp. have this structure (Leslie & Summerell, 2007).

As the macro-morphology of the fungi differ according to the media used for fungi culture, therefore a combination of macro-morphology and micromorphology are needed for a more accurate species identification.

Although Leslie and Summerell (2007) claimed that the fungi macroconidia can be used for species identification in *Fusarium* spp. However, species identification through colony morphological features are less accurate as compared to molecular identification as the description for the features are subjective (Prakash *et al.*, 2017).

2.5.2 Molecular Identification

In comparison to morphological identification, molecular identification is faster and more sensitive (Robles-Carrión *et al.*, 2016). Specific molecular markers are designed for various studies. Multiple information such as fungal variation, genetic relationship, taxonomy as well as population structure among the fungi can be generated from the genetic marker system (Cooley, 1991). (Zubi, 2016) states that molecular identification is essential in *Fusarium* spp. identification as many species in the species complex shared similar morphological characteristics, such as colony appearance and macroconidia features.

Fusarium spp. identification and phylogenetic studies nowadays commonly utilise the sequence of internal transcribed spacer (ITS) region (Chehri *et al.*, 2015; Kim *et al.*, 2016; Robles-Carrión *et al.*, 2016). ITS region sequences are highly variable in *Fusarium* as unit containing genic and spacer regions are repeated by ribosomal DNA (Zubi, 2016). A copy of 18S-5.8S and 28S-like rDNA and two spacers can be found in each repeat unit (**Figure 2.3**). Intergenic transcribed spacer region which are known as ITS 1 are located between the 18S small subunit (SSU) and 5.8S, while ITS 2 are located between 5.8S and 28S large subunit (LSU) genes (**Figure 2.3**). Hence, ITS regions sequences is also the most employed marker to infer lower levels taxonomy in fungi, where the rRNA genes are highly conserved in all species of fungi tested to date (Shahnazi *et al.*, 2014). Primers were designed for the amplification of one repeat unit of ITS. Among all the region in the repeat unit, ITS 1 and ITS 2 are among the useful region in the study of *Fusarium* systematic and taxonomy at species level due to their variability (Park & Min, 2005).

Schoch *et al.* (2012) claims that the ITS region showed highest success rate in identification of wide range of fungi among the regions of the ribosomal cistron, with the clearest defined barcode gap between inter- and intraspecific variation. Hence, ITS was chosen as the official barcode for fungi by a consortium of mycologists in Consortium for the Barcode of Life (Schoch *et al.*, 2012). However, Kebabc *et al.* (2014) claimed that there is limitation for ITS sequence to identify closely related members in species complex. The exist area of paralogous or xenologous segments in ITS 2 sequences had restricted the species identification process (O'Donnell & Cigelinik, 1997).

Although there is some restriction in species identification by ITS sequences, many researchers from all around the world still use this method for the species identification and phylogenetic study of *Fusarium*. In Korea, (Kim *et al.*, 2016) use ITS region sequences to identify the *Fusarium* spp. isolated from *Fusarium* wilt of safflower. On the other hand, (Cruz *et al.*, 2018) used ITS sequences to study the molecular diversity of *F. oxysporum* isolates from common bean in Brazil. (Soren *et al.*, 2015) utilise ITS region sequences to study the population distribution and genetic relatedness of *Fusarium udum* in India. Similarly in Malaysia, researchers such as Chehri *et al.* (2015), Low *et al.* (2011), and Shahnazi *et al.* (2012) used ITS region sequences to identify the *Fusarium* spp. isolated from Malaysia.

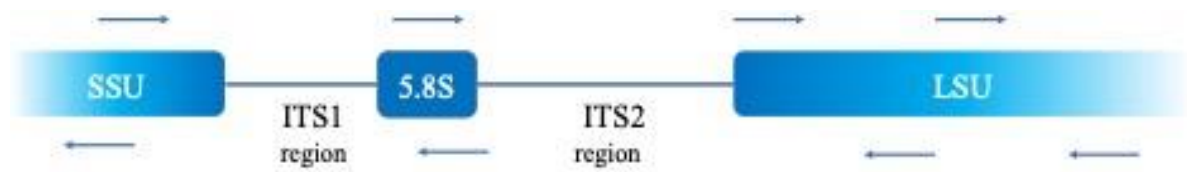


Figure 2.2: Map of Nuclear Ribosomal RNA Genes and Their ITS Regions (Modified from Toju *et al.*, 2012). SSU is 18S Small Subunit Gene; whereas LSU is 28S Large Subunit Gene. Positions of Forward (Right-pointing Arrow) and Reverse (Left-pointing Arrow) Primers.

2.6 Phylogenetic Analysis and Genetic Relatedness Study

An effective disease management requires genetic characterization of plant pathogens variants established in certain area (Shahnazi *et al.*, 2014). Therefore, phylogenetic analysis is essential as it involves the study of evolutionary relationship among different organisms from ancestors to the descendent (Pavlopoulos *et al.*, 2010). The analysis is usually presented in a two-dimensional representation of the relatedness among different biological species. The line drawing provides indication of the time series origin and the group of species or sequences. The phylogenetic trees are formed according to the model that is suitable for building the dendrogram for each study, either with distance based methods or character based methods (Zubi, 2016). There are several methods to infer the phylogenies, ie. neighbour-joining (NJ), unweighted pair group method with arithmetic (UPGMA), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference method.

The phylogeny analysis among *Fusarium* spp. is usually represented with monophyletic lineage (Roy *et al.*, 2014), where most of the same species of *Fusarium* tend to group under same clade (Shahnazi *et al.*, 2012; Chehri *et al.*, 2015). However, there are variations in substitution rate among the species. The longer the substitution rate indicates that the organism mutate more along the evolution process (Bromham, 2020).

‘Species complex’ referred as an informal assemblage of taxa, whose members are related phylogenetically and shared morphological similarities (Sousa-Paula *et al.*, 2021). DNA sequences of ITS regions can significantly present the evolutionary relationships among *F. solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC) (Aoki *et al.*, 2003; O’Donnell *et al.*, 2008). DNA sequences of ITS regions can significantly present the evolutionary relationships among *F. solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC). (O’Donnell, 2000; Aoki *et al.*, 2003).

F. solani and *F. oxysporum* usually have the most complex phylogenetic tree which caused them commonly known as *F. solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC). For example, both *F. solani* and *F. oxysporum* isolates retrieved from tobacco wilt and rot root were grouped under same cluster in the phylogeny tree of previous study by (Berruezo *et al.*, 2018). However, it is also possible to find isolates of the same species that do not group together in a cluster as presented for *F. solani* isolates in Chehri *et al.* (2015). From the phylogeny analysis, the genetic relatedness among the isolates can be studied based on their grouping of clusters and their substitution rates.

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview

The study started off with sample collection from fifteen randomly selected pepper farm along the highway from Kuching to Betong. Then, the samples were proceed for fungal isolation and isolate purification. Then, the isolates were pre-identified (as *Fusarium* spp.) based on their spores and grouped together based on their morphological appearance. After that, 25 *Fusarium* isolates were randomly selected, representing each morphological group and different sampling location for species identification via fungal ITS sequence. Then, morphological characterization based on macromorphology, micromorphology, mycelial growth rate and sporulation rate was done on 24 isolates as 1 isolate had poor sequence data for species identification. After that, nine *F. solani* isolates were selected to study the effect of different media pH & different nutrient media. Phylogeny analysis on the 24 *Fusarium* isolates was done to study the genetic relatedness among the *Fusarium* spp.. Lastly, nine *F. solani* isolates were selected to construct another phylogenetic tree to compare comparing with *F. solani* isolates from Asia countries.

3.2 Sample Collection

Samples of diseased root of black pepper were collected from 15 pepper farms located in Kuching, Serian, Sri Aman and Betong division of Sarawak (**Table 3.1**). Three diseased root samples were collected from each farm.

The collected root samples were kept in separate plastic bags and labelled according to the sampling site. The opening of the plastic bags was left open to avoid high humidity environment in the plastic bags. Collected root samples were brought to laboratory and sealed separately with zipper bag. All the samples were stored in refrigerator at ca. 4°C until further process.

Table 3.1: The Geographical Coordinate of Each Pepper Farm Where Three Diseased Root Samples Were Collected

Farm code	Location	Division	GPS coordinate
JHF	Stass	Kuching	N01°23'11.259", E109°58'27.6384"
SMG	Semenggok	Kuching	N01°23'35.2356", E110°19'35.637"
JMF	Paon Gahat	Serian	N00°56'58.1352", E110°39'7.8408"
JDF	Linsat	Serian	N01°03'41.4642", E110°43'21.594"
CXF	Linsat	Serian	N01°03'15.6558", E110°43'37.0842"
RNF	Linsat	Serian	N01°02'48.015", E110°43'13.4214"
BLF	Jaong	Sri Aman	N01°04'44.1228", E110°59'23.6652"
JG1	Jaong	Sri Aman	N01°05'38.9256", E110°59'16.3422"
JG2	Jaong	Sri Aman	N01°05'47.6628", E110°59'24.1758"
PTU	Pantu	Sri Aman	N01°05'9.7476", E111°06'40.9098"
GMF	Gua Ming	Sri Aman	N01°04'30.5358", E111°17'10.1796"
SDM	San Demam	Sri Aman	N01°07'13.9794", E111°35'25.875"
JLK	Jelukong	Sri Aman	N01°10'4.6704", E111°39'32.3784"
BTG	Saka	Betong	N01°23'15.8208", E111°33'0.0426"
JNF	Saka	Betong	N01°22'49.1334", E111°33'53.3232"

3.3 Isolation of *Fusarium* spp. Associated with *Fusarium* Wilt Disease of *Piper nigrum* L.

3.3.1 Fungal Isolation

Isolation method based on Shahnazi *et al.* (2012) was used. The root samples were cleaned under running tap water to remove excess dirt and soil. Sodium hypochlorite (1% v/v) were used for surface sterilisation of the cleaned samples for 5 minutes. The samples were rinsed three times with sterile distilled water and dried using paper towel. After that, cross section of each diseased root was thinly sliced. The root slices with lesion (dark patches) were selected for fungal isolation. Three slices of each samples were placed on the surface of water agar (WA) and incubated at room temperature (ca. 25°C) in dark condition and observed daily for the growth of fungal mycelia. On the next day, plugs of mycelia from the edge of growing colony of the predominant fungus (most noticeable fungus) growing from the infected area was transferred onto the surface of separate fresh potato dextrose agar (PDA) plates for the fungal pure culture establishment in the next step.

3.3.2 Pure Culture Establishment and Pre-identification

Hyphal fragment isolation method as described by (Leyronas *et al.*, 2012) were used for the establishment of pure culture. A tiny block of well separated hypha grown from a colony was transferred onto new PDA plate for each isolate under a dissecting microscope. The plates were incubated at room temperature (ca. 25°C) in dark condition and observed daily for any contamination. The steps were repeated until pure culture of each isolate was established, where the circular colony radiate from single centre point (Noman *et al.*, 2018)

3.4 *Fusarium* Identification

All *Fusarium spp.* isolated from diseased root samples of *Piper nigrum* L. were pre-identified based on the spore morphology on PDA media. The isolates were then grouped based on the colony morphological characteristics, such as texture, margin, colony elevation and pigmentation. Molecular identification using internal transcribed spacer (ITS) sequence and morphological characterization were done on a total of 25 randomly selected isolates (Table 3.2).

Table 3.2: *Fusarium spp.* isolates selected for molecular identification included.

Group	Number of sampling farm	Number of isolates selected from each groups	Remark
1	3	2	
2	4	2	
3	8	6	Included most of the sampling farm
4	2	2	
5	5	4	1 isolate sequence damaged
6	4	2	
7	3	3	
8	2	2	
9	2	2	
Total		25	

3.4.1 DNA Extraction

Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle, 1987) with modification was used for the extraction of fungal genomic DNA. The mycelia of four days old culture were scrapped and transferred into an Eppendorf tube. Then, the mycelia were ground together with 500 μ l of CTAB solution and incubated in 65°C water bath for an hour. The sample was spun down after incubation. Chloroform isoamyl alcohol (CIA) at the volume of 500 μ L were added and mixed well. The mixture was then spun at 13,500 rpm for 15 minutes. The supernatant was transferred into a new Eppendorf tube. Proper amount of isopropyl alcohol was added and mixed well before stored overnight at ca. 4°C.

On the following day, 1 mL of 70% ethanol were used to wash obtained DNA pellet, and centrifuged for another 15 minutes at 13,500 rpm. The DNA pellet was air-dried after the ethanol was discarded. Sterile distilled water at the volume of 50 μ L was added to dissolve the DNA pellet. The dissolved DNA was stored in -20°C before use. Spectrophotometer (IMPLEN NanoPhotometer® P-Class) was used to determine the quality and concentration of extracted genomic DNA.

3.4.2 PCR Amplification

DNA samples were used for internal transcribed spacer amplification with universal primer pair ITS-1 (5'-TCC GTA GGT GAA CCT GCG-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR was performed in a total volume of 25 μ L reaction mixture, containing 1 $\times$ PCR buffer, 0.2mM dNTP mixture, 1.5mM MgCl₂, 1U/ μ L Taq DNA polymerase (First Base), 0.1 μ mol of forward and reverse primers and template DNA in a Sensoquest labcycler (Sensoquest, Gottingen, Germany) with the following conditions: pre-denaturation at 95°C for 2 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minutes; and final extension at 72°C for 10 minutes.

3.4.3 DNA Purification and BLAST Analysis

The PCR products were electrophoresed together with 100 bp DNA ladder on a 1% agarose gel with 1 $\times$ TAE buffer, at 75volt for 30 minutes. Gel staining was done using ethidium bromide (ETBr) before visualizing the PCR products under UV-transilluminator.

QIAquick® Gel Extraction Kit (QIAGEN) was used for PCR products purification. Briefly, a mixture consisted of 60 µL PCR product, 180 µL Buffer QG and 60 µL of isopropanol were added into 2 mL spin column Eppendorf tube provided in the extraction kit and centrifuged for 1 minute at 13,500 rpm. Then, Eppendorf tube with flow-through was discarded and replaced with new tube. The mixture was centrifuged for 1 minute at 13500 rpm after another 500 µL Buffer QG was added in. The Eppendorf tube with flow-through was discarded and replaced with new tube. Then, 500 uL Buffer PE was added and incubated for 5 minutes before the tube was centrifuged for 1 minute at 13500 rpm. The Eppendorf tube with flow-through was discarded and replaced with new tube. The tube was centrifuged again for 1 minute at 13500 rpm. Then, the spin column was placed into new Eppendorf tube and incubate for 10 minutes to allow the alcohol to evaporate before 30 uL Buffer EB was added and incubated for 4 minutes. Lastly, the tube was centrifuged for 1 minute at 13500 rpm to obtain purified PCR product.

The purified PCR product was electrophoresed on a 1% agarose gel with 1× TAE buffer at 75 volt for 30 minutes. EtBr was used for gel staining before visualizing the purified PCR products under UV-transilluminator. The purified PCR products were sent for direct sequencing (Apical Scientific Sdn. Bhd.). ITS sequences of the isolates were BLAST against sequences in NCBI (National Centre for Biotechnology Information) database. Species verification was done based on the percentage of identity.

3.5 Morphological Characterisation

3.5.1 Macromorphology and Micromorphology Study

The *Fusarium* isolates morphological identification was carried out based on the description by (Nelson *et al.*, 1981) and (Leslie & Summerell, 2007). Three replicates were done for each isolate. Texture, margin, colony elevation and pigmentation were the colony characteristics observed and recorded for three replicates of each isolates. The description of colony morphology characteristics was based on UKEssays, (2018). A compound microscope (Nikon Eclipse E100) was used to study the shape of macroconidia and microconidia.

3.5.2 Growth Rate

Lines were drawn on Petri dishes to divide the plates into four equal sections (A, B, C and D). After the fungal plug was inoculated onto each Petri dish, colony growth rate from the three replicate plates for each isolate was observed daily. Ruler was used to measure the colony radius from centre of each Petri dish to the edge of mycelium and recorded. Observation end when the edge of mycelium touched the wall of Petri dish (full plate). The mycelium growth rate was calculated based on **Equation 3.1** and **Equation 3.2**.

$$\text{Average mycelium growth (D)} = \frac{\text{Length of (A+B+C+D)mm}}{4} \quad \text{Equation 3.1}$$

$$\text{Mycelium growth rate} = \frac{[(D2-D1)+(D3-D2)+(D4-D3)+\dots]}{\text{No. of days of growth}-1} \quad \text{Equation 3.2}$$

3.5.3 Sporulation Rate

Spores from a twelve-day old colony were scrapped off by adding 5mL of distilled water to the colony and transferred the spores to sterile 10mL falcon tubes before serial dilution was carried out until the concentration of 1×10^{-2} . The macro- and microspore were found in the solution. The concentration of the two different spores was estimated using a haemocytometer (LW Scientific). Quantity of both macroconidia and microconidia were calculated and recorded. The steps were repeated for three times (three separate plates) for each isolate. The sporulation rate was calculated using **Equation 3.3**:

No. of spore per 5 mL

$$= (A + B + C + D + E) \times \text{dilution factor} \times \text{magnification power} \quad \textbf{Equation 3.3}$$

Note: No. of spore per 5mL=(A+B+C+D+E) x 2000 x 100, where number of conidia counted from four outer and center squares of haemocytometer, multiplied by dilution factor and light microscope magnification power.

