CHARACTERIZATION OF *Fusarium mangiferae* AND *F. proliferatum,* AND MANAGEMENT OF MANGO MALFORMATION DISEASE

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CHARACTERIZATION OF *Fusarium mangiferae* AND *F. proliferatum,* AND MANAGEMENT OF MANGO MALFORMATION DISEASE

by

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LIST OF SYMBOLS

α	Alpha
β	Beta
bp	Base pair
℃	Degree celcius
cm	Centimetre
g	Gram
kb	Kilo base
L	Litre
K	Kilo
M	Molar
min	Minutes
mA	Micro ampire
μl	Microlitre
ml	Mililitre
mm	Millimetre
n	Total number
rpm	Rate per minute
v	Volt
$E£$	Egypt pound
%	Percent
/	Or
US$	United state dollar

p.s.i.	Pound per square inch
™	Trademark
®	Registered trademark
mM	Milli molar
×	Times
=	Equal
±	Plus minus
$p<0.05$	P-value less than 0.05
Ppm	Part per million
µg	Micro gram

LIST OF ABBREVIATIONS

MMD	Mango malformation disease
VM	Vegetative malformation
MI	malformed inflorescence
FM	Floral malformation
CLA	Carnation-leaf agar
CM	Complete medium
FM	Floral malformation
MI	Malformed inflorescence
MM	Minimal medium
MMD	Mango malformation disease
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PPA	Peptone pentachlorobenzene agar.
PSA	Potato sucrose agar
RT	Room temperature (27℃ ±1)
VM	Vegetative malformation
WA	Water agar
UV light	Ultraviolet light
HX	Hypoxanthine
NH_4	Ammonium nitrate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
IPM	integrated pest management

MAHA	Malaysia agriculture, horticulture and agrotourism
USA	The united states of America
TEF	translation elongation factor
BLAST	Basic local alignment search tool
NRRL	ARS Culture Collection
CBS	CBS-KNAW Biodiversity Centre
FRC	Fusarium Research Center
Zn	Zinc
FFSC	*Fusarium fujikuroi* Species Complex
KH_2PO_4	Potassium dihydrogen phosphate
PCNB	Pentachloronitrobenzene
$MgSO_4 \cdot 7H_2O$	heptahydrate sulfate mineral epsomite
dH_2O	Distilled water
MgCl	Magnesium chloride
dNTP	Deoxynucleoside triphosphate
TBE	Tris-Borate EDTA
MLST	Multilocus sequence typing
NJ	Neighbor-Joining
ML	Maximum Likelihood
DNA	deoxyribonucleic acid
rDNA	Ribosomal DNA
FIESC	*Fusarium incarnatum-equiseti* Species Complex
FOSC	*Fusarium oxysporum* species complex
BCAs	biological control agents
PIRG	Percentage inhibition of radial growth

GIC	growth inhibition category
BERNAMA	Pertubuhan Berita Nasional Malaysia

PENCIRIAN *Fusarium mangiferae* DAN *F. proliferatum*, DAN PENGURUSAN PENYAKIT MALFORMASI MANGGA

ABSTRAK

Mangga ialah salah satu buah kegemaran yang digemari oleh rakyat Malaysia. Malaysia menghasilkan lebih kurang seratus dua kilo tan mangga setahun. Bagaimanapun, permintaan pasaran tempatan terhadap mangga amat tinggi, maka Malaysia masih mengimport buah tersebut dari negara lain seperti Thailand dan Australia. Kejadian penyakit malformasi mangga (MMD) telah membantutkan pengeluaran mangga. Penyakit malformasi mangga telah lama dikenali sejak tahun 1891 di India dan beransur-ansur merebak ke negara penanaman mangga termasuk Malaysia. Sejak itu, beberapa spesies *Fusarium* telah dilaporkan menyebabkan penyakit ini di negara lain termasuk Egypt dan China. Namun, tiada laporan yang disahkan di Malaysia. Penyakit ini telah menyebabkan malformasi pada bunga dan bahagian vegetatif pokok, menjadikannya sebagai salah satu penyakit yang merosakkan pokok mangga. Penyakit ini memberi kesan pada pengeluaran buah, daripada hasil yang kurang kepada tiada hasil jika tanaman dijangkiti secara parah kerana bunga yang termalformasi tidak menghasilkan buah. Kajian ini memfokuskan kepada penyelesaian untuk mencari penyebab penyakit dan menilai keberkesanan racun kulat dan agen biologi dalam mengawal penyakit tersebut. Di Malaysia, beberapa spesies *Fusarium* telah dipencilkan daripada sampel yang terjangkit (panikel, dedaun dan bunga) seperti *F. mangiferae, F. proliferatum,* dan *F. subglutinans* tetapi ujian kepatogenan

mereka masih belum dilengkapkan. Pengutipan sampel telah dilakukan di ladang mangga di sekitar semenanjung Malaysia iaitu di Perlis, Kedah, Pulau Pinang, Perak, Melaka, Negeri Sembilan, Pahang dan Johor. Pencilan spesies *Fusarium* yang diperoleh daripada sampel berpenyakit (panikel, dedaun dan bunga) telah dikenalpasti menggunakan pendekatan morfologi dan molekul. Hasilnya, 103 pencilan *Fusarium* telah dikenalpasti sebagai *Fusarium concentricum, F. incarnatum, F. mangiferae, F. oxysporum, F. proliferatum, F. pseudocircinatum,* dan *F. verticillioides*. Berdasarkan ujian kepatogenan, hanya *F. proliferatum* dan *F. mangiferae* menghasilkan simptom malformasi. Walaupun sampel dikutip dari kesemua negeri di semenanjung Malaysia, hanya kebun di kawasan utara sahaja yang menunjukkan simptom malformasi. Untuk mengawal penyakit ini, ujian racun kulat dan agen kawalan biologi telah dilakukan menggunakan kaedah *in-vitro*. Kesemua racun kulat dan agen kawalan biologi yang diuji telah dibuktikan mempunyai kesan ke atas spesis *Fusarium*. Hasil daripada kajian ini dijangkakan dapat menolong saintis, penanam dan institusi yang terlibat dalam industri mangga untuk memahami penyakit ini dan meningkatkan hasil buah mangga.

