CHARACTERIZATION AND PATHOGENICITY OF FUNGI ASSOCIATED WITH STEM-END ROT OF MANGO FRUIT

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CHARACTERIZATION AND PATHOGENICITY OF FUNGI ASSOCIATED WITH STEM-END ROT OF MANGO FRUIT

by

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LIST OF SYMBOLS

%	Percentage
&	And
®	Registered
µl	Microlitre
µm	Micrometre
g	Gram
L	Litre
ml	Millilitre
mm	Millimetre
psi	Pounds per square inch
™	Trademark
V	Voltage

LIST OF ABBREVIATIONS

ACT	Actin
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CAL	Calmodulin
C_2H_5OH	Ethanol
CLA	Carnation leaf agar
dNTP	Deoxynucleotide triphosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ITS	Internal transcribed spacer
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
min	Minute
ML	Maximum likelihood
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth

rDNA	Ribosomal DNA
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
STR	Short tandem repeat
TBE	Tris-Borate-EDTA
TEF1-α	Translation elongation factor 1-α
WA	Water agar

PENCIRIAN DAN KEPATOGENAN KULAT YANG BERASOSIASI DENGAN PENYAKIT REPUT TANGKAI BUAH MANGGA

ABSTRAK

Mangga (*Mangifera indica* L.) merupakan salah satu tanaman buah-buahan yang popular di Malaysia dan penyakit reput tangkai buah mangga merupakan penyakit lepas tuai yang serius. Kajian ini dijalankan untuk menentukan patogen penyebab penyakit reput tangkai buah mangga. Buah mangga diperoleh daripada dusun, pasar malam dan pasar raya di negeri utara Semenanjung Malaysia. Pencilan kulat telah dipencilkan daripada lesi reput tangkai buah mangga dari kultivar Chok Anan, Falan, Golek, 'Black Gold', Harumanis, 'Waterlily', Raja dan 'Apple Mango'. Pencilan kulat telah dikenalpasti menggunakan ciri morfologi dan ciri molekul. Ciri-ciri morfologi digunakan untuk mengelompokkan pencilan kulat tujuh spesies Botryosphaeriaceae (*Lasiodiplodia theobromae, L. pseudotheobromae, L. iranensis, L. mahajangana, Neoscytalidium dimidiatum* dan *Pseudofusicoccum violaceum*), *Diaporthe* spp., dua spesies *Colletotrichum* (*C. asianum* dan *C. siamense*) dan *Beltrania rhombica* telah dikenal pasti. *Lasiodiplodia theobromae* (n=149), *L. pseudotheobromae* (n=9), *L. iranensis* (n=5) dan *L. mahajangana* (n=1) yang dikenal pasti secara morfologi telah disahkan berdasarkan jujukan Penjarak Transkripsi Dalaman (ITS), gen Faktor Pemanjangan Translasi 1-α (TEF1-α) dan gen β-tubulin. Identiti *Ne. dimidiatum* (n=26) dan *P. violaceum* (n=2) disahkan berdasarkan jujukan ITS dan TEF1-α. Pencilan yang dikenalpasti secara tentatif sebagai Botryosphaeriaceae telah dikenalpasti secara molekul sebagai *Neofusicoccum ribis* (n=55), *N. parvum* (n=12), *Botryosphaeria scharifii* (n=11), *B. dothidea* (n=6), dan *B.*

ramosa (n=1) berdasarkan jujukan ITS, TEF1-α dan β-tubulin. Analisis filogenetik menggunakan gabungan jujukan ITS, TEF1-α dan β-tubulin telah mengesahkan pencilan *Diaporthe* yang dikenalpasti secara morfologi sebagai *D. arecae* (n=9), *D. eugeniae* (n=13), *D. pascoei* (n=13), *D. perseae* (n=3), dan *D. ueckerae* (n=11). Jujukan dan analisis filogenetik menggunakan gabungan ITS, gliseraldehid-3-fosfat dehidrogenase (GAPDH) dan gen Aktin (ACT) telah mengesahkan identiti pencilan yang telah dikenalpasti secara morfologi sebagai *Colletotrichum asianum* (n=1) dan *C. siamense* (n=2). Spesies *Be. rhombica* yang dikenalpasti secara morfologi telah disahkan berdasarkan jujukan ITS dan analisis filogenetik. Pencilan kulat terpilih dari setiap spesies digunakan dalam ujian kepatogenan dan menunjukan kepatogen terhadap kultivar Chok Anan, Falan dan Waterlily luka kecuali *D. eugeniae* dan *Be. rhombica.* Pencilan *D. arecae*, *D. perseae* dan *N. ribis* didapati hanya patogenik terhadap kultivar rawatan luka yang tertentu. Hanya pencilan *Colletotrichum* (*C. asianum* dan *C. siamense*) dan *Neofusicoccum* (*N. parvum* dan *N. ribis*) didapati patogenik menggunakan rawatan tanpa luka. Hasil kajian ini menunjukkan bahawa terdapat beberapa spesies kulat yang berasosiasi dengan penyakit reput tangkai buah mangga dengan lapan spesies adalah pertama yang dilaporkan termasuk *L. mahajangana, B. ramosa, N. ribis, P. violaceum, D. arecae, D. pascoei, D. perseae,* dan *D. ueckerae.*