The data collected for both growth rate and sporulation rate were analysed using one-way ANOVA followed by Tukey HSD post hoc test ($p < 0.05$) using IBM SPSS Statistics 25 software.

3.6 Effect of pH and Different Nutrient Media on the Growth of *F. solani*

3.6.1 Media Preparation

For pH experiment, Potato dextrose agar (PDA; Merck) media were prepared. PDA media was selected as it provides favourable growth condition for most endophytic and pathogenic fungi (Guo *et al.*, 1998; W.-C. Li *et al.*, 2007). Besides that, PDA was used to culture and characterise the isolated *F. solani*. Hence, the morphological characterisation on media could serve as a reference to observe the morphological changes across different pH on PDA media. The pH of PDA media was adjusted to pH 5, pH 6, pH 7, pH 8 and pH 9, respectively, using 1.0 M NaOH to increase the pH value and 1.0 M HCl to decrease the pH value. The PDA media were then sterilized and poured onto Petri dishes having lines drawn on the back, separating the dish into four equal sections.

For studying the effect of different nutrient media on the growth of *F. solani*, five different media were prepared according to the recipe listed in **Table 3.2** and sterilized. Similarly, the sterilized media were poured onto Petri dishes having lines drawn on the back, separating the dish into four equal sections. The pH value for each nutrient medium was adjusted around pH 5 ± 0.5 (Sánchez-Rangel *et al.*, 2018).

Table 3.3: Recipe for Five Different Media Used to Observe the Growth of *F. solani*

Media	Material	Preparation	Justification
Oatmeal agar (OMA) (Koley & Mahapatra, 2015)	40 g Oat 15 g agar	40 g of oatmeal and 15 g of agar were added into clean media bottle. Distilled water was added to top up the mixture until 1 L.	Supply source of carbon
<i>Piper nigrum</i> L. leaf agar (PNA) (Iqbal <i>et al.</i> , 2005)	20 g healthy pepper leaves 15 g agar	20 g of healthy <i>Piper nigrum</i> L. leaves were grounded using a blender. The grounded leaves were then quickly transferred into media bottle to prevent further oxidation of the leaves. 15g of agar were added. Distilled water was added to top up the mixture until 1 L.	Natural host leaf. It might support fungal growth
<i>Piper colubrinum</i> agar (PCA) (Iqbal <i>et al.</i> , 2005)	20 g healthy <i>Piper colubrinum</i> leaves 15 g agar	20 g of healthy <i>Piper colubrinum</i> leaves were grounded using a blender. The grounded leaves were then quickly transferred into media bottle to prevent further oxidation of the leaves. 15 g of agar were added. Distilled water was added to top up the mixture until 1 L.	<i>P. colubrinum</i> is grouped under <i>Piper</i> genus. It might have similar nutrient to <i>P. nigrum</i> which supports fungal growth
Carnation leaf agar (CLA) (Iqbal <i>et al.</i> , 2005)	20 g carnation leaves 15 g agar	20 g of healthy carnation leaves were grounded using a blender. The grounded leaves were then quickly transferred into media bottle to prevent further oxidation of the leaves. 15 g of agar were added. Distilled water was added to top up the mixture until 1 L.	Mostly used as media for <i>Fusarium</i> identification (Iqbal <i>et al.</i> , 2015)

Table 3.2 continued

PDA (Merck)	39 g of PDA powder	39 g of Merck PDA powder were top up to 1L with distilled water	Provides favourable growth condition for most endophytic and pathogenic fungi (Guo <i>et al.</i> 1998; Li <i>et al.</i> 2007).
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For both experiments above, the morphological characterisation followed the description in section 3.1.5 above, except for sporulation rate, which was not observed (due to Movement Control Order for COVID-19). Growth rate of the colonies were analysed using one-way ANOVA followed by Tukey HSD post hoc test ($p < 0.05$) using IBM SPSS Statistics 25 software.

3.7 Phylogeny Analysis of *Fusarium* spp.

Twenty-four *Fusarium* spp. isolates used to construct phylogenetic tree as one isolate had poor sequence. Ten isolates of *F. solani*; three of *F. oxysporum* and 11 of *F. proliferatum* together with 10 isolates of *F. solani* and *F. proliferatum*, respectively from (Shahnazi *et al.*, 2012) to show the relationship of the *Fusarium* spp from Malaysia.

Another phylogenetic tree was constructed by using the ITS sequences of the 10 isolates of *F. solani* from this study together with the ITS sequences of *F. isolani* isolated from different countries. From NCBI, only ITS sequences of *F. isolani* isolates with known location (country) were selected (**Table 3.3**).

ITS sequences were aligned using Clustal W (MEGA X). Neighbour joining (NJ) phylogenetic tree generated using MEGA X. *F. staphyleae* strain NRRL22316 was taken as outgroup species as it is close to *F. solani*, *F. oxysporum* and *F. proliferatum* (Chehri *et al.*, 2015; Šišić *et al.*, 2018). Phylogenetic dendrogram with the bootstrap value, which illustrated 1000 replicate heuristic search was generated.

Table 3.4: List of *Fusarium* Isolates Used in Phylogenetic Dendrogram Construction

Country	Location	Accession number	Label in phylogeny dendrogram	Reference
Malaysia	Sarikei	JF322996	Fs-E2	(Shahnazi <i>et al.</i> , 2012)
		JF322997	Fs-F5	
		JF322998	Fs-H4	
		JF322999	Fs-H5	
		JF323000	Fs-H6	
		JF323001	Fs-H7	
		JF323002	Fs-H8	
		HQ830366	Fp-F3	
		HQ830367	Fp-F5	
		HQ830368	Fp-G2	
		HQ830369	Fp-G3	
		HQ830370	Fp-H6	
		HQ830371	Fp-H8	
		HQ830372	Fp-H9	

Table 3.3 continued

Malaysia	Sibu	JF323005	Fs-B3	
		HQ662410	Fp-A1	
		HQ662411	Fp-B1	
		HQ830365	Fp-D2	
	Kulai	JF323003	Fs-K2	
		JF323004	Fs-K6	
Taiwan	Taipei	EU719658	Fs-LW-1	(Wang <i>et al.</i> , 2010)
	Yuchi	AB551701	FsCymFs-3	(Chung <i>et al.</i> , 2011)
	Pingtung	AB551681	Fs-SLuY-01c	
	Chayi	AB551696	Fs-YS31b	
	Changhua	AB551708	Fs-53	
		AB551709	Fs-54	
		AB551710	Fs-55	
		AB551712	Fs-61	
	Taichung	AB551704	Fs-FS02p	
		AB551711	Fs-57	
	Tainan	AB551713	Fs-64	
		AB551684	Fs-SM1a	
Thailand	N/A	LC332026	Fs-RYG1	(Tiwatwananikul <i>et al.</i> , 2019)
		LC332025	Fs-RYG2	
		LC332023	Fs-RYG3	
		LC332024	Fs-RYG4	

Table 3.3 continued

India	Arunachal Pradesh	LR735965	Fs-Sil-4	
	Assam	LR735964	Fs-Jor-3	(Pappachan <i>et al.</i> , 2020)
	West Bengal	LR735963	Fs-Pip-2	
		LR735900	Fs-Kar-1	
	N/A	KR071141	Fs-Fs-1	(Kandan <i>et al.</i> , 2015)
	Marathwada	LC370340	Fs-S3	(Choudhary & Kareppa, 2016)
Indonesia	Mataram	LC055792	Fs-IPBBCC -7.525	(Tunarsih <i>et al.</i> , 2015)

CHAPTER 4

RESULTS

4.1 Sample Collection

A total of 15 pepper farms in North-western region of Sarawak ie. Kuching, Serian, Sri Aman and Betong were visited. Forty-five diseased root samples in total were collected from pepper plants with defoliation and rotten root (3 samples per farms). Briefly, soil beside the plants were removed to expose the plant roots. Roots with *Fusarium* wilt disease symptoms such as root rot or darken root were collected as sample. There were 93 *Fusarium* spp. isolates were successfully isolated from 45 diseased root samples (**Table 4.1**).

Table 4.1: Number of *Fusarium* spp. Isolated from 15 Pepper Farms in North-western Region of Sarawak

Farm code	Location	Division	GPS coordinate	Number of <i>Fusarium</i> spp. isolated
JHF	Stass	Kuching	N01°23'11.259", E109°58'27.6384"	15
SMG	Semenggok	Kuching	N01°23'35.2356", E110°19'35.637"	7
JMF	Paon Gahat	Serian	N00°56'58.1352", E110°39'7.8408"	9
JDF	Linsat	Serian	N01°03'41.4642", E110°43'21.594"	8
CXF	Linsat	Serian	N01°03'15.6558", E110°43'37.0842"	5
RNF	Linsat	Serian	N01°02'48.015", E110°43'13.4214"	11
BLF	Jaong	Sri Aman	N01°04'44.1228", E110°59'23.6652"	2
JG1	Jaong	Sri Aman	N01°05'38.9256", E110°59'16.3422"	5

Table 4.1 continued

JG2	Jaong	Sri Aman	N01°05'47.6628", E110°59'24.1758"	11
PTU	Pantu	Sri Aman	N01°05'9.7476", E111°06'40.9098"	8
GMF	Gua Ming	Sri Aman	N01°04'30.5358", E111°17'10.1796"	2
SDM	San Demam	Sri Aman	N01°07'13.9794", E111°35'25.875"	6
JLK	Jelukong	Sri Aman	N01°10'4.6704", E111°39'32.3784"	1
BTG	Saka	Betong	N01°23'15.8208", E111°33'0.0426"	3
JNF	Saka	Betong	N01°22'49.1334", E111°33'53.3232"	Nil
Total				93

4.2 Fungal Isolation and Pure Culture Establishment

The 93 *Fusarium* spp. isolated from diseased root samples were divided into nine groups primarily according to their colony morphological characteristics, such as surface appearance, margin, colony elevation and pigmentation cultured on PDA media. The conidia of *Fusarium* isolates displayed similar appearance. This makes the conidia features of *Fusarium* isolates cannot be used to differentiate between isolates. Hence, the conidia were not used for the grouping of *Fusarium* spp. isolates. Six out of nine groups ie. G3, G4, G6, G7, G8, and G9, showed round, uniform margin. Five out of nine groups ie. G1, G2, G3, G5 and G9 of the isolates exhibit fluffy, raised elevation (**Table 4.2**).

Table 4.2: Colony Characteristics Variation Among *Fusarium* spp. Isolates

Group	Surface appearance	Margin	Colony elevation	Pigmentation (Reverse view)
G1	Cloud-like mycelia distribution. Curly white, short mycelia.	Flare margin	Fluffy, raised elevation	Violet
G2	Uniform mycelia distribution. Curly, short, violet-white mycelia	Flare margin	Fluffy, raised elevation	Pale violet
G3	Uniform mycelia distribution. Curly, short white mycelia.	Round, uniform margin	Fluffy, raised elevation	Creamy white
G4	Uniform mycelia distribution with vein-like pattern radiate from middle. Fine, short, white mycelia.	Round, uniform margin	Flat elevation	Violet-white
G5	Cloud-like mycelia distribution. Fine, straight short mycelia.	Flare margin	Fluffy, raised elevation	Colourless
G6	Flat, uniform mycelia distribution. Fine, straight, short mycelia	Round, uniform margin	Flat elevation	Brown to white
G7	Uniform mycelia distribution. Curled, white mycelia.	Round, uniform margin	Flat elevation	Yellow to white
G8	Uniform mycelia distribution. Fine, short, white mycelia.	Round, uniform margin	Flat elevation	White

Table 4.2 continued

G9	Uniform mycelia distribution. Curled, long, white mycelia	Round, uniform margin	Fluffy, raised elevation	Peach white	to
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4.3 Species Identification

4.3.1 Molecular identification

A total of 24 isolates were randomly selected from each group for molecular identification using ITS sequence to represent different morphology from different sampling location (**Table 3.2**). Isolates pre-identified as *Fusarium* based on spores were selected representing morphological variations and sampling locations. The amplified ITS fragments were approximately 500 bp (**Figure 4.1**). After sequencing, the ITS sequence of the 24 isolates ranged from 517 bp to 525 bp. After blasting the ITS sequences against the *Fusarium* sequences in NCBI database, high percentage of identity ranging 99.2-100% was obtained. Three species, viz. *Fusarium solani*, *Fusarium oxysporum* and *Fusarium proliferatum* were identified. There were ten isolates of *F. solani*; three of *F. oxysporum* and eleven of *F. proliferatum* (**Table 4.3**).

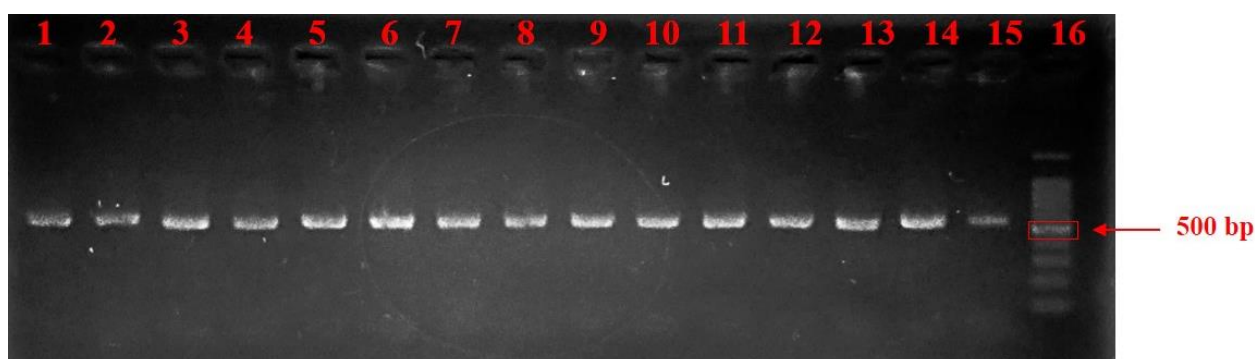


Figure 4.1: PCR Products Amplified from 15 *Fusarium* Isolates Using ITS1-ITS4 Primers. PCR Products of *Fusarium* Isolates (1)Fs-7SMG, (2)Fs-9T1, (3)Fs-6RN, (4)Fs-8JD, (5)Fs-8JM, (6)Fs-5JG, (7)Fs-JOG1, (8)Fs-JOG2, (9)Fs-JOG3, (10)Fs-6SDM, (11)Fo-JHF, (12) Fo-2BL, (13)Fo-4RN, (14) Fp-1JH, (15)Fp-3JH; Lane 16: 100 bp DNA Ladder.

Table 4.3: Identification of *Fusarium* spp. Based on ITS Region Sequencing

<i>Fusarium</i> spp.	Isolate code	Sampling farm code	Location	Accession number	Sequence with best match	Max identity (%)	E - value
<i>F. solani</i>	Fs-7SMG	SMG	Semonggok, Kuching	MT730093	LC055798	99.60	0.0
	Fs-9T1	SMG	Semonggok, Kuching	MT730096	JQ922211	99.22	0.0
	Fs-6RN	RNF	Linsat, Serian	MT730091	LC055798	99.60	0.0
	Fs-8JD	JDF	Linsat, Serian	MT730094	HQ696788	99.23	0.0
	Fs-8JM	JMF	Paon Gahat, Serian	MT730095	KY776027	99.61	0.0
	Fs-5JG	JG1	Jaong field 1, Sri Aman	MT730090	MH517359	99.61	0.0
	Fs-JOG1	JG1	Jaong field 1, Sri Aman	MT730097	MH517359	99.81	0.0
	Fs-JOG2	JG1	Jaong field 1, Sri Aman	MT730098	MH517359	99.61	0.0
	Fs-JOG3	JG1	Jaong field 1, Sri Aman	MT730099	MH517359	100	8e - 167
	Fs-6SDM	SDM	San Demam, Sri Aman	MT730092	MF579583	99.81	0.0

Table 4.3 continued

<i>F. oxysporum</i>	Fo-4JH	JHF	Stass, Kuching	MT730112	JN859834	100	0.0
	Fo-4RN	RNF	Linsat, Serian	MT730113	JN859834	100	0.0
	Fo-2BL	JG2	Jaong field 2, Sri Aman	MT730111	JN859834	100	0.0
<i>F. proliferatum</i>	Fp-1JH	JHF	Stass, Kuching	MT730101	MH715405	100	0.0
	Fp-3JH	JHF	Stass, Kuching	MT730105	MH715405	100	0.0
	Fp-9T2	SMG	Semonggok, Kuching	MT730110	MG543732	100	0.0
	Fp-2RN	RNF	Linsat, Serian	MT730102	MH715405	100	0.0
	Fp-3CX	CXF	Linsat, Serian	MT730104	MH715405	100	0.0
	Fp-3RN	RNF	Linsat, Serian	MT730108	MH715405	100	0.0
	Fp-3JM	JMF	Paon Gahat, Serian	MT730106	MH715405	100	0.0
	Fp-5JM	JMF	Paon Gahat, Serian	MT7301019	MH715405	100	0.0
	Fp-1GM	GMF	Gua Ming, Sri Aman	MT730100	MG543732	100	0.0

Table 4.3 continued

<i>F.</i>	Fp- 3PTU	PTU	Pantu, Aman	Sri	MT730107	MH715405	100	0.0
<i>proliferatum</i>	Fp- 3BTG	BTG	Saka, Betong		MT730103	MH715405	100	0.0

4.3.2 Morphological Characterisation

4.3.2.1 *Fusarium solani*

After molecular identification, the 24 isolates were characterised morphologically. The characteristics includes the shape of the colony (form), colony margin, mycelium elevation, colony surface, and the pigmentation on both upper view and reverse view. The *F. solani* isolates were mostly circular form except for isolate Fs-6RN which was filamentous in shape (**Figure 4.2a**). The colony margin of *F. solani* isolates were curled except that of isolate Fs-9T1 which had piliiform margin (**Figure 4.2b**). Isolate Fs-6RN and Fs-9T1 were also the only two isolates with raised colony elevation while the other *F. solani* isolates had flat colony (**Table 4.4**).