CHARACTERIZATION OF *Fusarium mangiferae* AND *Fusarium proliferatum*, AND MANAGEMENT OF MANGO MALFORMATION DISEASE

ABSTRACT

Mango is one of the most favourable fruits by Malaysians. Malaysia produced at an estimated of a hundred and two kilo tonnes of mango yearly. However, the local market demand towards mango is very high. Thus, Malaysia still imports the fruits from other countries such as Thailand and Australia. The occurrence of mango malformation disease (MMD) has been a stumbling block to mango production. Mango malformation disease has been a well-known disease since 1891 in India and gradually spread to other mango growing countries including Malaysia. Since then, several species of *Fusarium* have been reported to cause this disease in other countries including Egypt and China. However, none was confirmed in Malaysia. The disease has caused malformation of the inflorescence and vegetative parts of the tree making it as one of the most destructive diseases to mango tree. The disease affected the fruit production, from less to no yield if the plant was severely infected as the malformed inflorescence did not produce fruits. This research focused to determine the causal agent of MMD and evaluated the effectiveness of fungicides and biological agent in controlling the disease. In Malaysia, several species of *Fusarium* have been isolated from infected samples (panicles, leaflets and flowers) such as *F. mangiferae, F. proliferatum,* and *F. subglutinans,* but their pathogenicity tests were not completed. Sample collections were conducted in mango orchards around peninsular

Malaysia (Perlis, Kedah, Pulau Pinang, Perak, Melaka, Negeri Sembilan, Pahang and Johor). Isolates of *Fusarium* species recovered from the symptomatic samples (panicles, flowers and leaflets) were identified using morphological and molecular approaches. As a result, 103 isolates of *Fusarium* were identified as *Fusarium concentricum, F. incarnatum, F. mangiferae, F. oxysporum, F. proliferatum, F. pseudocircinatum,* and *F. verticillioides.* Based on the pathogenicity test, only *F. proliferatum* and *F. mangiferae* produced the malformation symptoms. Though samples were collected in all states in peninsular Malaysia, only orchards in northern region showed symptoms of MMD. To control the disease, fungicide testing and biological control agent were done using *in vitro* All fungicides and biological control agent tested were proven to have impact on MMD pathogens. The outcome of this study was expected to help researchers, growers and institutions involved in mango industries to understand the disease and increase the yield of mangoes.

CHAPTER 1

INTRODUCTION

Mango malformation disease (MMD) was firstly reported in India in 1891 and progressively spread to mango growing countries including Malaysia (Kumar *et al*., 1993; Tharanathan *et al.*, 2006). To date, mango is the only host for this disease (Department of Employment, 2011). This disease could be disruptive to the crop as it infects important parts of the tree which are inflorescences and vegetative parts. The infection will subsequently contribute to the plant canopy developments and floral phase in which the effect will limit and cause less production (Ploetz, 2001; Freeman *et al.*, 2014d)**.** Moreover, this disease has been a threat to the seedlings that are used as rootstocks especially in orchards (Ploetz *et al.*, 2002).

Kumar *et al.* (1993) reported that MMD has caused a lot of yield loss which can reach up to 83%. In Egypt, mango has been a major food as it is categorized as their National Food Basket which worth 26.94$ million yet, MMD has decreased their yield as much as 90% (Ploetz *et al.*, 2002). In India, it has been reported that the maximum yield loss is 86% (Kumar *et al.*, 1993; Rymbai & Rajesh, 2011; Avneet & Nirmaljit, 2018). Even so, South Africa recorded that the disease has been imputed yield loss up to 20% (Schoeman & Botha, 2015; Schoeman *et al.*, 2018a). To date, Malaysia does not have any record for the loss of mango yield.

The causal agent of MMD remains unknown although reports are available worldwide. According to previous reports, MMD was caused by several species of *Fusarium* namely *F. mangiferae, F. sterilihyphosum, F. sacchari, F. subglutinans,* and *F. proliferatum* (Kumar *et al.*, 1993; Britz *et al.*, 2002; Ploetz *et al.*, 2002; Lima *et al.*, 2009) with most mango growing countries reported *F. mangiferae* as the main causal

agent (Kumar *et al.*, 2016a; Veldman *et al.*, 2018; Katoch, *et al.*, 2019). However, due to multiple species associated with MMD from different countries, the diseaseis rather complicated to be resolved. Until now, only a few *Fusarium* species were reported to complete Koch's postulates in certain mango growing countries.

Following personal communication with growers and agricultural staffs during sampling period, the symptoms and incidence of MMD were not a concerning issue to them. The growers were also unaware of the symptoms and the effects of the disease. It was largely ignored due to limited exposure concerning the disease in Malaysia. Thus, it was less discussed by authorities responsible in agricultural sectors as the causal agent of the disease in Malaysia was still unclear. Therefore, there were no suggested management measure introduced in Malaysia. To our knowledge, limited reports have been made by either those who actively involved in mango growing industries or small scale growers in Malaysia. As stated in the book of "Diseases and Disorders of Mango in Malaysia", MMD in Malaysia was rather infrequent. The incidence of the disease was likely to hit on the variety that originates from India (Kwee & Chong, 1994). However, the variety was unnamed. According to agricultural authorities in Malaysia, MMD was currently not one of the listed diseases that needs to be monitored. In 2013, the distribution of *Fusarium* species isolated from suspected MMD samples were recorded to be dispersed in northern and southern regions of Malaysia (Mohamed Nor *et al.*, 2013).

The current statistics showed that Malaysia ranked 33rd in the world as one of the importers with 102.05K metric tons production volume and gradually increase in demand from year to year (Mango Production in Malaysia (Tridge), n.d). This high market values might be interrupted by the infection of the disease especially for Harumanis and Chok Anan varieties that are preferable by Malaysian. Therefore, a

study needs to be conducted to improve MMD data in Malaysia, especially, being one of mango growing countries as it has become a major fruit that is currently has high demands in Malaysia.