CHARACTERIZATION AND PATHOGENICITY OF FUNGI ASSOCIATED WITH STEM-END ROT OF MANGO FRUIT

ABSTRACT

Mango (*Mangifera indica* L.) is one of the popular fruit crops in Malaysia and stem-end rot is a serious post-harvest disease of mango fruits. The present study was conducted to determine the stem-end rot fungal pathogens. Mango fruits were collected from orchard, night market and supermarket at northern states of Peninsular Malaysia. Fungal isolates were isolated from lesion of stem-end rot from cultivars Chok Anan, Falan, Golek, Black Gold, Harumanis, Waterlily, Raja and Apple Mango. The fungal isolates were identified using morphological and molecular characteristics. Morphological characteristics were used to group the isolates into seven species of Botryosphaeriaceae (*Lasiodiplodia theobromae*, *L. pseudotheobromae*, *L. iranensis*, *L. mahajangana*, *Neoscytalidium dimidiatum* and *Pseudofusicoccum violaceum*), *Diaporthe* spp., two species of *Colletotrichum* (*C. asianum* and *C. siamense*) and *Beltrania rhombica* were identified. Morphologically identified *Lasiodiplodia theobromae* (n=149), *L. pseudotheobromae* (n=9), *L. iranensis* (n=5) and *L. mahajangana* (n=1) were confirmed based on Internal Transcribed Spacer (ITS) region, Translation Elongation Factor 1-α (TEF1-α) and β-tubulin sequences. The identity of *Ne. dimidiatum* (n=26) and *P. violaceum* (n=2) were confirmed based on ITS and TEF1-α sequences. Isolates that were tentatively identified as Botryosphaeriaceae were molecularly identified as *Neofusicoccum ribis* (n=55), *N. parvum* (n=12), *Botryosphaeria scharifii* (n=11), *B. dothidea* (n=6), and *B. ramosa* (n=1) based on ITS, TEF1-α and β-tubulin sequences. Phylogenetic analysis using combined sequences of ITS, TEF1-α and β-tubulin showed morphologically

identified *Diaporthe* isolates were phylogenetically identified as *D. arecae* (n=9), *D. eugeniae* (n=13), *D. pascoei* (n=13), *D. perseae* (n=3), and *D. ueckerae* (n=11). Sequence and phylogenetic analyses of ITS, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and Actin (ACT) genes confirmed the morphological identified *Colletotrichum asianum* (n=1) and *C. siamense* (n=2), whereas morphologically identified *Be. rhombica* (n=2) were confirmed using ITS sequences and phylogenetic analysis. Pathogenicity test of selected isolates from each species were pathogenic towards cultivars Chok Anan, Falan and Waterlily on wounded treatment except *D. eugeniae* and *Be. rhombica*. There were also isolates including *D. arecae*, *D. perseae* and *N. ribis* that only pathogenic on certain mango cultivars on wounded treatment. Only isolates of *Colletotrichum* (*C. asianum* and *C. siamense*) and *Neofusicoccum* (*N. parvum* and *N. ribis*) were pathogenic on unwounded treatment. In the present study, diverse fungal species were found to be associated with stem-end rot of mango with eight species were first reported including *L. mahajangana*, *B. ramosa*, *N. ribis*, *P. violaceum*, *D arecae*, *D. pascoei*, *D. perseae*, and *D. ueckerae*.