All the *F. solani* colonies were zonate where there was formation of ring on the colony surface which was covered by aerial mycelia varying from fine and sparse to dense and woolly appearance (**Figure 4.2a – 4.2j**). Uniquely, aerial mycelia of isolate Fs-6RN formed feather-like pattern radiating from centre of the colony (**Figure 4.2a**), while isolate 9T1 have wool-like surface (**Figure 4.2b**). Most isolate formed creamy and slimy sporodochia on the surface after day 14. Clusters of perithecia were found on upper view of the colony for both isolates Fs-8JD and Fs-6SDM, which were molecularly identified as *Nectria haematococca*, a sexual form of *F. solani* (**Figure 4.2d and Figure 4.2g**)

The pigment of colony of *F. solani* generally varied from brown to greyish colour on upper view, some isolates such as Fs-5JG and Fs-JOG1 have brown patches found on the edge of the colony (**Figure 4.2f and Figure 4.2h**). On the contrary, isolate Fs-6RN had violet hue instead of brown (**Figure 4.2a**). Isolate Fs-8JD and Fs-6SDM had orange ring near the edge of the colony (**Figure 4.2d and Figure 4.2g**). The isolates mostly exhibited brown to creamy white pigment on the reverse view, except isolate Fs-6RN which had violet hue pigment (**Figure 4.2a**). Brown patches were observed on the reverse view for those isolates which had brown patches on upper view (**Figure 4.2h and Figure 4.2r**). Yellow background, with tiny orange dots that looks like chia seed was uniquely found on the reverse colony of isolate Fs-8JD and Fs-6SDM (**Figure 4.2d and Figure 4.2g**).

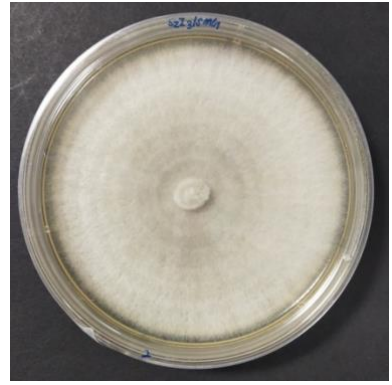
The shape of macroconidia of *F. solani* were mostly curved with pointed apical cell. Three to five septa, but mostly five, were found in *F. solani* macroconidia (**Figure 4.2u**). The microconidia were mostly oval, ellipsoidal or reniform in shape. Microconidia with nil to one septum were observed (**Figure 4.2v**).



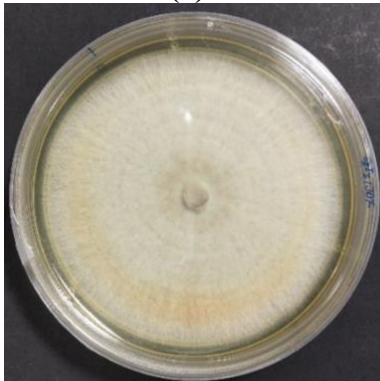
(a)



(b)



(c)



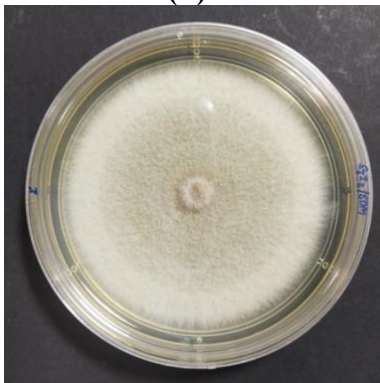
(d)



(e)



(f)



(g)



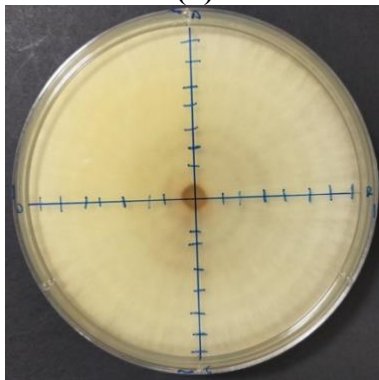
(h)



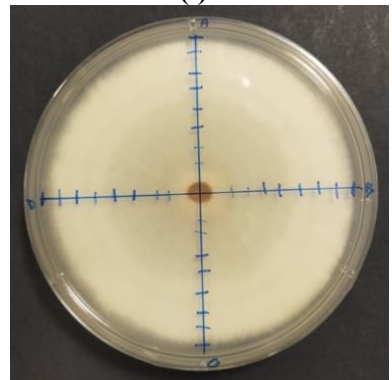
(i)



(j)



(k)



(l)

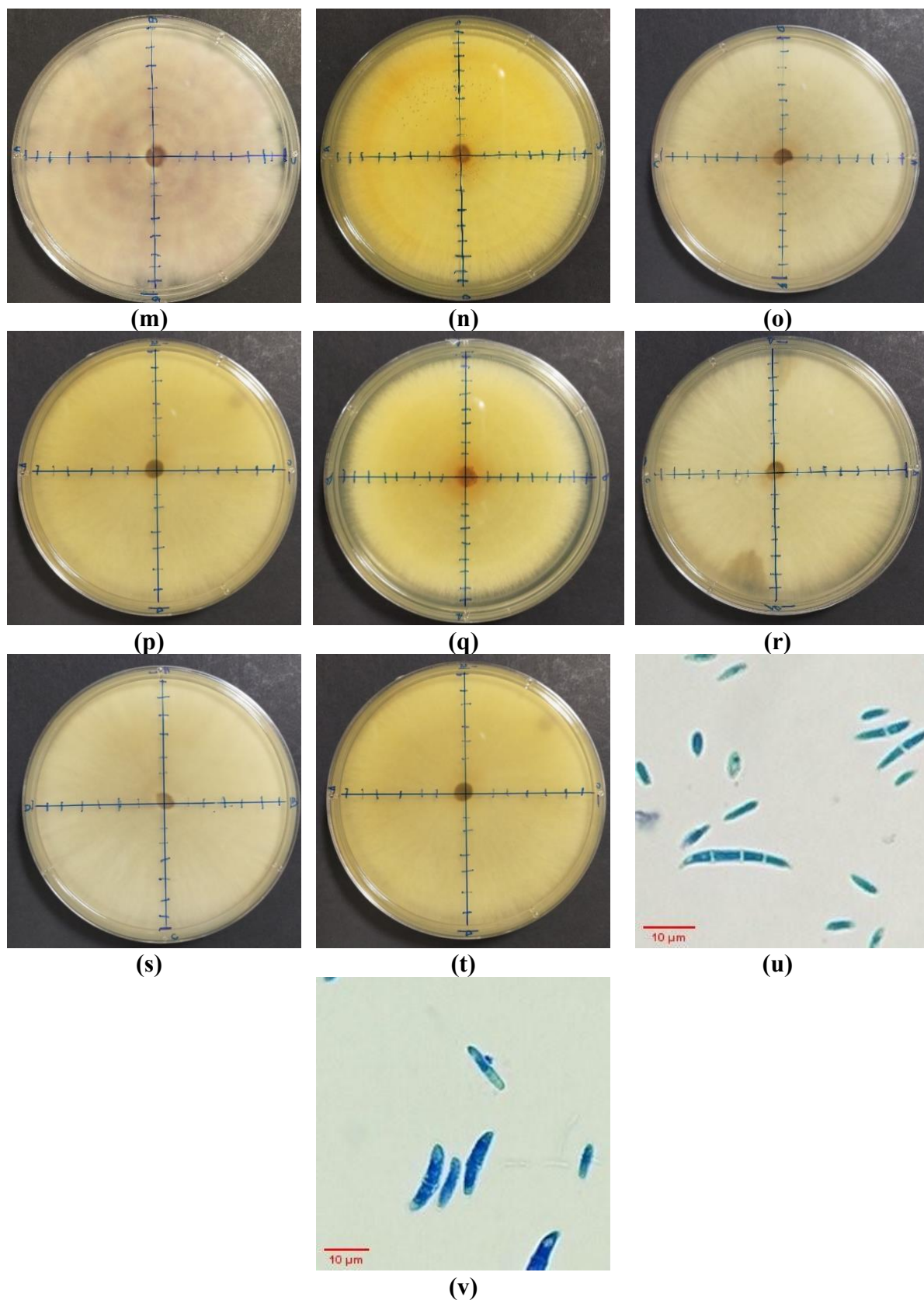


Figure 4.2: Macromorphology and Micromorphology Under Microscope at 40x Magnification of *F. solani* isolates (a) Upper View of Isolate Fs-6RN (b) Upper View of Isolate Fs-9T1 (c) Upper View of Isolate Fs-7SMG (d) Upper View of Isolate Fs-8JD (e) Upper View of Isolate Fs-8JM (f) Upper View of Isolate Fs-5JG (g) Upper View of Isolate

Fs-6SDM (h) Upper View of Isolate Fs-JOG1 (i) Upper View of Isolate Fs-JOG2 (j) Upper View of Isolate Fs-JOG3 (k) Reverse View of Isolate Fs-7SMG (l) Reverse View of Isolate Fs-9T1 (m) Reverse View of Isolate Fs-6RN (n) Reverse View of Isolate Fs-8JD (o) Reverse View of Isolate Fs-8JM (p) Reverse View of Isolate Fs-5JG (q) Reverse View of Isolate Fs-6SDM (r) Reverse View of Isolate Fs-JOG1 (s) Reverse View of Isolate Fs-JOG2 (t) Reverse View of Isolate Fs-JOG3 (u) Macroconidia of *F. solani* (Bar=20µm) (v) Microconidia of *F. solani* (Bar=10µm)

4.3.2.2 *Fusarium oxysporum*

The colony form of *F. oxysporum* isolates was divided into filamentous and circular (Table 4.4). Most of the *F. oxysporum* colonies were found to have entire margin except for isolate Fo-2BL with undulate margin (Figure 4.3a), and isolate Fo-4JH with curled margin (Figure 4.3b). The colony elevation of all the isolates were raised (Table 4.4).

The surface of *F. oxysporum* colonies were covered with abundant aerial mycelia ranged from sparse to fluffy texture. Distinctively, colony of isolate Fo-2BL and Fo-4JH was zonate with aerial mycelia grown and formed feather-like pattern on the colony surface (Figure 4.3a – 4.3b), while isolate Fo-4RN expanded randomly in a vein-like pattern from the culture plug (Figure 4.3c).

All the *F. oxysporum* isolates had greyish white upper view. However, the colour of the colonies' reverse view varied. The reverse view of isolate Fo-2BL and Fo-4JH showed violet colour from the centre and gradually turn to white at the edge of the colony (Table 4.4), whereas the reverse view of isolate Fo-4RN was found to be pinkish white in colour (Figure 4.3c).

The macroconidia of *F. oxysporum* were curved and slender with three to five septa were observed. Hook shape was observed on the apical cell of *F. oxysporum* macroconidia (Figure 4.3g). Microconidia of *F. oxysporum* were quite similar that of *F. solani* which they were oval, ellipsoidal or reniform in shape with nil to one septum (Figure 4.3h).

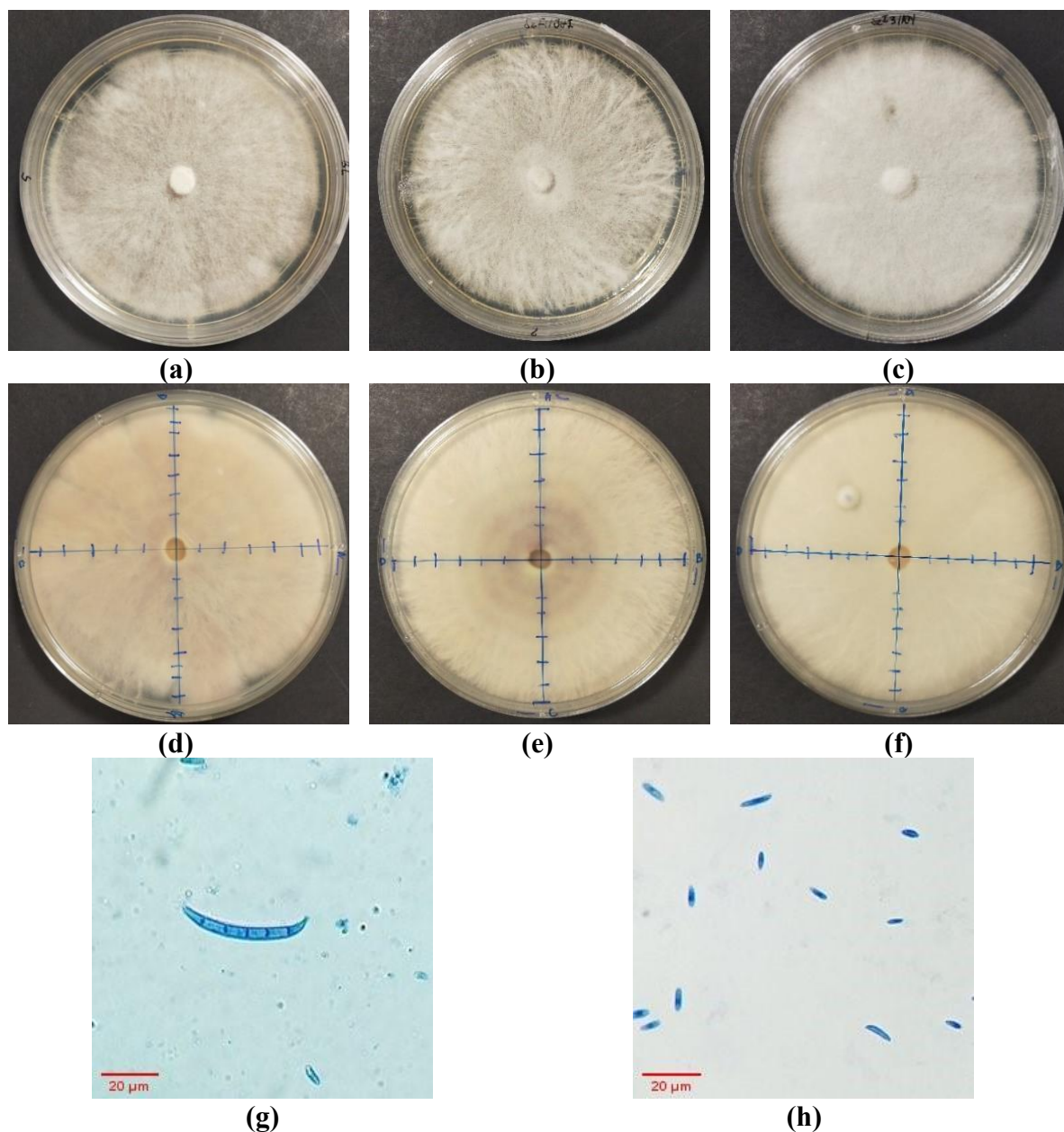


Figure 4.3: Macromorphology and Micromorphology Under Microscope at 40x Magnification of *Fusarium* Isolates. (a) Upper View of Isolate Fo-4JH (b) Upper View of Isolate Fo-4RN (c) Upper View of Isolate Fo-2BL (d) Reverse View of Isolate Fo-4JH (e) Reverse View of Isolate Fo-4RN (f) Reverse View of Isolate Fo-2BL (g) Macroconidia of *F. oxysporum* (Bar=20μm) (h) Microconidia of *F. oxysporum* (Bar=20μm)

4.3.2.3 *Fusarium proliferatum*

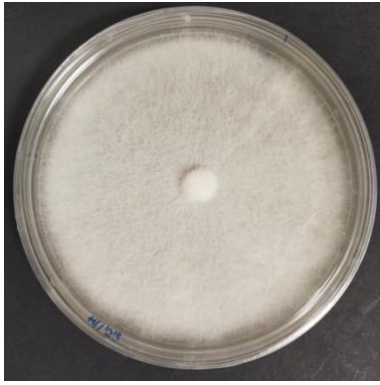
All *F. proliferatum* isolates were found to have colony with circular form, except for isolate Fp-1GM and Fp-3RN which had filamentous form (**Figure 4.4i and Figure 4.4d**) and isolate Fp-2RN with irregular form (**Figure 4.4g**). Four types of colony margin were found among the *F. proliferatum* isolates. Eight isolates had entire margin; one isolate with curled margin; one with piliform and one with undulate margin (**Table 4.4**). All *F. proliferatum* isolates had colony with raised elevation except for isolate Fp-9T2 which had flat colony (**Figure 4.4c**).

The colony of *F. proliferatum* was generally zonate and appeared to have woolly texture (**Figure 4.4**). The aerial mycelia grew out randomly in vein-like pattern in most of the *F. proliferatum* isolates. Isolates with distinctive surface features were observed. Isolate Fp-2RN had colony covered with fluffy aerial mycelia without vein-like pattern (**Figure 4.4g**). On the other hand, isolate Fp-1GM was found to be zonate, and aerial mycelia grew into feather-like pattern that resembled *F. oxysporum* isolates (**Figure 4.4i**), whereas isolate Fp-9T2 resembled majority of *F. solani* isolates with sparse to dense aerial mycelia (**Figure 4.4c**).