Since MMD was not considered as an important disease to mango in Malaysia, growers are depending solely on chemical treatment to treat wide-range diseases such as benomyl, dithane and thiophanate methyl (Woo *et al.*, 2014). Furthermore, the farmers and agricultural authorities do not suggest or use any biocontrol agents for disease management of mango. It is known that chemical control could carry negative effects to the plants by inhibiting plant development or agro-ecosystem that can shy away beneficial microbial communities as well as effect to the end user. It is also known that chemical agent could cause resistance to the pathogen in long run usage (Leroux, 1996; Woo *et al.*, 2014).

In 2009, European countries have been promoting integrated pest management (IPM) for crops which includes the use of biological control as alternative to chemicals to reduce the risk and effect of chemicals to human health and environment (Woo *et al.*, 2014). To this date, *Trichoderma* spp. have been widely used as a biocontrol agent, biofertilizer and plant growth regulator as they have the ability to protect plants and enhance vegetative growth as well as ability to improve soil nutrient, decomposition and biodegradation as soil amendments (Vinale *et al.*, 2008; Woo *et al.*, 2014). Generally, *Trichoderma* spp. are targetted to control soilborn pathogens either directly as mycoparasitisms or indirectly by promoting plant growth, competing for nutrients and space, or act as plant defensive mechanisms (Benítez *et al.*, 2004). The species that are commonly use as biocontrol are *T. asperellum* ,*T. atroviride* ,*T. harzianum, T. polysporum,* and *T. viride*. This alternative to control disease is also capable of managing wide-range pathogen. In fact, Asia has been disclosed to be the largest

distribution of *Trichoderma* spp. as bioproduct (Woo *et al.*, 2014). In this study, we intended to propose a chemical and biological treatment to manage MMD in Malaysia.

Thus, the objectives of this study were:

a) to determine *Fusarium* species associated with MMD and their distribution in Malaysia.
b) to determine the causal agent of MMD by conducting pathogenicity test.
c) to evaluate the effectiveness of fungicides and biological agent to control growth of *Fusarium* spp.

CHAPTER 2

LITERATURE REVIEW

2.1 Mango (*Mangifera indica* L.)

2.1.1 Origin and distribution

Mangifera species are largely found in Asia such as in peninsular Malaysia, Indonesia archipelago, Thailand, and Indo-China (Kumar *et al.*, 2011). It is known that at least 27 species are edible among all other *Mangifera* species. Mango (*Mangifera indica*) belongs to the family Anacardiaceae (Cashew family) and the order of Sapindales. It consists of thousands of cultivated varieties introduced in warm countries (Freeman *et al.*, 2014b; Jahurul *et al.*, 2015).

It has many common names throughout the world such as Aam for India, Mangga for Malaysia, Mangueira for Portugal, Mangot for French, and Manja for German (Tharanathan *et al.*, 2006; Freeman *et al.*, 2014a; Shah *et al.*, 2010). Among these, 80% of the varieties are found in India (Krishnan *et al.*,2009; Avneet & Nirmaljit, 2018). *Mangifera indica* is commonly known as mango while the other edible *Mangifera* species are referred as wild mango and are low in fruit quality (Bally, 2006). By far, there are approximately 1500 mango varieties have been documented. Among these, Alphonso is the most favourable variety in India (Tharanathan *et al.*, 2006), while Harumanis, Chok Anan, Masmuda and MAHA are popular varieties in Malaysia (Phebe & Khairul, 2013).This is due to their strong aroma, fleshy delicious taste and high nutritional value such as vitamin C, β-carotene and minerals.

Mango was originated from India 4000 years ago and was gradually introduced to several countries especially in Asia countries such as Malaysia, Bangladesh, China and Indonesia. Meanwhile Tharanathan *et al.* (2006) claimed that mango was originated from Indo-Burmese. World Conservation Union and World Conservation Monitoring Centre (1998) stated that wild populations of mango can be found in Assam, India and Myanmar, especially in the Assam-Chittagong Hills.

Due to its significant value and high demand from other countries, the production of mango has been dramatically increased by 50% between 1971 and 2001. The mango fruit has been exported to numerous countries including Central and South America, Africa, Hawaii, Egypt, and Southeast USA. It is currently the fifth important fruit crop worldwide (Tharanathan *et al.*, 2006).

2.1.2 Botanical description of mango

In general, mango tree grows as a large shady tree. This tree grows rapidly with sufficient heat surrounding. The fact that mango trees can live up to 300 years old, it can also grow up to 40 m in height (Yadav *et al.*,2018). However, mango tree in orchards usually kept at 6-9 m at most to stimulate optimum fruit production. Interestingly, the leaf starts growing in tan-red and become matured in shiny green on upper leaf and pale green below leaf. In early stage, the leaves omit a strong turpentine and the smell reduces as the leaves mature. Even so, some of the cultivars do not omit smell (Yadav *et al.*, 2018). Other than having leaves that remain green for a long time, the leaves contain significant amount of mangiferin which contain a remarkable xanthone that can be used to treat cancer (García-Rivera *et al.*, 2011).

Mango flowers consist of panicles that form pyramid shape bearing flowers. Flowers are usually male with some of the flowers are bisexual which can bear fruit.

The pollination of mango trees is greatly assisted by insects such as flies, wasps and bees. The fact that only 25% of the flowers are hermaphrodite (imperfect flowers) make an inflorescence (a bunch of flowers) to have a low chance to produce fruits. The high incidence of perfect flowers (male flowers) for an inflorescence reduced the fruit production of an inflorescence as it can't produce any pollens and thus incapable of producing fruits (Mango, 2016).

In reality, mango tree requires a significant demand of management as the tree usually needs chemical to promote flowering and fruiting. Contrasting to other fruit crops, mango needs chemical to stimulate the proportion of hermaphrodite flowers in order to increase the fruit production. The probability of pollination of mango flowers can be significantly reduced with high humidity and rain. Thus, explains why the flowers are hard to be pollinated (Mango, 2016).