CHAPTER 1

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most cultivated fruit after durian (*Durio* sp.) and rambutan (*Nephelium lappaceum*) in Malaysia, mainly grown in Sabah, Sarawak, Perlis and other northern states of Peninsula Malaysia (Department of Agriculture, 2017). The availability of the large area of land, suitable soil condition, temperature and high precipitation contributed to mango cultivation in all these states (Nik Rozana et al., 2017). Although, the area of mango planting is increasing, the production of mango fruit had dropped over the years (Department of Agriculture, 2018). The possible reasons that caused the downfall of yield are poor maintenance and lack of fertilizer usage as well as watering in the orchard resulted in the spread of diseases (Department of Agriculture, 2016).

Mango orchard with poor maintenance has a higher risk to be infected by fungi and bacteria as well as insect infestation which lead to occurrence of diseases on various parts of mango plant. Post-harvest diseases primarily showed the symptoms after harvesting and this affect the quality as well as the marketability of the fruits. Stem-end rot is one of the main post-harvest diseases of mango fruits and can cause considerable losses, and thus, a threat to local and export market (Johnson et al., 1991b; Johnson et al., 1993). Stem-end rot symptoms start from the stem end as fruit ripens and spread systemically till the other end of the fruit within 2-3 days, makes the fruit inedible (Pathak & Srivastava, 1969). Infection of the fungal pathogen causing stem-end rot begins with endophytic colonization at flowering or branches and twigs of the tree, developed through pedicels and turned pathogenic as the fruit starts to ripen (Johnson et al., 1992; Hartill & Everett, 2002).

A few fungal genera are associated with stem-end rot of mango such as Botryosphaeriaceae species (*Lasiodiplodia theobromae*, *Botryosphaeria dothidea*, *Neofusicoccum parvum*, and *Neoscytalidium dimidiatum*) (Johnson et al., 1991a; 1991b; Marques et al., 2013a; 2013b), *Diaporthe* spp. (Johnson et al., 1993), *Cytosphaera mangiferae* (Johnson et al., 1992), *Alternaria alternata*, *Cladosporium cladosporioides*, and *Pestalotiopsis* sp. (Johnson et al., 1991b). However, in Malaysia, only *L. theobromae* ws reported as causal pathogen of mango stem-end rot (Lim & Khoo, 1985). In consideration of the number of species associated with mango diseases worldwide particularly stem-end rot (Johnson et al., 1991a; 1991b; 1992; 1993; Marques et al., 2013a; 2013b), it is very likely that several fungal genera or species would be associated with stem-end rot mango in Malaysia. Thus, the main aim of the present study was to identify the fungi associated with stem-end rot of mango as the correct identity of the fungal pathogen is important to find suitable or proper post-harvest disease management to extend the shelf-life of mango fruits.

Morphological identification mainly based on macroscopic and microscopic characteristics is a conventional method for identification of plant pathogenic fungi. Several characteristics such as colony appearance, presence of pycnidia or acervuli, conidial shapes and sizes are often used to identify the fungal isolate to genus or species levels (Humber, 1997). However, not many fungal genera can be identified solely based on morphological characteristics especially cryptic species or species within a species complex as they are closely related and the morphological characters are often overlapping or similar (Hebert et al., 2003; Phillips et al., 2013). Moreover, many species never produce spores or other microscopic structures during cultural studies (Jeewon et al., 2013). Therefore, molecular approach and phylogenetic analysis of certain gene or region are often applied for robust species identification.

The most common region used for molecular identification is ITS region as it is a barcode marker for identification of fungi and is widely used for preliminary identification (Schoch et al., 2012; Stielow et al., 2015). Species identification using only single marker such as ITS is known to be much more accurate and reliable than morphological identification. However, for some fungal genera such as *Diaporthe*, *Colletotrichum* and *Neofusicoccum*, ITS region is not sufficient for identification of many species within the genera as the occurrence of cryptic species or species complex. Therefore, protein-coding gene such as β-tubulin, TEF1-α and GAPDH are useful markers for molecular identification as well as for phylogenetic analysis (Pavlic et al., 2009a; 2009b; Udayanga et al., 2012; Weir et al., 2012). For robust phylogenetic analysis particularly for confirmation of species, multi-locus phylogenetic analysis of combined sequences are recommended as this approach increased the informative sites and provide significant outcome in species delineation (Rintoul et al., 2012).

The taxonomic status of species associated with stem-end rot of mango particularly Botryosphaeriaceae, *Diaporthe* spp. and *Colletotrichum* spp. was also revised using molecular methods and phylogenetic analsyis (Everett, 2014). Thus, this study was conducted in order to identify and characterize stem-end rot pathogens based on current species recognition concept.