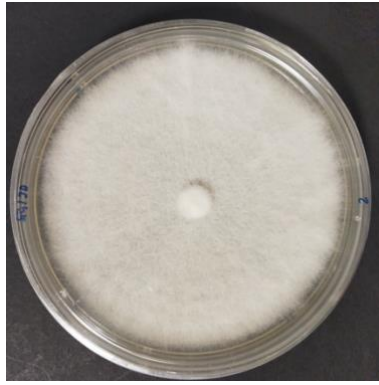
The upper view colony pigment of *F. proliferatum* isolates varied from greyish white to white hue except for isolate Fp-9T2 which was brownish white in colour (**Figure 4.4c**). On the reverse side, most of the isolates were found to be pinkish white in colour (**Table 4.4n**). Isolate Fp-1GM and Fp-2RN had violet colour at the centre on the reverse view and gradually become white at the edge (**Figure 4.4i and Figure 4.4g**). For isolate Fp-9T2, the reverse view had brownish white in colour. Isolate Fp-3CX had no pigmentation on the reverse side (**Figure 4.4p**).

The macroconidia of *F. proliferatum* were curved with pointed apical cell. Generally, three to four septa were found in the macroconidia (**Figure 4.4w**). The microconidia were

pyriform and club-shaped with flattened base. In general, nil to one septum was found on microconidia for some isolates (**Figure 4.4x**).



(a)



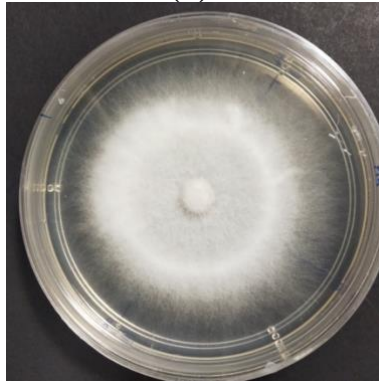
(b)



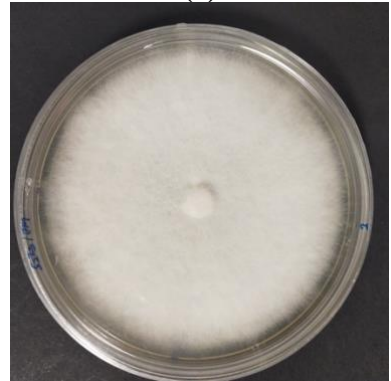
(c)



(d)



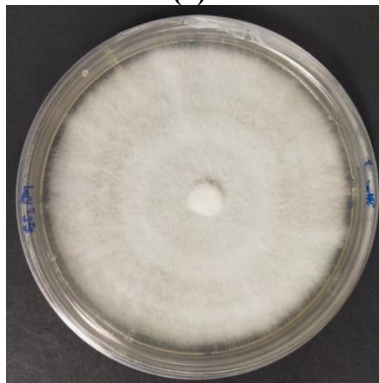
(e)



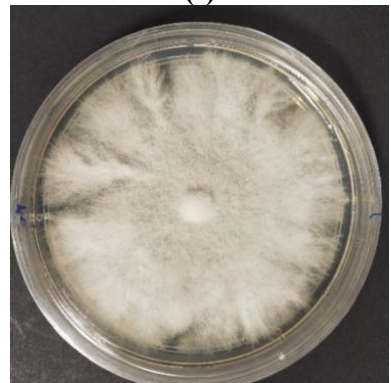
(f)



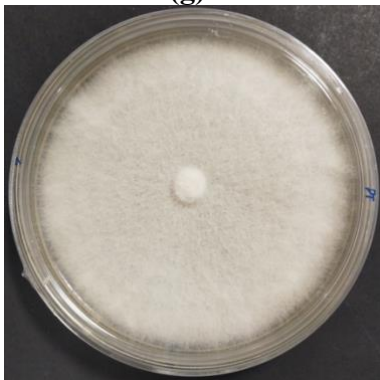
(g)



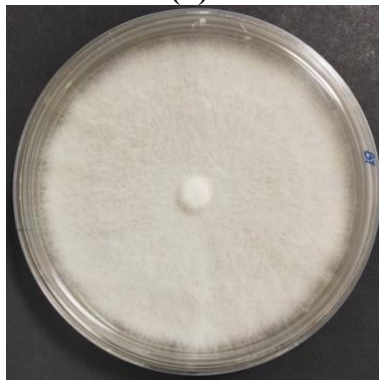
(h)



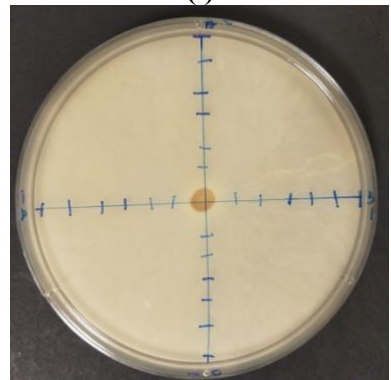
(i)



(j)



(k)



(l)

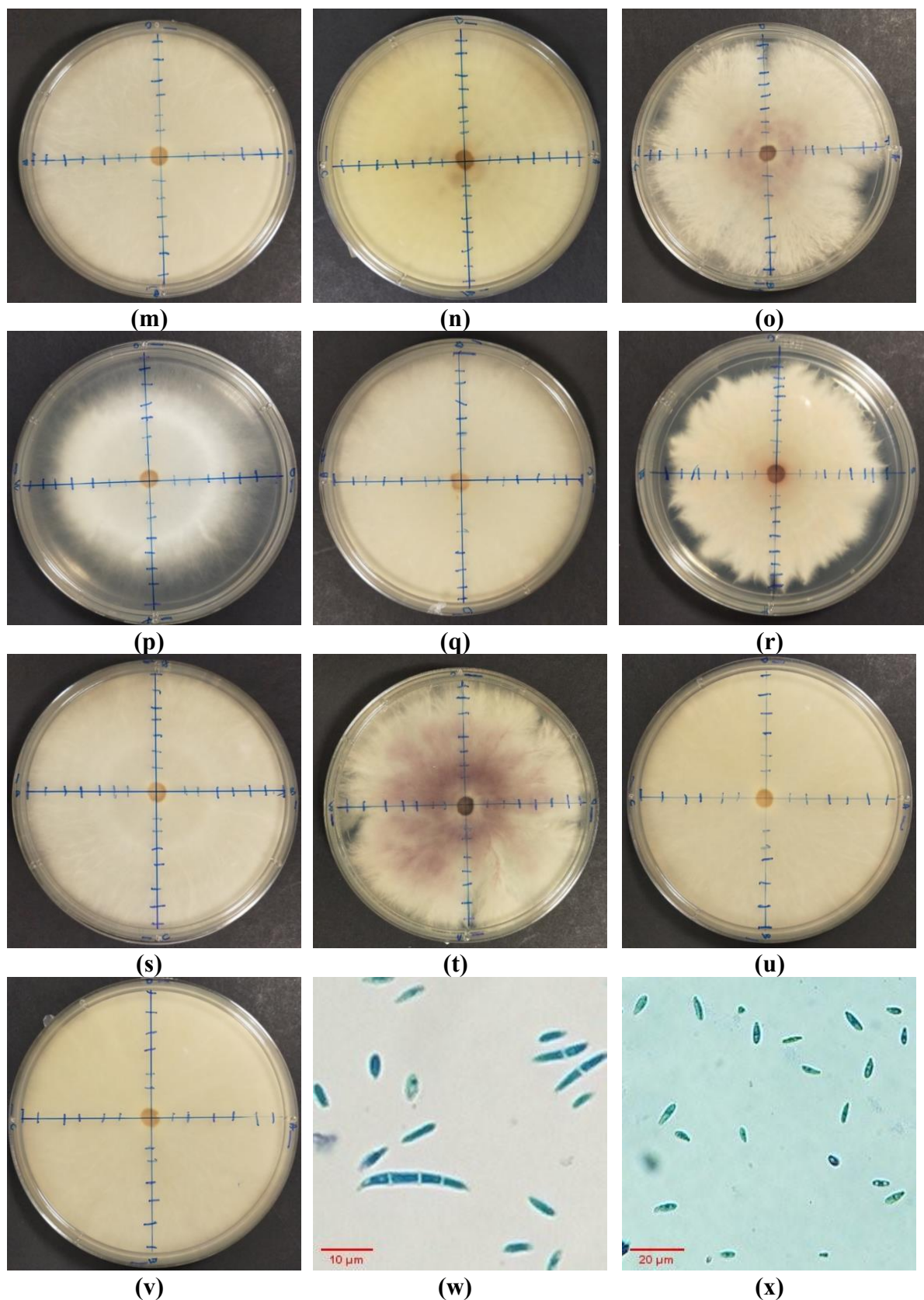


Figure 4.4: Macromorphology and Micromorphology Under Microscope at 40x Magnification of *F. solani* isolates (a) Upper View of Isolate Fp-1JH (b) Upper View of Isolate Fp-3JH (c) Upper View of Isolate Fp-9T2 (d) Upper View of Isolate Fp-3RN (e) Upper View of Isolate Fp-3CX (f) Upper View of Isolate Fp-3JM (g) Upper View of Isolate

Fp-2RN (h) Upper View of Isolate Fp-5JM (i) Upper View of Isolate Fp-1GM (j) Upper View of Isolate Fp-3PTU (k) Upper View of Isolate Fp-3BTG (l) Reverse View of Isolate Fp-1JH(m) Reverse View of Isolate Fp-3JH (n) Reverse View of Isolate Fp-9T2 (o) Reverse View of Isolate Fp-3RN (p) Reverse View of Isolate Fs-3CX (q) Reverse View of Isolate Fp-3JM(r) Reverse View of Isolate Fp-2RN (s) Reverse View of Isolate Fs-5JM (t) Reverse View of Isolate Fp-1GM (u) Reverse View of Isolate Fp-3PTU (v) Reverse View of Isolate Fp-3BTG (w) Macroconidia of *F. proliferatum* (Bar=10µm) (x) Microconidia of *F. proliferatum* (Bar=20µm)

From the observations, the colony morphology characteristics of the isolates were not much related to their origins. There was no unique feature of colony morphology found to be specific to certain locations.

Table 4.4: Summary of Colony Morphological Characteristics of 24 *Fusarium* spp. Isolates

<i>Fusarium</i> spp.	Division	Isolate	Form	Margin	Elevation	Surface appearance	Pigmentation	
							Upper view	Reverse view
<i>F. solani</i>	Kuching	Fs-7SMG	Circular	Curled	Flat	Fine mycelia	Maroon-grey to white	Brown to cream white
		Fs-9T1	Circular	Piliform	Raised	Fine, woolly texture	Grey to white	Greyish pink to white
	Serian	Fs-6RN	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to white
		Fs-8JD	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed	Yellow-grey to white. Orange outer ring formed	Yellow with tiny orange dots
		Fs-8JM	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
	Sri Aman	Fs-5JG	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown with brown patches

Table 4.4 continued

<i>F. solani</i>		Fs-6SDM	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed			Yellow-grey to white. Orange outer ring formed	Yellow with tiny orange dots
	Sri Aman	Fs-JOG1	Circular	Curled	Flat	Fine mycelia			Greyish white with brown patches	Pale brown with brown patches
		Fs-JOG2	Circular	Curled	Flat	Fine mycelia			Brown to greyish white	Pale brown to cream white
		Fs-JOG3	Circular	Curled	Flat	Fine mycelia			Brown to greyish white	Pale brown to cream white
<i>F. oxysporum</i>	Kuching	Fo-4JH	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre			Greyish white	Violet to white
	Serian	Fo-4RN	Circular	Entire	Raised	Fine mycelia with vein-like pattern radiate from centre			Greyish white	Pinkish white
	Sri Aman	Fo-2BL	Filamentous	Undulate	Raised	Fine mycelia with feather-like pattern radiate from centre			Greyish white	Violet to white

Table 4.4 continued

<i>F. proliferatum</i>		Fp-1JH	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
	Kuching	Fp-3JH	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-9T2	Circular	Curled	Flat	Fine mycelia	Brownish white	Brown to cream white
		Fp-2RN	Irregular	Piliform	Raised	Fine, woolly texture	White	Violet to white
		Fp-3CX	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	White	Colourless
	Serian	Fp-3JM	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-3RN	Filamentous	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
		Fp-5JM	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
	Sri Aman	Fp-1GM	Filamentous	Undulate	Raised	Fine mycelia with feather-like pattern radiate from centre	White	Violet to white

Table 4.4 continued

<i>F.</i>	Sri	Fp-3PTU	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white
<i>proliferatum</i>	Aman	Fp-3BTG	Circular	Entire	Raised	Fine, woolly texture with vein-like pattern radiate from centre	Greyish white	Pinkish white

In general, *F. oxysporum* showed least morphological variations. *F. solani* isolates exhibited the most variations among the three species. *F. solani* showed a total of twenty-one types of variations. On the other hand, *F. oxysporum* and *F. proliferatum* showed a total of 11 and 20 types of variations, respectively on the six morphological characteristics studied (**Table 4.5**).

There were two variants for the colony form, margin, and elevation, respectively, found among the *F. solani* isolates. Four variants of colony surface were found among *F. solani* isolates. The most diverse morphological characteristic among *F. solani* isolates was pigmentation, regardless of the colony upper view or reverse view of the colony. Six variants were observed for pigmentation on the upper view of colony, ranging from greyish-white to brownish-grey. Five variants ranging from greyish-pink to brownish-white on reverse view (**Table 4.5**).

On the other hand, *F. oxysporum* isolates showed two variants of the colony form, filamentous and circular. Three variants of colony margin were observed. No polymorphism was observed for elevation, and upper view pigmentation. The characteristic of colony surface among the *F. oxysporum* isolates was divided into two types. There were two variants of pigments observed on the reverse view of the colony, which were violet white and pinkish white (**Table 4.5**).

For *F. proliferatum* isolates, there were three variants of form. Four variants of colony margin, and two variants of elevation were observed among the isolates of *F. proliferatum*. The *F. proliferatum* isolates four variants of colony surface. The pigment variations among *F. proliferatum* isolates were less compared to *F. solani* isolates but more than *F. oxysporum* isolates. Three pigment variants ie. white, greyish-white and brownish-white were observed on the upper view of the *F. proliferatum* colony, while the reverse view of the colony displayed four variants of pigment, from colourless, pinkish white, violet white to brownish white (**Table 4.5**).

Table 4.5: Number of Variations Observed on the Colony Morphological Characteristics of the Isolated *Fusarium* Spp.

<i>Fusarium</i> spp.	Variations of colony morphology characteristics						Total variation
	Form	Margin	Elevation	Surface appearance	Upper view pigmentation	Reverse view pigmentation	
<i>F. solani</i>	2	2	2	4	6	5	21
<i>F. oxysporum</i>	3	3	1	1	1	2	11
<i>F. proliferatum</i>	3	4	2	4	3	4	20

Note: Total variation means the total number of different morphology characteristic displayed by the isolates colony.

4.3.3 Colony growth rate and sporulation rate

The growth rate varied among the species. The growth rate generally ranged from 4.00 to 4.90 mm/day for *F. solani*, 4.90 to 5.30 mm/day for *F. oxysporum* and 4.10 to 5.60 mm/day for *F. proliferatum*. Significant difference in the growth rate was observed for isolates of *F. solani* and *F. proliferatum* but not for those of *F. oxysporum*. Isolate Fs-7SMG, Fs-8JM and Fs-JOG3 of *F. solani* grew significantly faster than isolates Fs-8JD and Fs-6SDM (**Table 4.6**), with the difference of approximately 2 days to full plate. The former covered the entire surface of the Petri dish within 9 days while the latter reached full plate around the 11th day. For *F. proliferatum* isolate Fp-1JH and Fp-3JM grew significantly faster than Fp-2RN and Fp-3CX, 8 days versus 12 days to fully cover the surface of PDA plates.

Macroconidia sporulation rate showed variation among the isolates, ranging from 3.3×10^5 to 3.1×10^7 for *F. solani*; 4.3×10^5 to 1.6×10^6 for *F. oxysporum* and 7.3×10^5 to 2.9×10^7 for *F. proliferatum* isolates. Both *F. solani* and *F. proliferatum* isolates showed significant difference in macroconidia sporulation rate among isolates but not between isolates of *F. oxysporum*. Isolate Fs-8JD significantly produced more macroconidia compared to other *F. solani* isolates, followed by isolate Fs-9T1 at the rate of 3.1×10^7 and 2.4×10^7 , respectively. Conversely, isolate Fp-3CX yielded significantly more macroconidia compared to other 10 isolates of *F. proliferatum*, at the rate of 2.9×10^7 .

On the other hand, microconidia sporulation rate for *F. solani* ranged from 3.0×10^7 to 4.4×10^8 , 6.6×10^7 to 4.2×10^8 for *F. oxysporum* and 7.0×10^7 to 4.7×10^8 for *F. proliferatum*. The microconidia sporulation rate of *F. solani* and *F. proliferatum* isolates was found to have significant difference between isolates but not for *F. oxysporum*.