2.1.3 Uses and nutritional values

For over 4000 years ago, mango has been regarded as one of the important herbs by the Ayurvedic and indigenous medical systems. Each part of the mango tree has its medicinal attribution (Shah *et al.*, 2010). The whole plants are very beneficial as they are used to treat diarrhea, insomnia, toothache, snakebite, miscarriage, heat stroke, blisters and any other purposes. Mango fruit is well-known for its pleasing aroma, sweet taste, and soft texture. Besides, it contains significant vitamins, micronutrients and other phytochemicals, and also often used to treat heat stroke (Tharanathan *et al.*, 2006; Shah *et al.*, 2010). The seeds are used in treatment of asthma as well as astringent, while the kernels are used to make flour. In addition, the leaves are used to make fumes to be inhaled as a relief from hiccups and sore throat. The gums of the bark are often used as dyes and are also used as dressing for cracked feet

and scabies (Shah *et al.*, 2010). Mangiferin, a bioactive xanthonoid has been treasured for its medicinal benefits especially for its strong antioxidants, wound healing, anti-degenerative, antidiabetic activities and also hypertensive (Shah *et al.*, 2010).

2.2 Mango Malformation Disease (MMD)

2.2.1 Distribution and symptoms of MMD

Mango malformation disease has been a critical disease to mango trees in many mango growing countries. This disease was first reported in Darbhanga (Bihar, India) by Marries as a fungal disease and a physiological disorder (Watt, 1891; Avneet & Nirmaljit, 2018). Since then, the disease has been noticed in countries such as Mexico, USA, Pakistan, Sudan, Australia and recently the United Arab Emirates as well as in its origin country, India (Kumar *et al.*, 1993). As suggested by Darvas (1987), MMD is classified as an airborne disease (Kumar *et al.*, 1993). It arises as it infects vegetative and floral parts of the tree which are crucial part of the plant for growth and production. The disease has been a serious concern especially to the growers as the infected inflorescence would not set fruit at one blow (Kumar *et al.*, 1993; Youssef *et al.*, 2007).

The severity of the disease may vary from a variety to others as well as with severity and symptoms among trees can vary up to 50-60% to 100% of crop damage though the disease spreads slowly in the orchards (Summanwar, 1967; Kumar & Beniwal, 1992; Youssef *et al.* 2007; Kumar *et al.*, 2011; Rymbai & Rajesh, 2011; Avneet & Nirmaljit, 2018). Due to this event, the disease has been inferred as a threat to mango production as it is destructive and endemic in which the infected trees would never recover (Kumar *et al.*, 1993; Kumar *et al.*, 2011).

The disease also spreads steadily from infected to healthy seedlings or trees (Kumar *et al.*, 1993). Vegetative malformation (VM) usually affects young trees, as early as in 3-4 months old seedlings with maximum effect (90.9%), in 4-8 years old trees and seldom on mature trees (Rymbai & Rajesh, 2011).

There are two distinct symptoms of MMD which are vegetative and floral malformations. Vegetative malformation (VM) affects seedlings and young trees in nurseries, especially when seedlings are planted beneath the affected trees. Nonetheless, mature trees are also reported to be infected by the disease (Youssef *et al.*, 2007). The symptoms of VM include tightly bunched young shoots with swollen apical and lateral buds and hypertrophied (Figure 2.2) (Freeman *et al.*, 2014c). Malformed symptoms on vegetative tissue that occur on mature trees are not as severe as in young seedlings. Severely infected seedlings with MMD cause stunting of growth (Queensland Government, n.d).

Figure 2.2: Vegetative malformation symptom (Marasas *et al.* 2006).

Symptoms of floral malformation (FM) show shortened, thickened, and highly branched panicles on the primary and secondary axes (Figure 2.3) (Freeman *et al.*, 2014c). All the affected flowers are male and infrequently bisexual. This perfectly

malformed or bisexual inflorescence abnormally enlarged and bears non-functional ovary leading to less fruit reproduction. The infected trees also exhibit symptoms such as inflorescence enlargement and abortion of fruit production at young stage leading to direct loss (Kumar *et al.*, 1993). Since the inflorescence does not bear fruits, FM is considered more severe than VM (Mahrous, 2004). Normally, FM will show symptoms on mature flowering trees (Marasas *et al.*, 2006). In some cases, healthy and malformed flowers may appear on the same panicle or shoot (Kumar *et al.*, 2011). Conventionally, malformed panicles can be differentiated as heavy, medium and light types based on the disease severity and panicle compactness. Heavy type shows that the enlarged flowers can be dried up and remain attach as brown discolored bunches with compact and overloaded due to large masses of flowers while medium type is less compact. However, they remain in contact with the plants for a longer period than the healthy panicles. In contrary, light type does not remain attach on plants and slightly more compact than the normal flowers (Krishnan *et al.*, 2009; Kumar *et al.*, 2011; Avneet & Nirmaljit, 2018). These affected inflorescences usually do not set fruits and cause loss in yield to the mango growers (Youssef *et al.*, 2007)

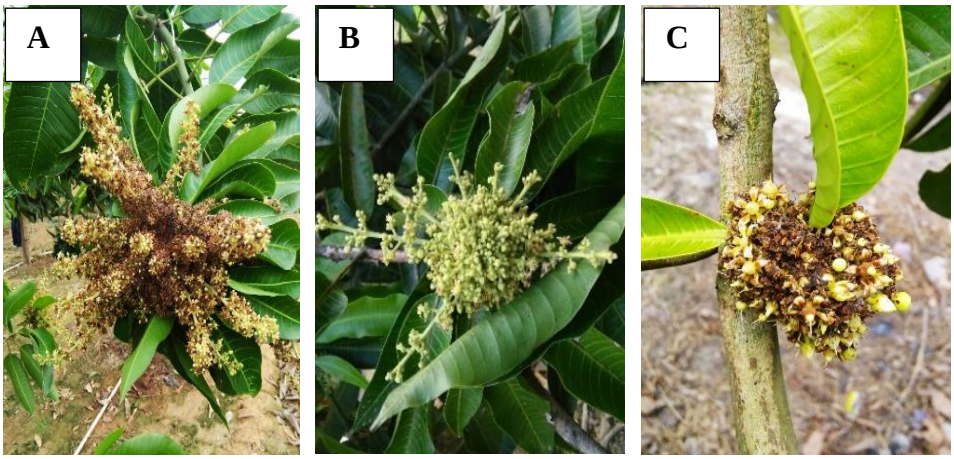

Figure 2.3: Symptoms of floral malformations. (A) Floral enlargement; (B) panicles are highly branched on primary axis; (C) brown compact bunches of flowers.