After identification, pathogenicity test is conducted to determine the causal pathogen and virulence of the fungal isolates recovered from the stem-end rot lesion. Many stem-end rot pathogens are opportunistic pathogens of which the fungi live as endophytes within the host tissues without causing apparent symptoms and the infections initiated only when the physical and chemical defence of the host against the pathogens is weaken (Tang et al., 2003; de Souza-Pollo & de Goes, 2017). The

endophytic characteristics of these pathogens have made the disease widespread and destructive (Prakash & Srivastava, 1987). Moreover, some of the fungal isolates in the lesion may occur as saprophytes of which play no role in causing this destructive disease. Therefore, it is important to determine the causal pathogens of stem-end rot of mango, as often different species may require different control methods (Rossman & Palm-Hernández, 2008).

Thus, the objectives of the present study were:

1. To identify the fungi associated with stem-end rot of mango fruit using morphological and molecular identification.
2. To determine the pathogenicity and virulence of the isolated fungi on mango fruit.

CHAPTER 2

LITERATURE REVIEW

2.1 Mango (*Mangifera indica* L.)

Mango is one of the stone fruits belong to Anacardiaceae which is a family of flowering plants that include cashew tree and poison ivy. Mango tree is perennial, erect with a wide evergreen crown that can grow and attains a great height provided the soil has balanced nutrition. However, mango plant possesses a high tolerance even to barren zone or poor soil condition. The shape of the leaves is simple and alternate that are pinkish-orange in colours when immature and gradually turned to dark, glossy red or maroon, then dark green as they mature (Litz, 2009).

As a flowering plant, the flower is an important reproductive structure in the development of mango fruit. The inflorescence of mango is a branched terminal panicle or determinate thyrse. A high number of flowers formed the inflorescence with the flower inside are either staminate or hermaphroditic (Ramírez & Davenport, 2016). Both types of flower could exist in one inflorescence with variable proportion that differs among cultivars. Besides, the types of flower proportion may also different even between the individuals of the same cultivar due to the plant planted in different regions as well as the ages of the tree (Litz, 2009). Pollination is crucial for a mango tree to fruit-set and conducted by pollinators which are insects from the orders Coleoptera, Diptera, and Lepidoptera (Ramírez & Davenport, 2016).

Mango fruits are different in sizes and the shapes could be round, oval, ovoid-oblong, the colours which could be green, yellow, or red. The flavour has different extent of sweetness and the seed is flattened, enclosed in a woody husk. The differences depend on the cultivars, matureness of fruits, environmental conditions

and the state during postharvest storage (Othman & Mbogo, 2009). Based on botanical aspects, the fruit can be divided into three parts which are exocarp, the outermost part that functions as fruit's protection; mesocarp, the fleshy edible yellow or orange pulp and also endocarp which is the thick and tough covering of the seed (Tharanathan et al., 2006; Ornelas-Paz et al., 2007).

Mango fruit is renowned around the world for its delicious or sumptuous flavour and high in nutrition. The fruit is low in fat, rich in carbohydrate especially dietary fibre with abundant of vitamins A and C. It is also rich in different types of minerals such as potassium, magnesium, sodium, phosphorus, and sulphur, which are essential for human health (Lebrun et al., 2008). Mango is seasonal fruit with short shelf-life which can only last three to four weeks at the longest at 10 to 15ºC. Therefore, part of the mango production will normally be processed into various packaged or canned food (Ajila et al., 2010).

The wild mango species under the genus *Mangifera* was believed to occur in the dense tropical forests of South East Asia including Malaysia, Laos, Cambodia, and Vietnam (Litz, 2009). On the other hand, according to Yadav & Singh (2017), wild form of *Mangifera indica* and other allied species also occurred in southern Asia which is eastern India, Burma, and the Andaman Islands (Indo-Burma region). Despite the origin of the genus *Mangifera* is inconclusive, the commercial cultivars of mango was predominantly originated in India (Litz, 2009; Yadav & Singh, 2017). India is the major production of mango fruit in the world. The fruit is even known as the king of fruits in the Indian sub-continent (Asif *et al.*, 2002; Tharanathan et al., 2006). China is the second country after India in major mango production and exportation while other cultivation areas are Central and South America, Australia, South-east Asia, Egypt, Israel and also South Africa (Tharanathan et al., 2006).