Table 4.6: Growth Rate and Spore Concentration of *Fusarium* spp. Isolates

<i>Fusarium</i> spp.	Division	Isolate	Growth rate (mm/day)	Macroconidia sporulation rate	Microconidia sporulation rate
<i>F. solani</i>	Kuching	Fs-7SMG	4.90 ^c	$4.4 \times 10^6 \pm 4.0 \times 10^5$ ^a	$4.0 \times 10^7 \pm 2.6 \times 10^5$ ^a
		Fs-9T1	4.30 ^{abc}	$2.4 \times 10^7 \pm 1.4 \times 10^6$ ^c	$4.2 \times 10^8 \pm 2.5 \times 10^7$ ^{cd}
		Fs-6RN	4.90 ^{bc}	$2.5 \times 10^6 \pm 8.8 \times 10^4$ ^a	$8.6 \times 10^7 \pm 1.7 \times 10^6$ ^{ab}
	Serian	Fs-8JD	4.00 ^a	$3.1 \times 10^7 \pm 3.1 \times 10^6$ ^d	$3.0 \times 10^8 \pm 2.3 \times 10^7$ ^{bcd}
		Fs-8JM	4.90 ^c	$1.0 \times 10^7 \pm 1.1 \times 10^6$ ^b	$4.4 \times 10^8 \pm 1.4 \times 10^8$ ^d
	Sri	Fs-5JG	4.40 ^{abc}	$3.3 \times 10^5 \pm 1.3 \times 10^5$ ^a	$2.0 \times 10^8 \pm 7.3 \times 10^6$ ^{abc}
	Aman	Fs-6SDM	4.00 ^a	$3.0 \times 10^6 \pm 2.3 \times 10^5$ ^a	$2.5 \times 10^8 \pm 7.5 \times 10^6$ ^{abcd}

Table 4.6 continued

<i>F. solani</i>	Sri Aman	Fs-JOG1	4.30 ^{ab}	$1.2 \times 10^6 \pm 2.7 \times 10^5$ ^a	$1.4 \times 10^8 \pm 1.6 \times 10^7$ ^{ab}
		Fs-JOG2	4.80 ^{bc}	$2.3 \times 10^6 \pm 4.9 \times 10^5$ ^a	$1.5 \times 10^8 \pm 1.1 \times 10^7$ ^{ab}
		Fs-JOG3	4.90 ^c	$1.9 \times 10^6 \pm 4.3 \times 10^5$ ^a	$3.0 \times 10^7 \pm 5.2 \times 10^6$ ^a
<i>F. oxysporum</i>	Kuching	Fo-4JH	4.90 ^a	$9.3 \times 10^5 \pm 8.8 \times 10^4$ ^{ab}	$7.1 \times 10^7 \pm 2.5 \times 10^6$ ^a
	Serian	Fo-4RN	5.10 ^a	$1.4 \times 10^6 \pm 2.9 \times 10^5$ ^b	$4.2 \times 10^8 \pm 4.4 \times 10^7$ ^c
	Sri Aman	Fo-2BL	5.30 ^a	$1.6 \times 10^6 \pm 1.9 \times 10^5$ ^b	$6.6 \times 10^7 \pm 7.7 \times 10^6$ ^a
		Fp-1JH	5.40 ^c	$4.2 \times 10^6 \pm 3.8 \times 10^5$ ^b	$4.6 \times 10^8 \pm 5.7 \times 10^6$ ^f
	Kuching	Fp-3JH	5.10 ^{bc}	$1.1 \times 10^6 \pm 1.7 \times 10^5$ ^a	$4.7 \times 10^8 \pm 2.4 \times 10^7$ ^f
<i>F. proliferatum</i>		Fp-9T2	4.40 ^{ab}	$9.8 \times 10^6 \pm 3.3 \times 10^4$ ^c	$2.3 \times 10^8 \pm 2.4 \times 10^7$ ^c
		Fp-2RN	4.10 ^a	$1.4 \times 10^6 \pm 3.5 \times 10^5$ ^a	$7.0 \times 10^7 \pm 2.8 \times 10^6$ ^a
		Fp-3RN	5.10 ^{bc}	$5.3 \times 10^6 \pm 2.0 \times 10^5$ ^b	$2.4 \times 10^8 \pm 5.9 \times 10^6$ ^c
	Serian	Fp-3JM	5.60 ^c	$5.4 \times 10^6 \pm 7.5 \times 10^5$ ^b	$2.0 \times 10^8 \pm 9.4 \times 10^6$ ^{bc}
		Fp-3CX	4.30 ^a	$2.9 \times 10^7 \pm 5.5 \times 10^5$ ^d	$1.6 \times 10^8 \pm 7.8 \times 10^6$ ^b
		Fp-5JM	4.90 ^{abc}	$2.0 \times 10^6 \pm 1.5 \times 10^5$ ^a	$3.3 \times 10^8 \pm 1.9 \times 10^7$ ^d
	Sri	Fp-1GM	4.90 ^{abc}	$2.0 \times 10^6 \pm 1.5 \times 10^5$ ^a	$3.9 \times 10^8 \pm 4.8 \times 10^6$ ^{de}
	Aman	Fp-3PTU	4.80 ^{abc}	$7.3 \times 10^5 \pm 2.6 \times 10^5$ ^a	$1.7 \times 10^8 \pm 9.6 \times 10^6$ ^b
	Betong	Fp-3BTG	4.90 ^{abc}	$4.0 \times 10^6 \pm 1.7 \times 10^5$ ^b	$4.5 \times 10^8 \pm 2.4 \times 10^6$ ^{ef}

Note: Comparison between species in terms of growth rate, macroconidia sporulation rate and microconidia sporulation rate with significant test done within column using variance analysis (one-way ANOVA) followed by Tukey HSD post hoc test ($p < 0.05$). The different alphabet within column indicates significant difference.

4.4 Effect of Different pH on the Growth of *F. solani* Isolates

Five pH value of PDA ranging from pH 5 to pH 9 were used to study the effect of pH on *F. solani* isolates. Colony morphological characteristics such as forms, margin, elevation, colony surface appearance and pigmentation for both upper view and reverse view were done for all *F. solani* isolates except for Fs-5JM. In general, all the observed characters across all the isolates had at least two variants.

There were two variants of colony form observed where all isolates had circular form except for isolate Fs-6RN which had filamentous form (**Table 4.7**). Three variants of colony margin were found among the *F. solani* isolates across pH (**Table 4.7**). The nine isolates either displayed one or two of the variants. The nine isolates of *F. solani* were found to have either raised or, flat or both variants of colony elevation. For colony surface appearance, five variants were observed across different pH (**Table 4.7**). Each isolate showed one to two of the five variants. There were 13 and 26 variants of pigmentation for upper and reverse view of the colony, respectively (**Table 4.7**). Each isolate showed at least one of the variants, with one isolate displayed up to four variants for upper view pigmentation and five for reverse view.

Table 4.7: Number of Variations Observed on the Colony Morphological Characteristics of the *F. solani* Isolates Across pH

Morphological characteristics	Form	Margin	Elevation	Surface appearance	Upper view pigmentation	Reverse view pigmentation
Number of variants	2	3	2	5	13	26

Note: Total variations is the total number of variations observed across different morphological characteristics displayed by the *F. solani* isolates across pH.

For each isolate, there were at least two morphological characters observed had variations across different pH, (**Table 4.8**), except for isolate Fs-6RN (**Figure 4.5**). For the colony form, the isolates were either circular or filamentous across different pH, there were no changes of the form for each isolate across pH (**Table 4.9**). This is not true for other characters (**Table 4.8**).

Piliform, curled and entire were the three types of colony margin found among the *F. solani* isolates. Isolate Fs-9T1, Fs-8JD and Fs-6SDM showed dimorphism (**Figure 4.5**), while the margin of the remaining isolates kept constant across different pH (**Table 4.8**). For isolate Fs-9T1, piliform margin were found on lower pH (pH 5 – 6), and portrayed entire margin when cultured on neutral to alkalic media (pH 7 – 9) (**Table 4.9**). For isolate Fs-8JD and Fs-6SDM, the colony margin was different on acidic and alkalic condition, however no clear trend of changes was observed on both isolates (**Table 4.9**).

The colony elevation of each isolate across different pH remained constant for all isolates (either raised or flat), except for isolate Fs-7SMG and Fs-8JD (**Table 4.8**). There was no change of colony surface appearance observed on all isolates across pH, with exception of three isolates (Fs-9T1, Fs-8JD and Fs-6SDM) (**Table 4.8**). For example, Isolate Fs-9T1 displayed zonate, fine woolly textured mycelia when cultured in slightly acidic environment (pH 5 and pH 6). These features were not observed in neutral to alkalic environment (pH 7 to pH 9) (**Table 4.9**).

The colony pigment on the upper view ranged from brown to grey to violet hue across different pH. Only two isolates (Fs-6RN and Fs-6SDM) kept constant pigment (**Table 4.8**). Isolate Fs-8JM have the most variant on the upper view colony pigmentation, followed by isolate Fs-9T1 and Fs-JOG1 each with three pigment variants on the upper view. The remaining isolates each showed two variants of pigment on the upper view of their colony.

On the reverse view, most of the isolates tend to show wide variety of colours across different pH. There were two isolates (Fs-6RN and Fs-6SDM) kept constant pigment on the reverse view across the pH. Isolate Fs-JOG2 showed two variants of pigmentation on the reverse view, followed by isolate Fs-9T1 which displayed three variants. Isolate Fs-8JM displayed the most colour variants (5) on their reverse view of the colony, while the remaining isolates showed four colour variants (**Table 4.8**).

Generally, the *N. haematococca* isolates (isolate Fs-6RN and Fs-6SDM) showed least morphological variation whereas isolate Fs-8JM and Fs-8JD showed the most morphological diversity. The polymorphisms observed within an isolate indicates there was a change in morphological characteristics on the different pH. Yet, there were no distinct differences of morphological characteristics for forms, margin, elevation, colony surface appearance and pigmentation for both upper view and reverse view on the colony compared to the previous observation in **Section 4.1.3 (Table 4.4)**.

Table 4.8: Number of Variations Observed on the Colony Morphological Characteristics of the *F. solani* Isolates Across pH

<i>F. solani</i>	Variations of colony morphology characteristics						Total variation
	Form	Margin	Elevation	Surface appearance	Upper view pigmentation	Reverse view pigmentation	
Fs-7SMG	1	1	2	1	2	4	11
Fs-9T1	1	2	1	2	3	3	12
Fs-6RN	1	1	1	1	1	1	6
Fs-8JD	1	2	2	2	2	4	13
Fs-8JM	1	1	1	1	4	5	13
Fs-6SDM	1	2	1	2	1	1	8
Fs-JOG1	1	1	1	1	3	4	11
Fs-JOG2	1	1	1	1	2	2	8
Fs-JOG3	1	1	1	1	2	4	10

Note: Total variations is the total number of variations observed across different morphological characteristics displayed by the *F. solani* isolates across pH

Table 4.9: Summary of Colony Morphological Characteristics of 9 *F. solani* Isolates on PDA with Different pH

Division	Isolate	Media pH	Form	Margin	Elevation	Colony surface	Pigmentation	
							Upper view	Reverse view
Kuching	Fs-7SMG	5	Circular	Curled	Flat	Zonate, fine mycelia	White with ring of maroon grey	Reddish orange to white
		6	Circular	Curled	Flat	Zonate, fine mycelia	Greyish white to white	Cream to white
		7	Circular	Curled	Raised	Zonate, fine mycelia	Greyish white to white	Greyish yellow to white
		8	Circular	Curled	Flat	Zonate, fine mycelia	Greyish white to white	Cream to white
		9	Circular	Curled	Flat	Zonate, fine mycelia	Greyish white to white	Orange cream to orange white
	Fs-9T1	5	Circular	Piliform	Raised	Zonate, fine woolly textured mycelia	White	Orange white to white
		6	Circular	Piliform	Raised	Zonate, fine woolly textured mycelia	White	Orange white to white

Table 4.9 continued

		7	Circular	Entire	Raised	Zonate, fine mycelia	Brownish white	Orange white to white
		8	Circular	Entire	Raised	Zonate, fine mycelia	White to brownish white	Cream to white
		9	Circular	Entire	Raised	Zonate, fine mycelia	White to brownish white	Pale yellow to cream
Serian	Fs-6RN	5	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to violet white
		6	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to violet white
		7	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to violet white
		8	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to violet white

Table 4.9 continued

	9	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to violet white
	5	Circular	Curled	Flat	Zonate, Fine mycelia with clusters of perithecia formed	White	Yellow to cream with tiny orange dots
	6	Circular	Entire	Raised	Zonate, Fine mycelia with less clusters of perithecia formed	White	Pale yellow to white with tiny orange dots
Fs-8JD	7	Circular	Entire	Raised	Fine mycelia with less clusters of perithecia formed	White	Yellowish white to white with tiny orange dots
	8	Circular	Entire	Raised	Zonate, Fine mycelia with less clusters of perithecia formed	White	Yellowish to white with tiny orange dots
	9	Circular	Curled	Flat	Zonate, Fine mycelia with clusters of perithecia formed	Brownish white	Orangish white with tiny orange dots

Table 4.9 continued

		5	Circular	Curled	Raised	Zonate, Fine mycelia with lines radiate from centre	Brown to brownish white	Brown to brownish cream
		6	Circular	Curled	Raised	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brown to white
Fs-8JM		7	Circular	Curled	Raised	Zonate, Fine mycelia with lines radiate from centre	Pale brown to greyish white	Pale brown to white
		8	Circular	Curled	Raised	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish white to white
		9	Circular	Curled	Raised	Zonate, Fine mycelia with lines radiate from centre	Brownish white	Orangish white
Sri Aman	Fs-6SDM	5	Circular	Curled	Raised	Zonate, Fine mycelia with clusters of perithecia formed	White	Cream with tiny orange dots

Table 4.9 continued

Sri Aman	Fs- 6SDM	6	Circular	Entire	Raised	Zonate, Fine mycelia with less clusters of perithecia formed	White	Cream with tiny orange dots		
		7	Circular	Entire	Raised	Zonate, Fine mycelia with less clusters of perithecia formed	White	Cream with tiny orange dots		
		8	Circular	Curled	Raised	Zonate, Fine mycelia with clusters of perithecia formed	White	Cream with tiny orange dots		
		9	Circular	Curled	Raised	Zonate, Fine mycelia with clusters of perithecia formed	White	Cream with tiny orange dots		
	Fs- JOG1	5	Circular	Curled	Flat	Zonate, Fine mycelia	Greyish with patches	white brown	Brownish with patches	white brown
		6	Circular	Curled	Flat	Zonate, Fine mycelia	Greyish with minor patches	white brown	Brownish with patches	white brown

Table 4.9 continued

Sri Aman	Fs- JOG1	7	Circular	Curled	Flat	Zonate, Fine mycelia	Greyish white with minor brown patches	Yellowish cream to cream
		8	Circular	Curled	Flat	Zonate, Fine mycelia	Greyish white	Cream to white
		9	Circular	Curled	Flat	Zonate, Fine mycelia	Greyish white with minor brown patches	Brown with brown patches
	Fs- JOG2	5	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white with brown patches	Brownish white with brown patches
		6	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish white
		7	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish white

Table 4.9 continued

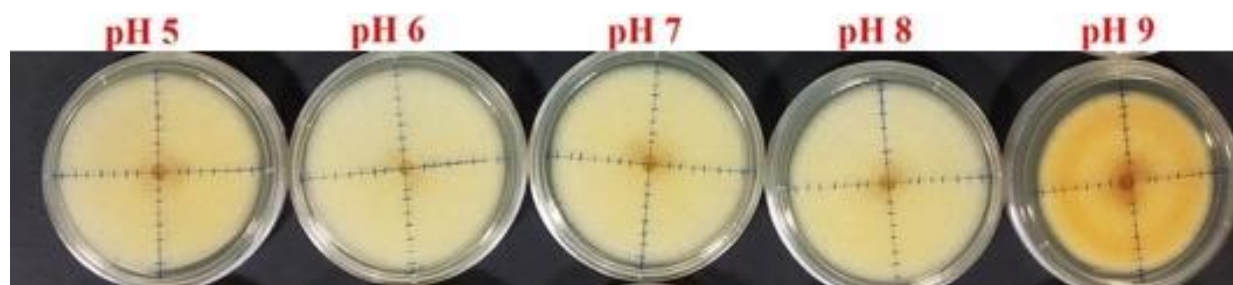
Sri Aman	Fs- JOG2	8	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish white
		9	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white with brown patches	Brownish white with brown patches
	Fs- JOG3	5	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white with brown patches	Brownish cream to cream with minor patches
		6	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish cream to cream
		7	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish cream to cream
		8	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Cream

Table 4.9 continued

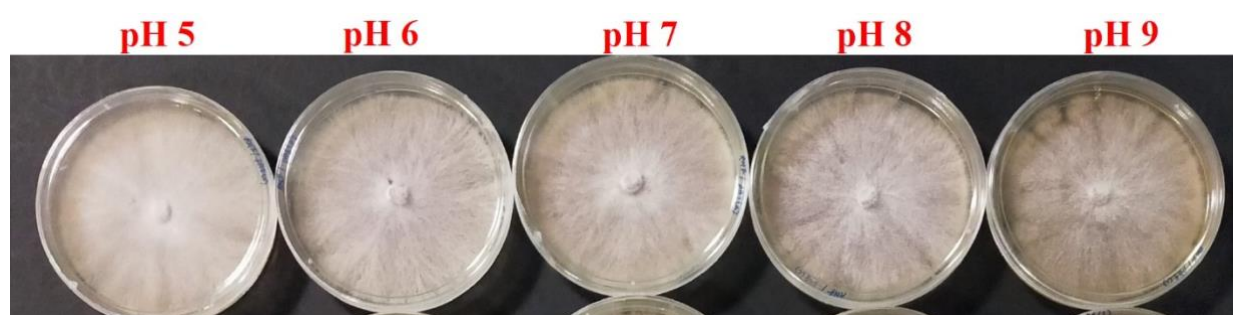
Sri Aman	Fs- JOG3	9	Circular	Curled	Flat	Zonate, Fine mycelia with lines radiate from centre	Brown to greyish white	Brownish cream
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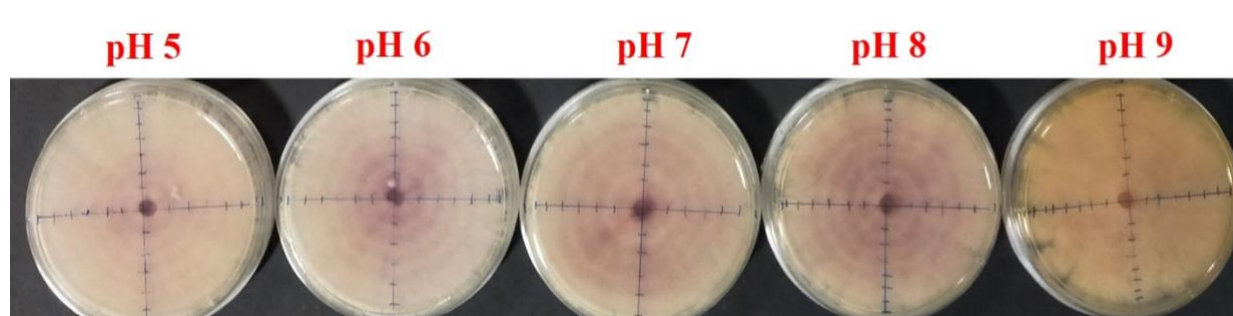
(a)



(b)



(c)



(d)

Figure 4.5: Colony Morphology of *F. solani* Isolates on Different pH (a) Upper View of Isolate Fs-8JD (b) Reverse View of Isolate Fs-8JD (c) Upper View of Isolate Fs-6RN (d) Reverse View of Isolate Fs-6RN

4.4.1 Colony Growth Rate of *F. solani* Isolates on Different pH

In general, all the *F. solani* isolates can generally grow well under neutral pH ranging pH 6 to pH 8 although six out of nine isolates of *F. solani* does not show clear preference towards specific pH statistically.