Internal symptoms can also be spotted such as the development of hyperplastic and hypertrophied cells in VM and FM parts, malformed flowers with inflated disks, and degenerating or undeveloped embryos in infected fruit (Kumar *et al.*, 1993).

2.2.2 Susceptibility, Production and Loss

The incidence of malformation disease in mango varieties is depending on various factors including temperature, time, age of tree and other abiotic factors (Kumar *et al.*, 2011). It is reported that the early blooming varieties are more receptive to the disease than the late blooming varieties (Krishnan *et al.*, 2009; Kumar *et al.*, 2011). In India, the majority of commercial mango varieties are severely infected including Alphonso variety (Chakrabarti, 2011; Ansari *et al.*, 2015). To date, experimental evidence for the variety susceptibility to this disease has not been published elsewhere (Kumar *et al.*, 2014).

In 2004, National Board of Horticulture reported that mango production is next to banana on the global basis (Krishnan *et al.*, 2009; Avneet & Nirmaljit, 2018). Back in 2016, Food and Agriculture Organization of the United Nations stated that global

mango production was 48.4 million tons with India as the leading producer next to China and Thailand. The production continues to increase to 50.6 million tons in 2017 (Food and Agriculture Organization, 2017).

Due to MI that produces malformed tissue and sterile bunches, mango production has experienced a great yield loss despite having large cultivation area. Though the production is high, the productivity is still low with 6.3 million tons per hectare due to various factors including biotic and abiotic factors affecting its growth (Kumar *et al.*, 2011; Avneet & Nirmaljit, 2018). The limitations can go up to 50-60% and even a total loss in severe cases (Chakrabarti, 2011; Ansari *et al.*, 2015; Avneet & Nirmaljit, 2018). In Mexico, mango growers reported yield reduction in the tropical, dry land growing regions, causing the loss up to 30 to 40% (Noriega-Cantú *et al*., 1999). In 1998, an approximate US$15 million loss was experienced by the growers due to this disease in Egypt and in more important producing countries (Ploetz *et al.*, 2002; Freeman *et al.*, 2014). The maximum economic loss reported in India is 86% (Kumar *et al.*, 1993; Rymbai & Rajesh, 2011; Avneet & Nirmaljit, 2018).

2.2.3 Pathogen in Relation to MMD

The causal MMD is still in question as the causal agent of MMD varies between countries. Steenkamp *et al.* (2000) stated that fungi are the most contributed factor responsible for the disease although abiotic factors such as temperature, plant growth regulators, malformin and mangiferin may also contribute to this disease too (Freeman *et al.*, 2014a). The infection of fungi is tremendous in malformed flowers and vegetative shoots, lower or non-existent in asymptomatic tissues, but infrequent in branches even when they supported malformed panicles or shoots (Youssef *et al*.,

2007). Youssef *et al. (*2007) further explained that conidium survival rapidly reduced in soil during the summer. However, the pathogen may get lengthy periods in soil within infected plant tissues (Youssef *et al.*, 2007).

A number of *Fusarium* species have been scientifically identified as causal agent of MMD (Marasas *et al.*, 2006). Back in 1993, *F. oxysporum* (as test fungus) and *F. subglutinans* has been confirmed as the causal agent of the mango malformation (Ploetz *et al.*, 1993). However, there was no recent reports of *F. oxysporum* that could cause MMD. According to a study, *F. subglutinans* was believed to have an association with mango malformation (Steenkamp *et al.*, 2000). Although the fungus has several synonyms in literature, it was redescribed as *F. mangiferae* in India, Sri Lanka, China, Oman, and Spain (Freeman *et al.*, 2014b). To date, many countries such as Egypt, Israel, Brazil, Mexico, and Spain reported that *F. mangiferae* is the causal agent of MMD (Summanwar, 1967; Kumar *et al.*, 1993; Youssef *et al.*, 2007; Lima *et al.*, 2009). According to the other report, *F. mangiferae* was found to be the most dominant species isolated from infected mango orchards located in South Africa with the percentage of 91.4%. However, pathogenicity test was not yet done to confirm the pathogenicity of *F. mangiferae* (Veldman *et al.*, 2018).

In 2006, Marasas *et al.* (2006) figured out that there were more causal agents of MMD are discovered from other mango growing countries. This has never been more compelling as majority of them are from *Fusarium* genus. But none of the reports showed any evidence of Koch's postulates were completed. Though Brazil has confirmed *F. mangiferae* as MMD pathogen, it was recently reported that *F. tupiense* is the other causal agent that is currently responsible for this disease (Lima *et al.*, 2012). *F. tupiense* was morphologically similar to *F. sterilihyphosum* but apparently

different phylogenetically from both *F. mangiferae* and *F. sterilihyphosum* as well as producing a unique teleomorph in the *F. fujikuroi* complex (Lima *et al.*, 2012).

Until now, only China has claimed that *F. proliferatum* is the causal agent with Koch's postulates completed (Zhan *et al.*, 2010; Lv *et al.*, 2013). In 2013, Mohamed Nor *et al.* reported *F. proliferatum* has been a potential causal agent of MMD in Malaysia, still, no pathogenicity test was conducted. In Mexico, a new species of *Fusarium*, *F. mexicanum* was identified to be associated with MMD, in which it was then confirmed to be a causal agent of MMD as Koch's postulates were completed (Otero-Colina *et al.*, 2010; Soto-Plancarte *et al.*, 2015). In another report, Freeman *et al.* (2014c) confirmed that *F. pseudocircinatum* could cause MMD in Mexico. A study by Summanwar *et al.* (1967) revealed that *F. moniliforme* (synonym: *F. verticillioides*) was accountable for the floral phase of MMD. Similarly, Varma *et* al. (1971) also found that *F. moniliforme* was also responsible for the vegetative phase of the disease. In South Africa and Brazil, *F. sterilihyphosum* was found to be associated with MMD (Britz *et al.*, 2002; Lima *et al.*, 2009). However, pathogenicity test for species other than *F. fujikuroi* species complex was not yet completed (Marasas *et al.*, 2006).