2.2 Mango production in Malaysia

The tropical climate in Malaysia is suitable for various fruit crops cultivation and has an advantage in boosting the exportation of tropical fruits. Mango is one of the selected fruit crops to be develop to cater for the domestic as well as foreign markets (Ding & Darduri, 2013; Zakaria & Rahim, 2014).

The different cultivars of mango available in Malaysia are introduced mainly from India and Indonesia. Favourable cultivars include Chok Anan, Harumanis, Apple Mango, Golek and others which are around 209 clones of mango that have been registered by the Department of Agriculture (Mirghani et al., 2009). Cultivar Chok Anan is the most widely cultivated in Malaysia and also the main cultivar that exported to Singapore, Hong Kong and Brunei (Ding & Darduri, 2013). On the other hand, cultivar Harumnis predominantly planted in Perlis is exported to Japan (Musa et al., 2010).

In general, mangoes are planted mainly in Sabah, Sarawak, and northern states of Peninsular Malaysia such as Perlis, Kedah, Pulau Pinang and Perak with cultivated area increased from 2015 to 2017 with 5773 Ha to 6048 Ha respectively. However, mango production was decreased from 2015 to 2017 with 22672 Mt to 16913 Mt respectively (Department of Agriculture, 2018). The possible reason of decrease in mango production may due to the set-in of different types of diseases caused by different types of pathogens. Thus, effective management of fruit crops disease should be implemented as mango provides not only as source of fruit and nutrition but also livelihood to the farmers.

2.3 Post-harvest disease

The post-harvest disease is a disease that occurs after the fruit crops was harvested and disease symptoms will gradually emerge during storage period (Prusky & Lichter, 2007). Post-harvest diseases are common on fruit crops and the common causal pathogens are mostly ascomycetes. The fungal pathogens including *Colletotrichum* that causes anthracnose (Diao et al., 2017), *Botrytis* causes grey mold (Servili et al., 2017), *Penicillium* causes blue mold (Li et al., 2017), *Alternaria* causes Alternaria rot (Li et al., 2018), *Monilinia* causes brown rot (Bernat et al., 2017), *Aspergillus* causes black mold rot (Parveen et al., 2014), *Lasiodiplodia* (Marques et al., 2013a), and *Phomopsis* (Davidzon et al., 2010) cause stem-end rot on different kinds of stone fruits.

The causal pathogens of post-harvest disease could invade the host in the orchard through natural openings such as lenticels or physical wound openings before the formation of fruits. Another method is when conidia or spores landed directly on the fruit surface during growing season or prior harvest, then enters a period of dormancy and activated again during fruit ripening. Infections and disease can also occur together after harvest due to the conidia or spores of pathogenic fungi landed on the wounded surface that was produced mechanically during harvest or by insect injury (Barkai-Golan, 2001).

The fungal pathogen that present in plant host or spores that landed on fruits surface may or may not go through a latent stage. The probable reasons for a fungal pathogen to enter latent stage are due to sugar insufficiency that needed for development, and the ability of the pathogens to produce cell wall degrading enzymes was inhibited as well as the plants may produce antimicrobial substances. The pathogens will only commence their active stage when the resistance of the

fruits started to decrease as it is ripening or the environmental conditions are favourable such as the temperature is optimal and relative humidity is high (Barkai-Golan, 2001). Activated fungal pathogen invades the fruit endophytically through stem end or the germinated spores penetrate the fruit cuticle using infecting hyphae or appressoria (Barkai-Golan, 2001; Prusky & Lichter, 2007). The pathogens will then cause blemishes, disfigure or rotting on the fruits. It also leads to the maceration of tissues of the fruits and foul smells emitted, render the reduction of their acceptance level to customer as well as reduce the market value (Barkai-Golan, 2001).

2.4 Post-harvest diseases of mango

The incidence of black spot decay that caused by fungal pathogen on stored mango fruit can be high. The types of rotting or diseases can be diagnosed based on the signs and symptoms that present either internal or on the surface of the fruits. There are several types of mango fruit rots including stem-end rot, anthracnose and insidious fruit rot which are considered as major rotting diseases as these diseases are possible causes of major economic loses (Johnson et al., 1991b; Tarmizi et al., 1993; Dinh, 2002; Prabakar et al., 2005) while Alternaria rot, black mold rot, Rhizopus rot, grey mold and blue mold are regarded as minor rotting diseases as no major loses have been reported (Sangchote, 1987; Prusky et al., 2002; Prabakar et al., 2005; Palou, 2014).