Isolate Fs-7SMG preferred environment close to neutral, ranging from pH 6 to pH 8. The isolate significantly grew the slowest on pH 5 and pH 9 media at the rate of 4.30mm/day (**Table 4.10**). Isolate Fs-6RN was more favourable to weak acidic to alkalic environment, ranging from pH 6 to pH 9. The isolate grew the slowest in pH 5 at the pace of 4.00mm/day (**Table 4.10**). In contrast, isolate Fs-6SDM least favourable to alkalic environment at pH 9 as they grew significantly slowest at the rate of 3.70mm/day (**Table 4.10**). It was found that three isolates, Fs-JOG1, Fs-JOG2, Fs-JOG3 have no preference to any specific pH. There was no significant different found on the growth rate of the three isolates from pH 5 to pH 9, ranging from 4.60mm/day to 5.20mm/day (**Table 4.10**).

Isolate Fs-9T1, Fs-8JD and Fs-8JM showed no clear preference of pH. Generally, isolate Fs-9T1 seems to prefer pH 8 to pH 9, pH 6 to pH 8 for isolate Fs-8JD and pH 7 to pH 9 for isolate Fs-8JM (**Table 4.10**).

Table 4.10: Colony Growth Rate of *F. solani* Isolates on PDA Media with Different pH Value

Isolate	Growth rate (mm/day)				
	pH 5	pH 6	pH 7	pH 8	pH 9
Fs-7SMG	4.30 ^a	4.60 ^b	4.70 ^b	4.90 ^b	4.30 ^a
Fs-6RN	4.00 ^a	5.80 ^b	6.10 ^b	5.80 ^b	5.80 ^b
Fs-6SDM	4.30 ^b	4.10 ^b	4.10 ^b	4.20 ^b	3.70 ^a
Fs-JOG1	4.60 ^a	4.60 ^a	4.60 ^a	4.60 ^a	4.60 ^a
Fs-JOG2	4.90 ^a	5.20 ^a	5.20 ^a	5.20 ^a	5.20 ^a
Fs-JOG3	4.90 ^a	5.20 ^a	5.20 ^a	5.20 ^a	5.30 ^a
Fs-9T1	4.10 ^a	4.30 ^{ab}	4.30 ^{ab}	4.40 ^{bc}	4.60 ^c
Fs-8JD	4.40 ^{ab}	4.60 ^b	4.60 ^b	4.60 ^b	4.30 ^a
Fs-8JM	4.30 ^a	4.60 ^b	4.80 ^{bc}	4.90 ^{bc}	4.90 ^c

Note: Comparison of isolate growth rate across different media pH with significant test done within column using variance analysis (one-way ANOVA) followed by Tukey HSD post hoc test ($p < 0.05$). The different alphabet indicates significant difference. Highlighted cells are the media pH more preferable by each *F. solani* isolate.

4.5 Effect of Different Nutrient Media on the Growth of *F. solani* Isolates

Five different nutrient media were used to study the effect of nutrient media on the growth of *F. solani* isolates. The pH value for each nutrient medium was adjusted around $\text{pH } 5 \pm 0.5$ (Sánchez-Rangel *et al.*, 2018).

Study on colony morphological characteristics such as forms, margin, elevation, colony surface and pigmentation for both upper view and reverse view were carried out. In general, there were variations found on all morphological characteristics of the colony which includes form, margins, elevation, colony surface appearance, pigmentations of the isolates across different nutrient media tested.

Across the different media, there were two variants of colony form observed, viz, circular and filamentous. All isolates were monomorphic across the different media except for Fs-6RN. For colony margin, three variants were observed. Eight isolates showed dimorphisms, only isolate Fs-9T1 showed all three variants. For colony elevation, there were also three variants observed. Three isolates showed monomorphic across the different media, four isolates were dimorphic, and two isolates showed all three variants. The colony surface appearance had 10 variants across the different media. Each isolate showed two to four of the variants. For upper and reverse view pigmentation, each had 11 variants were observed across the different media. The isolates showed three to four of the total variants, mostly four (**Table 4.11**, **Table 4.12**).

For each isolate, there were at least four morphological characters observed had variations across different media (**Table 4.12**), except for isolate Fs-6RN which had variations for all six morphological characters. For colony form, all isolates showed circular form in all media, except for isolate Fs-6RN which showed filamentous form on PDA (**Table 4.13**). For colony margin, eight isolates showed dimorphism of curled and entire margin on the different media. Fs-9T1 was the only isolate showed all three variants of margin, with piliform margin on PDA media, curled margin on OMA media and entire margin for the others.

For colony elevation, the isolates were found to have either raised, raised to flat (raised at the centre flat the edge) or flat colony (**Table 4.11**). Isolate Fs-9T1 and Fs-6RN exhibited all 3 types of colony elevation across different media (**Table 4.12**). Two different types of colony elevation were found on isolate Fs-7SMG, Fs-8JD and Fs-8JM (**Table 4.12**). No variation of elevation across different media was observed for the remaining isolates (**Table 4.12**).

Table 4.11: Number of Variants Between *F. solani* Isolates Across Nutrient Media

Morphological characteristics	Form	Margin	Elevation	Surface appearance	Upper view pigmentation	Reverse view pigmentation
Number of variants	2	3	3	10	11	11

All the isolate showed change of surface appearance when cultured on different media, except when cultured on PNA and PCA media. Isolate Fs-8JD and Fs-6SDM showed dimorphism surface appearance across the nutrient media, whereas isolate Fs-7SMG and Fs-8JM showed trimorphic on their colony surface appearance. The other isolates displayed up to four variants of surface appearance (**Table 4.12**).

Pigmentation on the upper view of the isolates ranged from colourless to brown to violet hue (**Table 4.13**). All the isolates shared the same pigmentation when cultured on PNA and PCA media (**Figure 4.6**). All the isolates exhibited four colour variants on upper view of the colony, only isolate Fs-9T1 and Fs-8JM exhibited three colour variants across the different media (**Table 4.12**). Similarly, the reverse view of the isolates shared same range of pigmentation with the upper view. Isolate Fs-8JM was the only isolate exhibited three variants of pigmentation on the reverse view. All the remaining of the isolates showed four variants of pigmentation on their reverse view when cultured on different nutrient media (**Table 4.12**).

Compared to the finding in **Table 4.4**, no remarkable differences were found on all the isolates cultured on PDA. However, the polymorphisms observed within an isolate indicates there was a change in morphological characteristics on different nutrient media.

Table 4.12: Number of Colony Morphological Characteristics Variation on *F. solani* Isolates Across Nutrient Media

<i>F. solani</i>	Variations of colony morphology characteristics						Total variation
	Form	Margin	Elevation	Surface appearance	Upper view pigmentation	Reverse view pigmentation	
Fs-7SMG	1	2	2	3	4	4	16
Fs-9T1	1	3	3	4	3	4	17
Fs-6RN	2	2	3	4	4	4	19
Fs-8JD	1	2	2	2	4	4	15
Fs-8JM	1	2	2	3	3	3	14
Fs-6SDM	1	2	1	2	4	4	14
Fs-JOG1	1	2	2	4	4	4	17
Fs-JOG2	1	2	1	4	4	4	16
Fs-JOG3	1	2	1	4	4	4	16

Note: Total variations is the total number of variations observed across different morphological characteristics displayed by the isolates of each *Fusarium* species.

Table 4.13: Summary of Colony Morphological Characteristics of 9 *F. solani* Isolates on Different Nutrient Media

Division	Isolate	Media	Form	Margin	Elevation	Colony surface	Pigmentation	
							Upper view	Reverse view
Kuching	Fs-7SMG	PDA	Circular	Curled	Flat	Fine mycelia	Maroon-grey to white	Brown to cream white
		OMA	Circular	Curled	Flat	Fine mycelia	Grey to white	Grey to white
		PNA	Circular	Curled	Flat	Fine, silky mycelia	Colourless	Colourless
		CLA	Circular	Curled	Raised to flat	Fine, discrete mycelia with web-like mycelia around plug	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
	Fs-9T1	PDA	Circular	Piliform	Raised	Fine, woolly texture	Grey to white	Greyish pink to white
		OMA	Circular	Curled	Flat	Fine mycelia with lines radiate from centre	Grey to white	Grey to white
		PNA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless

Table 4.13 continued

Kuching	Fs-9T1	CLA	Circular	Entire	Raised to flat	Fine, discrete mycelia with web-like mycelia around plug	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
Serian	Fs-6RN	PDA	Filamentous	Curled	Raised	Fine mycelia with feather-like pattern radiate from centre	Greyish violet	Violet to white
		OMA	Circular	Entire	Flat	Fine mycelia	Grey to brownish white	Grey to light pink
		PNA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
		CLA	Circular	Entire	Raised to flat	Fine, discrete mycelia with web-like mycelia around plug	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless

Table 4.13 continued

Serian	Fs-8JD	PDA	Circular	Curled	Raised	Fine mycelia with clusters of perithecia formed	Yellow-grey to white. Orange outer ring formed	Yellow with tiny orange dots
		OMA	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed	Grey with white ring formed	Grey to white
		PNA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
		CLA	Circular	Entire	Flat	Fine, silky mycelia	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
	Fs-8JM	PDA	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
		OMA	Circular	Entire	Raised	Fine mycelia	Grey with white ring formed	Grey to white

Table 4.13 continued

Serian	Fs-8JM	PNA	Circular	Entire	Flat	Fine, silky mycelia with lines radiate from centre	Colourless	Colourless
		CLA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
Sri Aman	Fs-6SDM	PDA	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed	Yellow-grey to white. Orange outer ring formed	Yellow with tiny orange dots
		OMA	Circular	Curled	Flat	Fine mycelia with clusters of perithecia formed	Grey with white ring formed	Grey to white
		PNA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
		CLA	Circular	Curled	Flat	Fine, silky mycelia	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless

Table 4.13 continued

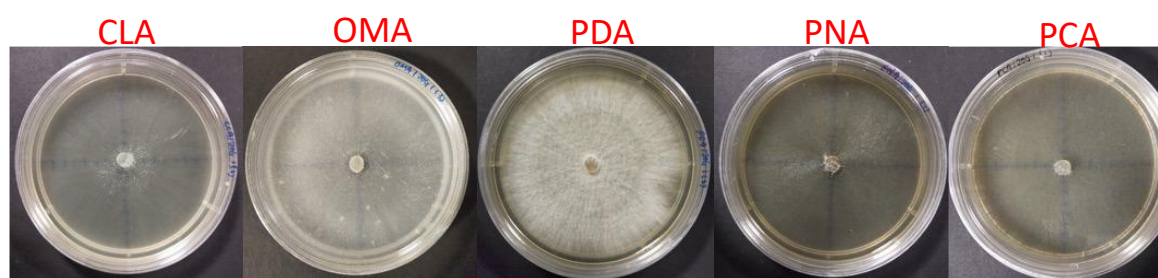
Sri Aman	Fs-JOG1	PDA	Circular	Curled	Flat	Fine lines radiate from centre	mycelia with cream to white with brown patches	Greyish
		OMA	Circular	Entire	Raised to flat	Fine lines radiate from centre	mycelia with Grey with ring formed	Grey to white
		PNA	Circular	Entire	Flat	Fine, silky mycelia with web-like mycelia on plug	Colourless	Colourless
		CLA	Circular	Entire	Raised to flat	Fine, silky mycelia with web-like mycelia on plug. Lines radiate from centre	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless

Table 4.13 continued

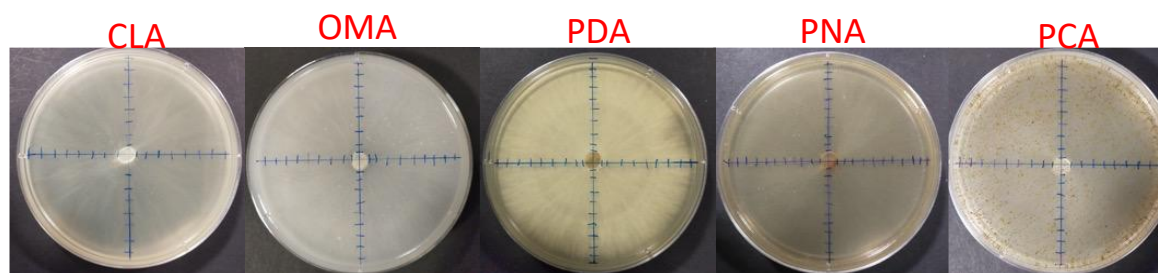
Sri Aman	Fs-JOG2	PDA	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
		OMA	Circular	Entire	Flat	Fine mycelia with lines radiate from centre	Greyish cream with brown patches	Greyish cream with brown patches
		PNA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
		CLA	Circular	Entire	Flat	Fine, silky mycelia with web-like mycelia on plug	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine, silky mycelia	Colourless	Colourless
	Fs-JOG3	PDA	Circular	Curled	Flat	Fine mycelia	Brown to greyish white	Pale brown to cream white
		OMA	Circular	Entire	Flat	Fine mycelia with lines radiate from centre	Greyish cream with brown patches	Greyish cream with brown patches

Table 4.13 continued

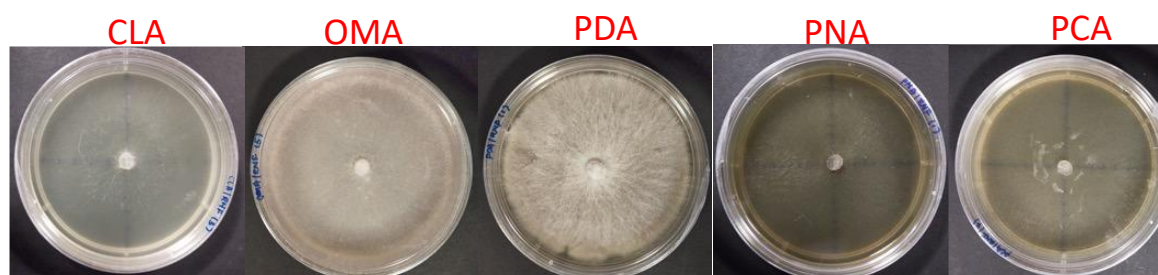
Sri Aman	Fs-JOG3	PNA	Circular	Entire	Flat	Fine, silky mycelia with web-like mycelia on plug	Colourless	Colourless
		CLA	Circular	Entire	Flat	Fine, silky mycelia with web-like mycelia on plug	Greyish white	Greyish white
		PCA	Circular	Entire	Flat	Fine mycelia	Colourless	Colourless



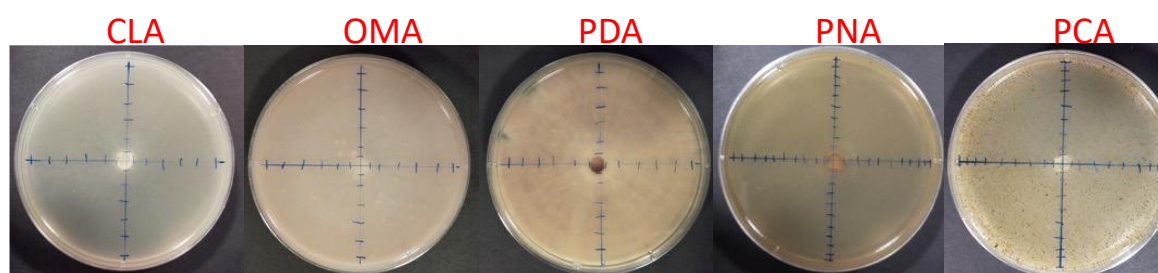
(a)



(b)



(c)



(d)

Figure 4.6: Colony Morphology of *F. solani* Isolates on Different Nutrient Media (a) Upper View of Isolate Fs-JOG1 on Different Nutrient Media (b) Reverse View of Isolate Fs-JOG1 on Different Nutrient Media (c) Upper View of Isolate Fs-6RN on Different Nutrient Media (d) Reverse View of Isolate Fs-6RN on Different Nutrient Media

4.5.1 Colony Growth Rate of *F. solani* Isolates on Different Nutrient Media

Generally, CLA was the media of preference by most if not all isolates based on colony growth rate. PNA and PCA are the two media less preferred by most if not all the isolates. Two isolates, Fs-9T1 and Fs-JOG1, preferred CLA over other media. Their growth was the fastest on CLA. Three isolates showed preference towards two media. Fs-8JM and Fs-6SDM grew the fastest on CLA and OMA, while Fs-8JD on OMA and PDA. Fs-6RN was the only isolate which grew equally well over three media, CLA, OMA and PDA. The other three isolates (Fs-7SMG, Fs-JOG2 and Fs-JOG3) showed no clear preference over which media. However, CLA and OMA seems to be more preferred by the three isolates because their growth rate on these media was significantly faster than at least one of the other media.