2.3 Identification of *Fusarium* species

2.3.1 Morphological Identification

The genus *Fusarium* is the most important pathogenic fungi with complicated and unstable taxonomic history (Geiser *et al.*, 2004). The shape and size of conidia, types of conidiogenous cells, and chlamydospores are among important characteristics for *Fusarium* species identification (Leslie & Summerell, 2006). The features of the colony including the pigmentation, as well as the presence of sporodochia on the media

are also important in assisting the species identification. Typical morphological identification of *Fusarium* consists of primary and secondary characteristics.

Macroconidia are the primary characters to observe when using morphological characteristics to identify the species. Macroconidia are found in sporodochia on carnation leaf agar (CLA). The macroconidia are found in sporodochia are commonly consistent in their shape and size. Apart from the shape and the size of macroconidia, the number of septate, and the shape of apical and basal cells are other features to identify *Fusarium* species. On the other hand, microcondia are more common on growing hyphae cultured on media in which they are usually abundant and diverse (Leslie & Summerell, 2006). For microconidia, the difference between the two conidiogenous cells of monophialides and polyphialides is often clearer to be observed under microscope in water mount slides rather than *in situ* observation. Monophialides have a single opening in the conidiogenous cell whilst polyphialides have two or more.

The most frequent method used for detecting secondary characters are odour and growth rate. Commonly, these are used to confirm a diagnosis based on other characters than to make an initial identification. Growth rates may be formed on linear growth in a race tube or on radial growth in a Petri dish. Other than that, secondary characters can be physiological data on toxins and other metabolites produced. However, these data are not available for routine diagnoses (Summerell *et al.*, 2003).

Chlamydospores are commonly found in older cultures either in the hyphae, on the surface of the media, or embedded in the media (Klotz *et al.*, 1988; Summerell *et al.*, 2003). True chlamydospores, pseudochlamydospores, and swollen cells may appear similar and can be easily misidentified. True chlamydospores have a thick wall,

a warty appearance and light coloration. Chlamydospores can be formed in chains, clumps, or single structure and can be found in the hyphae either on or below the agar.

2.3.2 Molecular Identification

Molecular identification using protein-coding genes such as Translation Elongation Factor-*1α* and β-tubulin and *α*-calmodulin have been regularly practiced in *Fusarium* research. In 2004, Geiser *et al.* (2004) has created *Fusarium* MLST to gather all available database of *TEF-1α* sequences. This is due to the limitations in *Fusarium* which has been the imprecise and vague primarily in term of species names and morphological species recognition. Users can initiate sequences using primers that are conserved across the genus leading to a correct identification of a known species and use the sequence as a query to BLAST the database. The database can be accessed at either http://fusarium.mycobank.org/ (Geiser *et al.*, 2004).

TEF-1α gene encodes an essential part of the protein translation machinery. It has been designed as universal marker that works across the phylogenetic breadth of the genus. Due to its highly informative region, it is especially helpful at the species level in *Fusarium*. In addition, *TEF-1α* has not detected any non-orthologous copies of the gene in the genus, making it is the best tool for phylogenetic utility. *TEF-1α* is firstly used as a phylogenetic marker to define species and generic level relationships among Lepidoptera (Cho *et al.*, 1995; Geiser *et al.*, 2004). Originally, the primers i.e. TEF1 and TEF2 were designed based on sites shared in exons between *Trichoderma reesei* (Hypocreales/Sordariomycetes/Pezizomycotina/Ascomycota) and *Histoplasma capsulatum* (Eurotiales/Eurotiomycetes/Pezizomycotina/Ascomycota). The sites were later on applied to a wide variety of filamentous ascomycetes.

In *Fusarium* species, *TEF-1α* marker amplifies an approximately 700 bp region of TEF that bounds three introns which total over half of the amplicon's length (Figure 2.4) (Geiser *et al.*, 2004). Over time, researchers found that this gene appears to be persistent single copy in *Fusarium*. It also shows a high level of sequence polymorphism among closely related species and even in comparison to other intron-rich portions of protein-coding genes such as calmodulin, beta-tubulin and histone H3. Up to now, *TEF-1α* has become the best marker used in *Fusarium* identification (Geiser *et al.*, 2004).

Phylogenetic species recognition in *Fusarium* has reckoned mostly on Genealogical Concordance Phylogenetic Species Recognition (Taylor *et al.*, 2000). The method pinpoints shared partitions among multiple gene genealogies as landmarks for species boundaries. Although two or more gene genealogies are required for recognizing species, species may be identified accurately using a single DNA sequence marker in as much as the background of phylogenetic analyses has been performed using the marker along with others (Geiser *et al.*, 2004). In 2015, at least 300 phylogenetically recognizable species have been resolved as genealogically exclusive lineages based on phylogenetic analyses of representative *Fusarium* in the ARS Culture Collection (NRRL), the CBS-KNAW Biodiversity Centre (CBS) and the Fusarium Research Center (FRC) (Ward *et al.*, 2015).

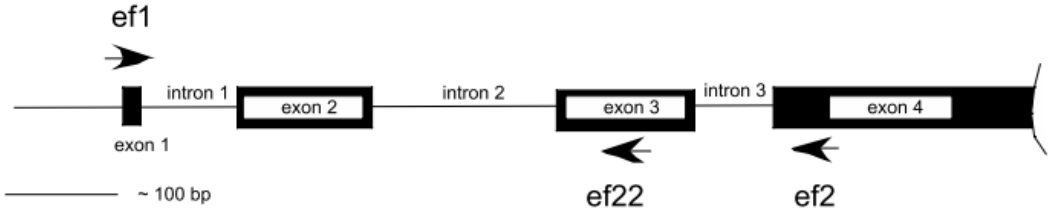

Figure 2.4: Map of the TEF gene region in *Fusarium* used in FUSARIUM-ID with primer locations used by Geiser *et al.* (2004).

2.4 Pathogenicity Test

Practically, pathogenicity test is the only reliable method to confirm the suspected causal agent in causing a disease or infection. A suitable host is required in order to carry out the pathogenicity test (Klement, 1963). According to Klement (1963), testing of woody plants through is especially difficult. This is due to its hard surface for infiltration and higher amount of suspension is required. Pathogenicity test of MMD is especially difficult to prove since there is no convincing method. Personal communication with MMD expert, Dr. Stanley Freeman (Department of Plant Pathology and Weed Research, The Volcani Center, Israel) revealed that symptom development of MMD on mango tree was inconsistent with less than 70% success rate. Further elaborated, the symptoms of MMD are difficult to scale as they can be varied in severity. Thus, the symptoms are usually mark as positive or negatively tested.

Mango malformation symptoms, particularly vegetative malformation happen by pierce inoculation, soil inoculation, and spraying seedlings/trees with spore suspension. In other cases, attempts to produce floral malformation have been extensively unsuccessful. As reported by Kumar *et al.* (1993), both stages of the disease are reproducible by spraying spore suspension of *F. subglutinans*. However, the factors that govern the development of typical symptoms of malformation have been unresolved (Kumar *et al.*, 1993).

Up to now, only *Fusarium* spp. have been tested for pathogenicity test of MMD. The interrelation of *Fusarium* species with malformed tissues has been poorly demonstrated in the pathogenicity test. Pathogenicity test of MMD took months to produce MMD symptoms on a tree. In recent study, typical malformation symptoms can be developed within 3 to 4 months. Most attempts were done on *F. subglutinans* (Recently re-describe as *F. mangiferae*) and *F. oxysporum* as test fungus since the

species were firstly identified as the probable causal agent for MMD (Kumar *et al*., 1993). However, those attempts were unsuccessful because inoculation method has been an issue to researchers as it did not work consistently (Kumar *et al.*, 1993).

Apart from *F. subglutinans*, another species namely *F. proliferatum* has also been associated with MMD. The association has been reported by mango growing countries such as Malaysia and China (Zhan *et al.*, 2010; Lv *et al.*, 2013; Mohamed Nor *et al.*, 2013). In 2013, Zhan *et al.* (2010) proved *F. proliferatum* could cause MMD in China. However, not all of the inoculated with *F. proliferatum* produced typical symptoms with incidence ranging from 10% to 70% (Ru-Lin Zhan et al., 2010).

Consistent with a study by Lima *et al.* (2012) found that the pathogenicity test of *F. tupiense* was difficult to be reproducible. Although they managed to complete Koch's postulates to both *F. tupiense* and *F. sterilihyphosum* but only a small number of plantlets presented malformation symptoms. Not only that, the symptoms were observed only after 6 months later (Lima *et al.*, 2009; Lima *et al.*, 2012).

2.5 Disease Control Management

To this date, little or no success is achieved in controlling the disease despite various methods have been introduced. The control measures between countries exhibited inconsistent results in orchards, nurseries, and other sites of mango growing (Chakrabarti, 1996; Freeman *et al.*, 2014b; Avneet & Nirmaljit, 2018). The control measures include the use of plant growth regulators, pruning of malformed areas, deblossming, insecticides, pesticides, and biopesticides (Kumar *et al.*, 2011). According to Rymbai & Rajesh (2011), the severity of the disease could be lessen by following integrated management packages such as sanitary pruning, adding organic

matter to the soil, weed control, irrigation management, control of vectors, balanced chemical fertilization, protection of new buds, and promoting anticipated blooming. The disease occurrence can also be minimized by selecting resistant cultivars than those of susceptible cultivars. The most likely reason is malformation is correlated with the occurrence and timing of flowering in plants (Kumar *et al.*, 2011; Avneet & Nirmaljit, 2018). Therefore, in epidemic prone areas, alternate bearing and late flowering varieties should be selected for cultivation (Pant, 2000; Rymbai & Rajesh, 2011; Avneet & Nirmaljit, 2018).

The most common technique used in India and Egypt is to remove and destroy the diseased tissue showing the symptoms of MMD to prevent transmission of the disease in the orchards. This includes nurseries and plant stocks must pathogen-free from the infected area and graft of diseased plants should not be used at any cost (Ploetz, 2001; Rymbai & Rajesh, 2011). In India, moderate pruning of 20 cm shoot bearing malformed panicles in panicle emergence state leads to the termination of the disease (Sirohi *et al.*, 2009). Generally, pruning comprises removal and burning of infected terminals and other subtending three nodes is considered effective given the methods is followed for 2-3 consecutive years (Muhammad *et al.*, 1999; Ploetz, 2001). This sanitation practice leads to the reduction in mango malformation by limiting the source of inocula. Nonetheless, it is laborious to apply on the large trees with panicles that are difficult to access. This control measure is commonly practiced in South Africa and the United States (Kumar *et al.*, 2011; Rymbai & Rajesh, 2011; Freeman *et al.*, 2014d; Avneet & Nirmaljit, 2018). South Africa has been suggesting to inspect the trees during October and November routinely when malformed panicles are easily noticeable to which the affected panicles were removed. However, these control

measures proved to be insufficient between 2010 to 2012 as malformation increased yearly (Schoeman *et al.*, 2018a).

The use of insecticides, fungicides, and plant growth regulators along with pruning might be needed to reduce the level of inoculum in the orchard (Ploetz, 2001; Rymbai & Rajesh, 2011). Thus, there is a need to establish new plantings with pathogen-free nursery stock (Ploetz, 2001; Rymbai & Rajesh, 2011). O'Donnell *et al.* (1998) and Kumar *et al.* (2011) reported that mango yields were significantly increased by an integrated management scheme that includes a removal of the terminal symptomatic shoots, sprays of different fungicides, and five applications of sulphur acaricide.