Table 4.14: Colony Growth Rate of *F. solani* Isolates on Different Media

Isolate	Growth rate (mm/day)				
	CLA	OMA	PDA	PCA	PNA
Fs-9T1	4.90 ^d	4.60 ^c	4.10 ^b	4.10 ^b	3.80 ^a
Fs-JOG1	4.90 ^c	4.40 ^b	4.30 ^{ab}	4.10 ^{ab}	4.00 ^a
Fs-8JM	5.30 ^b	5.30 ^b	4.60 ^a	4.60 ^a	4.30 ^a
Fs-6SDM	5.30 ^b	4.90 ^b	4.00 ^a	4.20 ^a	3.80 ^a
Fs-8JD	4.40 ^b	4.90 ^c	4.90 ^c	4.10 ^{ab}	4.00 ^a
Fs-6RN	6.50 ^c	6.50 ^c	6.10 ^c	5.20 ^b	4.20 ^a
Fs-7SMG	4.90 ^d	4.70 ^{cd}	4.40 ^{bc}	4.10 ^{ab}	3.80 ^a
Fs-JOG2	4.90 ^{bc}	5.30 ^c	4.60 ^{ab}	4.70 ^{ab}	4.40 ^a
Fs-JOG3	5.30 ^c	5.30 ^c	5.20 ^{bc}	4.60 ^{ab}	4.20 ^a

Note: Comparison of isolate growth rate across different nutrient media with significant test done within column using variance analysis (one-way ANOVA) followed by Tukey HSD post hoc test ($p < 0.05$). The different alphabet indicates significant difference. Highlighted cells are the media pH more preferable by each *F. solani* isolate.

4.6 Phylogeny Analysis of *Fusarium* spp.

4.6.1 Phylogeny Study of *F. solani* within Malaysia

The ITS sequences of the twenty-four selected *Fusarium* spp. (viz. ten isolates of *F. solani*; three isolates of *F. oxysporum* and eleven isolates of *F. proliferatum*), together with 10 isolates of *F. solani* and *F. proliferatum*, respectively from Shahnazi *et al.* (2012) were used to construct a phylogenetic dendrogram. All *Fusarium* spp. isolates were clearly separated into three distinct clades (**Figure 4.7**). Each of the clade represents the three identified species. Isolates with similar colony morphology were grouped together within their respective clade, under the same lineage regardless of their origins.

All the *F. proliferatum* isolates were grouped together in single clade with bootstrap value of 93%. This is expected because the 11 *F. proliferatum* isolates from this study portrayed minor morphological variation among them (**Table 4.6**). Isolates originated from Sibuan and Sarikeni have the tendency to group together.

Both *F. proliferatum* and *F. oxysporum* showed sister relationship as displayed in molecular phylogeny. The sister relationship between the two species indicates that they shared more recent common ancestor. A 100% of homology of morphology among the *F. oxysporum* isolates were observed even though their origins were from three different divisions of Sarawak (Kuching, Serian and Sri Aman). The *F. oxysporum* clade was supported with 91% of bootstrap value.

Lastly, the clade of *F. solani* formed two subclades. Wide range of phylogenetic affinities among the members of *F. solani* isolates was found in the clade. The isolates in Subclade I exhibited high morphological similarity among each other, whereas higher variations of colony morphological characteristics were observed among the *F. solani* isolates grouped under Subclade II (**Table 4.7**).

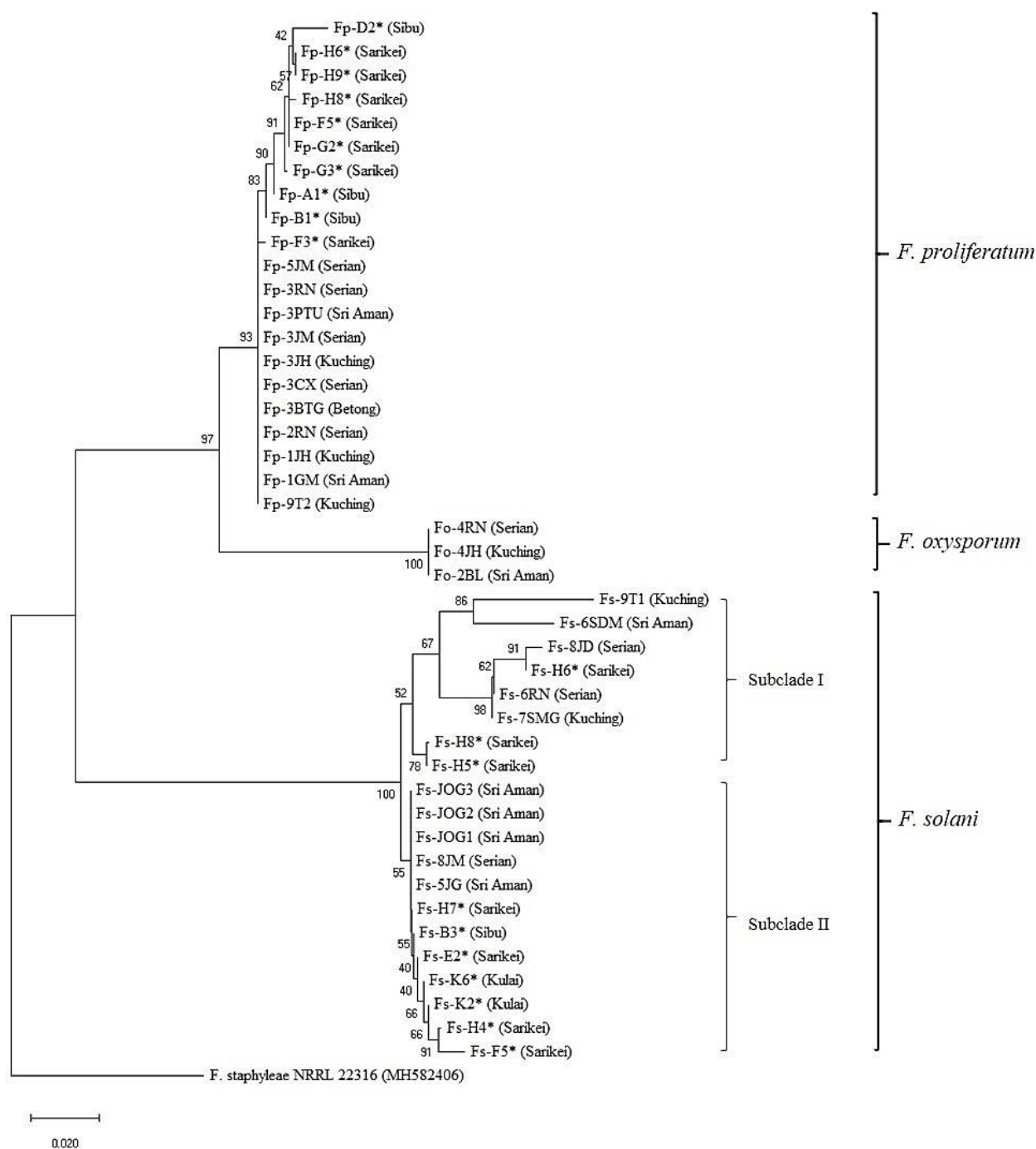


Figure 4.7: Neighbour Joining (NJ) Phylogenetic Tree of *Fusarium* Isolates from Malaysia Using Maximum Likelihood Method Supported by Bootstrap Test 1000 Replicates, with 0.02 Unit of Base Substitution per Site. Isolates from Shahnazi *et al.* (2012) Study Were Marked with *.

4.6.2 Phylogeny Study of *F. solani* within Asia

A phylogenetic diagram was constructed using the ITS sequence of nine *F. solani* isolates, together with 33 isolates of *F. solani* from other countries in Asia from previous study, with *F. staphyleae* as the outgroup (Chehri *et al.*, 2015) in the dendrogram. The 33 isolates of *F. solani* consisted of 10 isolates from Malaysia (Shahnazi *et al.*, 2012), six isolates from India (Choudhary & Kareppa, 2016; Pappachan *et al.*, 2020), four isolates from Thailand (Tiawatwanikul *et al.*, 2019), 12 isolates from Taiwan (Chung *et al.*, 2011; Wang *et al.*, 2010), and one isolate from Indonesia (Tunarsih *et al.*, 2015).

In general, the *F. solani* isolates were distinctively divided into 2 clades. It was found that the origin of the *F. solani* isolates does not affect directly on the relatedness of the isolates genetically. Majority of the *F. solani* isolates grouped under Subclade I, which means that they shared the same recent common ancestors. There was more branching in Subclade I compared to Subclade II, which indicated that more evolution process takes placed among the members in Subclade I (**Figure 4.8**).

It was found that the Malaysian isolates were split into 4 groups in the phylogenetic dendrogram. Most Malaysian isolates were grouped under clade I-subclade I, except for isolate Fs-6SDM and Fs-9T1. Those 12 Malaysian isolates in clade I-subclade I, were more closely related to one isolate from India (Fs-Jor-3) and one isolate from Thailand (Fs-RYG3) with bootstrap value of 87% (**Figure 4.8**). There were two Malaysian isolates (Fs-H8 and Fs- H5) in clade I-subclade I, which were more related to one isolate from India, three isolates from Thailand and seven isolates from Taiwan with 100% molecular affinity.

In two clade I-subclade II, one Malaysia isolate (Fs-6SDM) were grouped together with two isolates from India, one isolate from Taiwan and Indonesia, respectively, supported with bootstrap value of 86%. This group of isolates is a sister clade to a clade consist of only the Malaysian isolate Fs-9T1.

In Clade II was formed by only four Malaysian isolates from Sarawak (Fs-8JD and Fs-6RN from Serian, Fs-H6 from Sarikei and Fs-7SMG from Kuching division). The result indicates the four isolates were genetically lesser related to the 13 isolates representing other Asian countries.

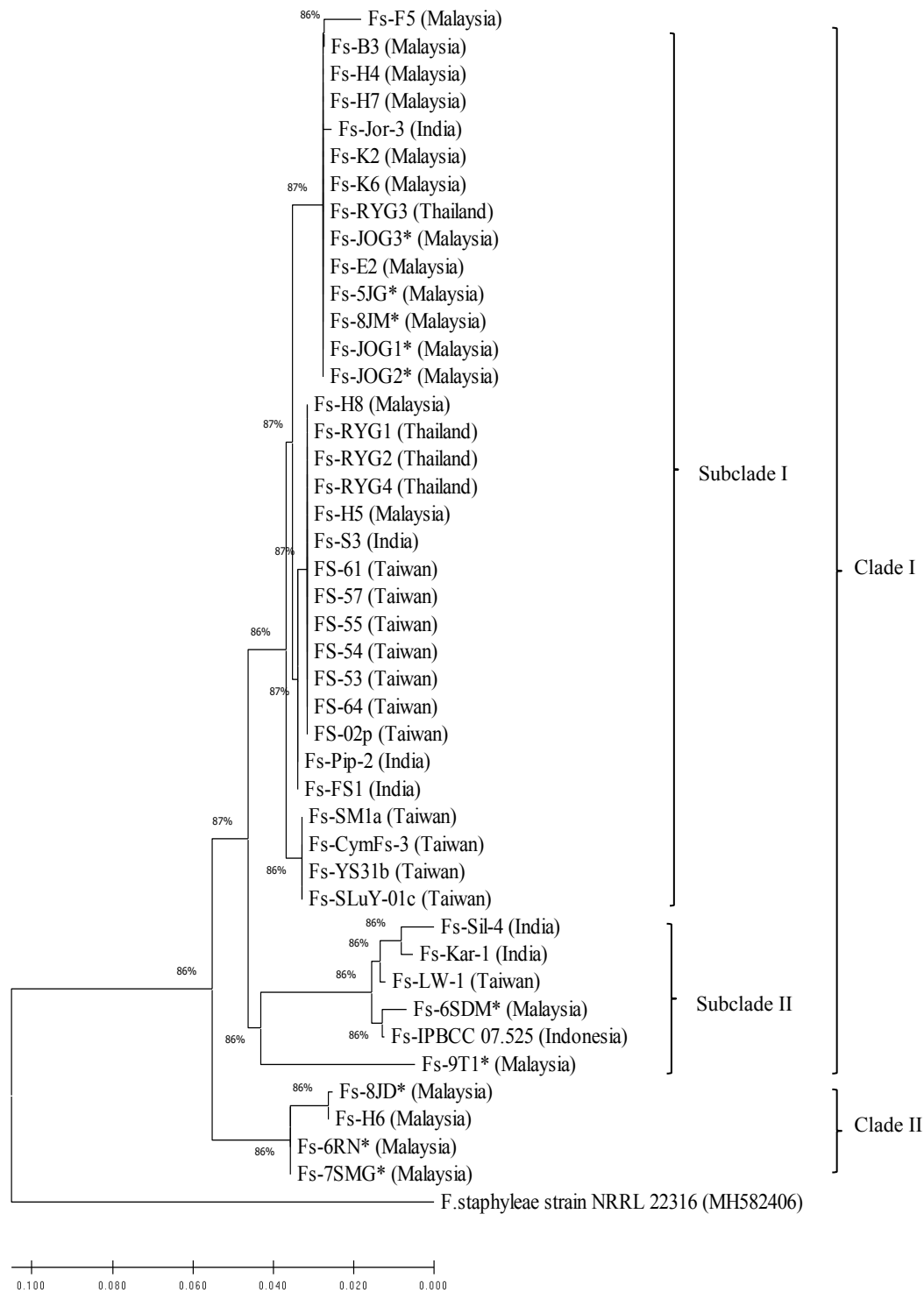


Figure 4.8: Neighbour Joining (NJ) Phylogenetic Tree of *Fusarium* Isolates from Asia Countries Supported by Bootstrap Test 1000 replicates, with 0.02 Unit of Base Substitution per Site. Isolates from Current Study Were Marked with *

CHAPTER 5

DISCUSSION

5.1 Overview

Documentation regarding *Fusarium* wilt disease of *P. nigrum* L. in Sarawak is rare. Hence, series of studies on the variations of *Fusarium* spp. associated with *Fusarium* wilt of *P. nigrum* L. from North-western region of Sarawak in terms of morphological and molecular characteristics hoped to deliver better understanding on *Fusarium* spp. associated to *P. nigrum* L. as the information generated from current study are essential for the development of disease control strategy in the future. For example, those field infected by *Fusarium* spp. with faster growth rate should be spray with fungicide to prevent the spreading of the disease. Other than that, molecular information of the pathogen can also use to develop molecular engineered *P. nigrum* L. cultivar cooperated with pathogen resistance gene.

5.2 Isolation of *Fusarium* spp. Associated with *Fusarium* Wilt Disease of *Piper nigrum* L. in North-western Region of Sarawak

Three *Fusarium* species associated with *Fusarium* wilt were identified from the samples collected. There were ten isolates of *F. solani*; three isolates of *F. oxysporum* and 11 isolates of *F. proliferatum* isolated from the 93 samples collected. However, the causal agent of *Fusarium* wilt disease among the three species is yet to be identified as pathogenicity test for the isolates were not carried out in this study due to the availability of host plant (*Piper nigrum* L. plantlet). *F. solani* as the causal agent of the disease was reported in previous studies (Hamada *et al.*, 1988; Silva *et al.*, 2014; Luz *et al.*, 2017). In contrast, *F. oxysporum* was also claimed as an important agent responsible for *Fusarium* wilt in *P.*

nigrum L. (Duarte *et al.*, 2001; Edward *et al.*, 2013). Shahnazi *et al.* (2012) reported *F. proliferatum* was not pathogenic to *P. nigrum* L. plants.

There were multiple species of *Fusarium* isolated from same sampling farm. For example, isolate Fs-6RN, Fo-4RN, Fp-2RN and Fp-3RN were isolated from RNF farm, Linsat. The finding was in accordance with previous study by Shanazi *et al.* (2012), where both *F. solani* and *F. proliferatum* isolates were discovered from the same field. Current study also found that multiple *Fusarium* spp. coexisted in the same field and diseased samples. For example, *F. solani* and *F. oxysporum* were found on same root sample. Hence, there is possibility of interaction between the *Fusarium* spp. which might affect plant health.