2.5.1 Biofungicide Control

The use of biopesticides was effective in limiting the growth of *Fusarium* species (Malik *et al.,* 2018). In India, three species of *Trichoderma* (*T. viride*, *T. virens*, and *T. harzianum*) were tested and were found effective against *F. moniliforme* (Kumar *et al.*, 2011; Avneet & Nirmaljit, 2018). However, the present study in India recorded that *T. viride* was the most effective against *Fusarium* species (Varma *et al.*, 1971). According to Malik *et al.* (2018), biopesticides could be the best disease management of MMD by utilizing various *Trichoderma* spp.. Up to the present, *T. harzianum* has been reported to give the best controlling effect by inhibiting 71% of *F. mangiferae* and other pathogenic fungi. These filamentous fungi are very familiar in nature, with huge population densities in soil and plant litters (Akrami & Yousefi, 2015; Estifanos *et al.*, 2018).

MMD is believed to be systemic in nature and specifies that vegetative malformation in nurseries may outspread through soil. Due to this hypothesis, application of bio-agents in nursery is highly recommended which may help in the control measure of vegetative malformation in nurseries as well as other control measures (Bhatnagar & Beniwal, 1977; Malik *et al.*, 2018). Utilization of *T. harzianum* has been used in other disease management to suppress other soil-borne diseases of mangoes in nurseries to which researchers come to conclusion that *Trichoderma* spp. has the properties to the control of the pathogenic fungi. Due to its ability, *Trichoderma* spp. was believed that if they have the benefit to control the disease *in vitro*, it will be a great achievement for the disease management in orchard environment (Malik *et al.*, 2018).

2.5.2 Chemical control

No effective chemicals are reported for disease control of infected mango trees. To this date, any specific fungicides have not been suggested in the MMD management routine MMD worldwide (Kumar *et al.*, 2014). In order to implement a new integrated management strategy, the effective fungicides to protect buds from conidia of *Fusarium* species must be identified. Disease can be reduced with the help of foliar spray as it delays or advances the commencement of flowering (Kumar *et al.*, 2011). Freeman *et al.* (2014b) conducted *in vitro* tests to determine the efficacy of several fungicides in inhibiting *F. mangiferae*. They later found that prochloraz is the most effective fungicide inhibited *F. mangiferae* compared to other tested fungicides such as carbendazim, pyraclostrobin, boscalid, and azoxystrobin. They also found that other fungicides did not inhibit *in vitro* fungal growth of the pathogen *in-vitro* such as bupirimate, flutolanil, tebuconazole, thiophanate methyl, triadimenol, and triforine. In

another experiment, a significant yield increase was observed when strict sanitation was combined with timely spray prochloraz treatment in 3 years' time, compared with the unsprayed treatment to which symptomatic tissue was removed only at harvest (Freeman *et al.*, 2014b). Recently, Schoeman *et al.* (2018b) have stated that the yield data was insignificant between treatments, however, there was a significant increase in yield from the first to the second year to which then the yield remained constant. The control measure involves spraying prochloraz-Zn in three-week intervals combined with elimination of FM at frequent intervals. This convince that strict sanitation using fungicide alone resulted in minimal increase in yields (Kumar *et al.*, 2014). Thus, the combination of chemical prochloraz and strict sanitation treatments are recommended as they significantly increased yield (Schoeman *et al.*, 2018b).

Prior to flower bud differentiation, application of naphthalene acetic acid and indole-3-butyric acid reduced the incidence of FM whilst foliar spray of napthalene acetic acid and application of benomyl to control of disease can reduce the disease incidence efficiently (Mahrous, 2004; Rymbai & Rajesh, 2011).

Back in those days, the use of benomyl was proved to inhibit MMD *in vitro* , but did not affect MMD development when sprayed *in situ* (Ibrahim *et al.*, 1975; Chada *et al.*, 1979; Dieckman *et al.*, 1982; Kumar *et al.*, 2014).

Fungicides such as benzimidazoles and Topsin-M when applied were reported to reduce MMD but statistical significance was not determined (Iqbal *et al.*, 2011; Freeman *et al.*, 2014b; Avneet & Nirmaljit, 2018). For instance, benzimidazoles have been tested frequently against MMD, but their impact was doubtful even when positive results have been reported. Topsin-M was reported to have effect with a single spray applied at the bud differentiation stage by reducing malformation in the non-treated control but statistical analysis was not included (Muhammad *et al.*, 1999). A study by

Golakiya *et al.* (2018) used different types of fungicides including systemic, non-systemic and fungicide combination to control MMD.

Although clear consensus does not exist regarding the efficacy of fungicides for MMD management, it is still considered as potential management tools especially to large orchards. For this reason, effective fungicides would need to be identified including effective application intervals that would optimize the usage as these could be toxic at high concentration and when used for longer durations (Kumar *et al.*, 2014).

CHAPTER 3

DISTRIBUTION AND IDENTIFICATION OF *Fusarium* spp. FROM MANGO MALFORMATION DISEASE

3.0 Introduction

Fusarium genus can cause a remarkable range of plant diseases such as crown and root rot, vascular wilt disease, and stalk rot on different plants including mango (Leslie & Summerell, 2006; Summerell *et al.*, 2011). Mango malformation disease is one of the most important diseases to mango. More than one species of *Fusarium* has been associated with MMD. The species may be a pathogen or saprophyte that can act as a secondary pathogen or even have no role in the disease development (Leslie & Summerell, 2006). The information of MMD in Malaysia is still lacking in which the causal agent is still unknown as well as its distribution. Based on previous reports, Malaysia is associated with *Fusarium* species from *Fusarium fujikuroi* species complex (FFSC), however, the pathogenicity test was not yet done (Mohamed Nor *et al.*, 2013).

The isolates of *Fusarium* can be isolated on PDA for identification purpose to distinguish their colony morphology, growth rates and growth culture as the medium is rather consistent. However, the morphology of *Fusarium* conidia is often more reliable on carnation leaf agar (CLA) as the macroconidia and microconidia appear more consistent and can induce more sporodochia than on PDA (Leslie & Summerell, 2006). In determining *Fusarium* species, macroconidia are the most important characteristics in defining the species besides microconidia and chlamydospores, yet, the size and shape of macroconidia can sometimes be confused due to environmental factors (Leslie & Summerell, 2006).