Colony morphological characteristic variations were observed among the *Fusarium* isolates. The findings were in accordance with previous studies whereby the different level of morphological variations shown by the isolates of the same species (Shahnazi *et al.*, 2012; Haapalainen *et al.*, 2016; Berruezo *et al.*, 2018). Generally, the colony of most of the *Fusarium* isolates were circular in shape and zonate surface appearance. The aerial mycelia of *F. solani* was observed to range from fine and sparse to dense and woolly textured; for *F. oxysporum* isolates, the aerial mycelia vary from sparse to fluffy textured. Aerial mycelia with woolly textured appearance were observed in *F. proliferatum* isolates. Five septa were mostly found in macroconidia of *F. solani* isolates; for *F. oxysporum* isolates, macroconidia were observed to have hook-shaped apical cell. *F. proliferatum* macroconidia was found to have 3-4 septa with curved pointed apical cell. The observation showed that most of the colony morphological characteristics of the 24 isolates in this study matched the description by Nelson *et al.* (1983) and Leslie & Summerell (2007). In addition, clusters of perithecia were found on upper view of the colony for both Fs-6SDM and Fs-8JD. The observation

matched with the description for *N. haematococca*, sexual form of *F. solani* in previous studies (Wu *et al.*, 2011; Vaz *et al.*, 2012).

No distinct characteristic was found to be specific to isolates from one division. It can be deduced that there was no relation between the isolate morphology and their origin. Similarities in the morphological characteristics were observed between isolates of the same species from different origin. The finding was similar to the previous studies on *F. proliferatum*, *F. oxysporum* and *F. solani* (Shahnazi *et al.*, 2012; Haapalainen *et al.*, 2016; Robles-Carrión *et al.*, 2016; Berruezo *et al.*, 2018). For example, previous study by Berruezo *et al.* (2018) found that isolate 12 from Ovejeria and isolate 15 from La Caravana were both identified as *F. oxysporum* and grouped together with 100% bootstrap. Both isolate 12 and 15 shared same colony characteristics, although they were from different origin.

Variations in growth rate and both macroconidia and microconidia sporulation rate of the isolates were observed within each species. For instance, PDA media was more suitable for the growth of isolates such as Fp-3JM compared to Fp-3CX (**Table 4.6**). The variations may be due to nutrient deficiency (Robles-Carrión *et al.*, 2016). Previous study by Mohsen *et al.* (2016) reported nitrogen, potassium and phosphorus are required by *F. solani* isolates to support their growth. However, the nutrients supported the growth performance isolates in current study are yet to be confirmed as the chemical composition of the media used were not investigated.

Meanwhile, no correlation between the growth rates, macroconidia sporulation rate, and microconidia sporulation rate was observed. The finding was in agreement with previous study by (Robles-Carrión *et al.*, 2016) on *F. oxysporum* in Ecuador and also other fungal pathogens such as *Pyricularia oryzae* of Sarawak Hussin *et al.* (2020), where the growth rate of the isolates does not affect the sporulation rate of macroconidia and microconidia and vice

versa. The information on the growth rate and sporulation rate of the pathogen are essential to design an efficient and sustainable strategies for disease control in the future (Robles-Carrión *et al.*, 2016).

Sporulation usually happen when growth rate decreased and slowed down under conditions that encourage rapid mycelial growth (Dahlberg & Etten, 1982). Kirk *et al.* (2008) mentioned that fungal spores are important for fungal dissemination and propagation. Hence, the information on the growth rate and sporulation rate of the pathogen are essential to design an efficient and sustainable strategies for disease control in the future (Robles-Carrión *et al.*, 2016). For example, disease control strategies such as removal of plants or application of fungicide should be done earlier on plants infected by fast growth rate and high sporulation phytopathogen compared to plants infected by slow growth rate and slow sporulation phytopathogen to prevent the pathogen disseminate to other non-infected area.

Diverse of pigmentation ranging from white to violet to brown were observed among the isolates. The observation may suggest variation in the virulence of the isolates in this study towards *P. nigrum* L. where different pigmentation of the pathogen may have different toxigenic activity (Duarte & Archer, 2003). The association of pigment production in pathogen with the increment in virulence towards the host was reported in previous study (Saviola, 2014). Previous study by Shahnazi *et al.*, 2012 on *F. solani* isolated from *P. nigrum* L. was also in agreement with the statement where stronger pathogenicity was showed in *F. solani* isolates with dark red pigmentation showed.

5.3 Effect of pH and Nutrient Media on the Growth of *F. solani*

F. solani was selected for further characterization. Both morphological characteristics and growth rate was observed among the *F. solani* isolates cultured on PDA media with different pH ranging from pH 5 to pH 9. No previous study on the effect of PDA pH on fungal morphology was reported. However, the morphological variation such as mycelial growth rate and colony pigmentation were observed across the pH was in accordance with Singh *et al.* (2017) on pH 5-8 of Czapek's dox agar. For example, two different colours ie. creamy white and cottony white *F. solani* isolate colony were found on pH 5, pH 7 and pH 6, pH 8, respectively in the study.

The isolates tend to show least colour intensity at pH 7 and gradually increase the pigmentation along the increment and decrement of the pH value. The findings matched with the statement by Ogbonna (2016) where environmental factors such as pH, temperature, light, moisture, agitation speed and dissolved oxygen concentration would affect the fungal pigments production. The change of pigmentation intensity on different pH suggested that there might have the possibility to alter the toxigenic activity of the pathogen by changing their environment pH as Shahnazi *et al.* (2012) reported *F. solani* isolates with darker pigments were more pathogenic than other isolates. However, this assumption yet to be confirm.

The *F. solani* isolates generally grew well in pH 6 to pH 8 on PDA media. The observation was similar with the finding by Patel, (2020) who reported the optimum pH for the growth of *F. solani* isolates is pH 7 to pH 8 on PDA media. Other study also reported the isolates of *F. solani* prefer pH ranging from 5 to 7 on potato dextrose broth media (Arunakumara & Satyanarayana, 2017). Hence, pH 7 to pH 8 of PDA media are recommended to culture *F. solani* for faster mycelial growth.

The observation suggested that different *F. solani* isolates have different preference of pH environment, which are in accordance with previous study (Abd Murad *et al.*, 2017) (Murad *et al.*, 2017). For example, isolate Fs-6SDM grew significantly slowest on pH 9 (**Table 4.10**). The observation deduce that the isolate was least favourable to alkalic environment, which was in accordance with previous study where increase of the pH resulted retardation effect on the fungi growth of *F. oxysporum* (Poorvasandhya, 2020). Similarly, low pH environment also does not support the colony growth of *F. solani* isolates. For example, the growth performance of isolate Fs-6RN was the worst on pH5 (**Table 4.10**). Previous study found that the growth of *Fusarium* spp. stunted when cultured in acidic pH was in line with the observation (Sánchez-Rangel *et al.*, 2018).

F. solani isolates also exhibited morphological variation when cultured on different nutrient media. Isolates cultured on PDA media generally exhibited wide variation of pigmentation among the isolates on both upper and reverse view, ranging from greyish-white to brownish-grey on the upper view and greyish-pink to brownish-white on reverse view (**Table 4.13**). On OMA, most *F. solani* isolates showed greyish shade pigmentation except for isolate Fs-JOG2 and Fs-JOG3 where brown patches were observed on OMA media. No pigmentation observed on isolates cultured on CLA, PCA and PNA media. The observation was in accordance with the finding in previous study by Iqbal *et al.*, (2005) where the fungi have different morphological appearance when cultured on different nutrient media. Therefore, it can be deduced that the performance of *F. solani* isolates can be affected by the nutrient provided. There could be possibility that nutritional factor such as carbon and nitrogen would affect the pigment production of the *F. solani* (Ogbonna, 2016).

Generally, all the *F. solani* isolates grow well in most of the nutrient medias, but there are certain media is more preferred by some of the isolates. The finding matched with previous study by Singh *et al.* (2017). For example, both isolate Fs-9T1 and Fs-JOG1 had their best growth performance only on CLA media. Besides that, isolate Fs-7SMG, Fs-8JM, Fs-JOG2 and Fs-6SDM performed the best in CLA and OMA media instead of PDA and OMA media. Meanwhile, both isolate Fs-6RN and Fs-JOG3 does not show significant different in terms of growth rate between CLA, OMA and PDA media.

Other than that, nutrient media such as PDA and OMA also preferred by certain isolate. For example, isolate Fs-8JD significantly performed better in both PDA and OMA media compared to other nutrient medias. The observation was in agreement with the statement by Iqbal *et al.*, (2005) where PDA supported better the growth of *Fusarium* spp. Iqbal *et al.* (2005) stated that carbohydrate content support high mycelia growth rate. The source of carbon is first being hydrolysed by the enzyme of the fungus and convert the complex carbon into their component sugar (Ravat, 2019). Besides that, PDA and OMA also preferred by other fungi species such as *Pyricularia oryzae* (Hussin *et al.*, 2020).

F. solani is soil borne and cause vascular system diseases (Eng, 2011). No previous studies reported *F. solani* infected the leaves of their host plants. The host leaf extract media did not support fungal mycelia growth well as there are possibility that nutrient requirements of the *F. solani* isolates were found mostly on the root of the host plant but not the leaf part. Media prepared from roots may content the nutrient needed to support fungal mycelia growth of *F. solani*. However, no previous studies found regarding the nutrition content of nutrient media that responsible for the mycelial growth of *Fusarium* spp.. Hence, host leaf extract media (PNA & PCA media) does not support the growth of *F. solani* isolates as compared to other isolates. This finding matched with previous study where *F. oxysporum* also

performed poorly in term of mean colony diameter when cultured in host leaf extract media in the study (Chittem & Kulkarni, 2008). Other fungus such as *Alternaria solani* also performed poorly when cultured in host leaf extract agar where it had the least mean radial growth among all the types of media tested (Koley & Mahapatra, 2015).

From the growth performance of the *F. solani* isolates on different media, it was found that CLA media is recommended nutrient media to culture *Fusarium* isolates as most of the isolates performed better in CLA media. However, this nutrient media is not suitable for colony morphological study as the appearance of the isolates were almost the same (**Table 4.13**). Sporulation rate of *F. solani* isolates were not documented in this study, but previous report by Iqbal *et al.*, (2005) claimed CLA media is the best medium to study both microconidia and macroconidia for fungal identification.

PDA media is recommended nutrient media to study the pigmentation of the *F. solani*. Wide range of colony appearance and pigmentation were exhibited by the fungus cultured on the PDA media (**Table 4.13**). Other than that, PDA media was able to induce the sporulation of the fungi, which are essential in study the taxonomy of the isolates (Koley & Mahapatra, 2015).

Compared to PDA media, OMA media was less recommended as the semi-opaque colour of the media increased the difficulty to observe the morphological characteristics of the isolates, especially on the colony pigmentation of the reverse side of the *F. solani* isolates. The true colours of the pigmentation being covered by the semi-opaque colour of the media, which might cause wrong colour characterisation. However, OMA are recommended for heavily pigmented fungi such as *P. oryzae* (Hussin *et al.*, 2020).

From this study, different pH and nutrient media showed impact on the morphology characteristics and growth rate of the *F. solani* isolates. Neutral to weak alkali nutrient media ranging pH 7 to pH 8 are more favourable to the growth of *F. solani* isolates. Different nutrient media should be used to culture *F. solani* isolates according to different purpose of study. CLA media is recommended for the rapid growth of the isolates; whereas PDA with pH ranging from 7 – 8 are more recommended to culture the *F. solani* isolates for colony pigmentation study.

5.4 Phylogeny Analysis of *Fusarium* spp.

All the *Fusarium* spp. isolates were grouped under 3 distinct clades representing three identified species, respectively (**Figure 4.5**). It was found that both *F. proliferatum* and *F. oxysporum* shared the same common ancestor, which in accordance to previous study (Haapalainen *et al.*, 2016; Kim *et al.*, 2016). Both *F. proliferatum* and *F. oxysporum* isolates showed simpler clade as they were grouped within one main branch whereas *F. solani* clade exhibited more branches which indicates that the *F. solani* isolates undergone further evolution process. The result indicates that the evolution time for both *F. proliferatum* and *F. oxysporum* were near to each other. It can be deduced that both *F. proliferatum* and *F. oxysporum* were genetically close to each other. Isolate Fp-D2 showed the longest branch length among the *F. proliferatum* isolates which indicates the isolate evolved at different substitution rate compared to other *F. proliferatum*. No branching for the *F. oxysporum* clade was observed. Low number of *F. oxysporum* isolates is insufficient to show the variation among the *F. oxysporum*.

On the other hand, *F. solani* isolates were further divided into two main lineages within the clade. This is also observed in Shahnazi *et al.* (2012). *F. solani* isolates does not share recent common ancestor with the other two *Fusarium* species. Instead, more lineages within the subclade were displayed as compared to the other clades (**Figure 4.5**) which matched with both Shahnazi *et al.* (2012) and Hafizi *et al.* (2013) study. However, the bootstrap value within *F. solani* clade was low which may indicate they are close to each other. Isolate Fs-9T1 showed longest branch length among all the *Fusarium* isolates, which can be deduced that isolate Fs-9T1 evolved at different timing compared to other isolates of *Fusarium* spp.. However, even though *F. solani* isolates portrayed wide variation, there was no correlation observed between the origin of the isolates and their genetic relatedness, in terms of molecular phylogeny. The observation indicates that the genetics of the isolates were closely related to each other despite their geological origin which fits with previous study (Hafizi *et al.*, 2013; Shahnazi *et al.*, 2012).

It was found that the clade of *F. solani* were divided into more branches forming a complex phylogenetic dendrogram after taking some isolates of *F. solani* from other country in Asia for comparison on the molecular phylogeny of the species (**Figure 4.6**). The findings in **Figure 4.6** showed that some of the isolates from other countries such as India, Taiwan and Indonesia are closely related to some of the Malaysia isolates as they shared common recent ancestor and grouped under subclade II. This indicated there was no correlation between the origin of the isolates and their genetic relatedness.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the objectives of this study were achieved. *F. solani*, *F. oxysporum* and *F. proliferatum* were the three species of *Fusarium* successfully retrieved from diseased root samples of *P. nigrum* L.. Twenty-four *Fusarium* isolates were identified viz. ten isolates of *F. solani*; three isolates of *F. oxysporum* and eleven isolates of *F. proliferatum* through their morphological characteristics and molecular characterization using ITS sequences. There were molecular character variations showed in **Table 4.3**, where different percentage of max identity was found in BLAST analysis of the *Fusarium* isolates. Interestingly, no distinctive morphological characteristic was found to be unique to certain location. Growth rate, macroconidia sporulation rate, and microconidia sporulation rate of the isolates also showed variations but they were not correlated.

Morphological characteristics of the isolates were affected by the pH values of PDA media in terms of margin, elevation, and pigmentation. The pigmentation intensity was the least at pH 7. The colour intensity gradually increased along the increment and decrement of the pH values. PDA media with pH 6 to pH 8 generally supported better mycelial growth of most of the isolates. Similarly, the colony morphology of the isolates was also affected by the nutrient media used to culture the isolates. Colony pigmentation of the isolates were observed on PDA and OMA media. Isolates cultured on CLA, PCA and PNA media were found to have transparent mycelia on both upper and reverse view of the colony. Among all the media used, most isolates performed the best in CLA media, followed by OMA and PDA media.

In terms of molecular phylogeny, the three *Fusarium* species were separated into three distinct clades representing each identified species. The genetic relatedness between the isolates within each species is depicted in the tree. More evolution process took place among the *F. solani* isolates as phylogeny dendrogram of the isolates displayed higher genetic variations among *F. solani* isolates with different substitution rates. On the other hand, the genetic relatedness of all the *F. solani* isolates were found to be not related with their place of origin after comparing to other *F. solani* isolates from Asia country. This study would contribute information on the variations of *Fusarium* spp. associated with *Fusarium* wilt of *P. nigrum* L. from North-western region of Sarawak.

6.2 Recommendations for Further Research

Data on the *Fusarium* spp. associated with *P. nigrum* L in North-western region of Sarawak, with their morphological variation in different pH and nutrient media, as well as the genetic relatedness among the *Fusarium* spp. was generated from this study. The information would provide better picture on the diversity of the *Fusarium* associated with *P. nigrum* L. in Sarawak.

Study on the level of pathogenicity of the *F. solani* and *F. oxysporum* isolated may be considered for further research as argument between both the fungus as the causal agent of *Fusarium* wilt disease are still remain unsolved. Different inoculation concentration for both the phytopathogen should be studied to estimate the threshold of pathogen population to cause disease on *P. nigrum* L. within specific time period after inoculation. Other than that, different combination of *F. solani* and *F. oxysporum* isolates in sequential inoculation or in combination can be studied as well to understand the reaction of the host with the presence of both the fungus.

Study on other environmental factors such as temperature and light intensity could be another aspect to be considered for further study to get a bigger picture on the pathogen behavior under different environmental conditions. This information might be useful in designing cultural practices in black pepper plantation to prevent or control the disease.

In terms of molecular characteristics, genetic relatedness among the *F. solani* from different country using other molecular markers such as ISSR marker, or designated markers should be done in future for clearer overview of the genetic diversity of the pathogen. Information on the pathogen genetic would be essential in the effort of disease control globally. If the pathogens are related to each other regardless their origin, this indicates the gene pool of the pathogen are small. Therefore, same disease control strategy such as development of disease resistant cultivar may be applied to control the pathogen population regardless their geological variation (Harry *et al.*, 2021)

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