2.4.1 Stem-end rot

Stem-end rot of mango rotting starts from the stem end as the fruit ripen. The pulp of the diseased fruits becomes brown, softer and watery as times goes by with the sign such as mycelium may appear in severe cases and rot completely within 2-4 days (Plates 2.1A & B) (Pathak & Srivastava, 1969; Lim & Khoo, 1985; Prakash & Srivastava, 1987; Johnson, 1993). Fruits with pedicels attached are less susceptible to infection than those without (Lim & Khoo, 1985). If the pedicel is attached on the fruit during the rotting process, the fruit may become dry instead of soft and watery. The pathogen may sporulate if the fruit is left under humid condition for a long period of time. The blackened fruit becomes mummified under arid condition (Pathak & Srivastava, 1969).

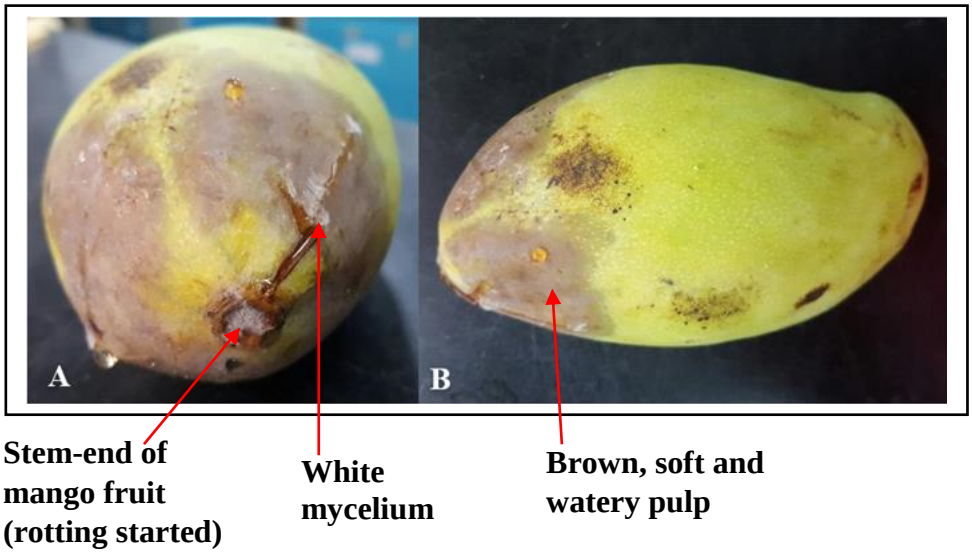

Plate 2.1: Symptoms of stem-end rot of mango fruit. **A:** Watery brown soft rot with the sign of white mycelium. **B:** Rotting starts from the stem end.

Stem-end rot is a serious post-harvest disease due to the causal pathogens which are mainly Botryosphaeriaceous fungi which tend to remain quiescent and cause no symptom on the surface of the fruit. The endophytic characteristics of these fungi can be seen after harvest or during storage (Johnson et al., 1991b; 1992; 1993). The symptoms that appeared affected the quality of the fruit during supply chain and caused serious loses of which the whole fruit rotted. There is no efficient control method available for stem-end rot although several attempts have been made to control the disease including radiation, fungicides spray and hot water treatment (Prakash & Srivastava, 1987; Gosbee et al., 1996; Terao et al., 2018).

2.4.1(a) Disease cycle of stem-end rot of mango

Stem-end rot pathogens invade mango tree through natural openings or physical wounds and caused primary infection (Figure 2.1A). The pathogens can also occur endophytically in mango inflorescence with the outgrowth of hyphae only limited to the vascular tissues (Figure 2.1B) (Gosbee et al., 1996). They become pathogenic earlier before fruit set causing pre-harvest symptoms such as canker or die-back of the mango trees (Figure 2.1C) provided the host is physiological or physical stressed. The residues of the infected plants such as dead twigs and bark could harbour the stem-end rot pathogens and served as primary inoculum (Figure 2.1D). The pathogens produce conidia on these dead panicles or leaves and the conidia spread through rain-splashed to other trees, leaves or flowers and cause secondary infection (Figure 2.1E) (Prakash & Srivastava, 1987; Johnson et al., 1992; 1993).

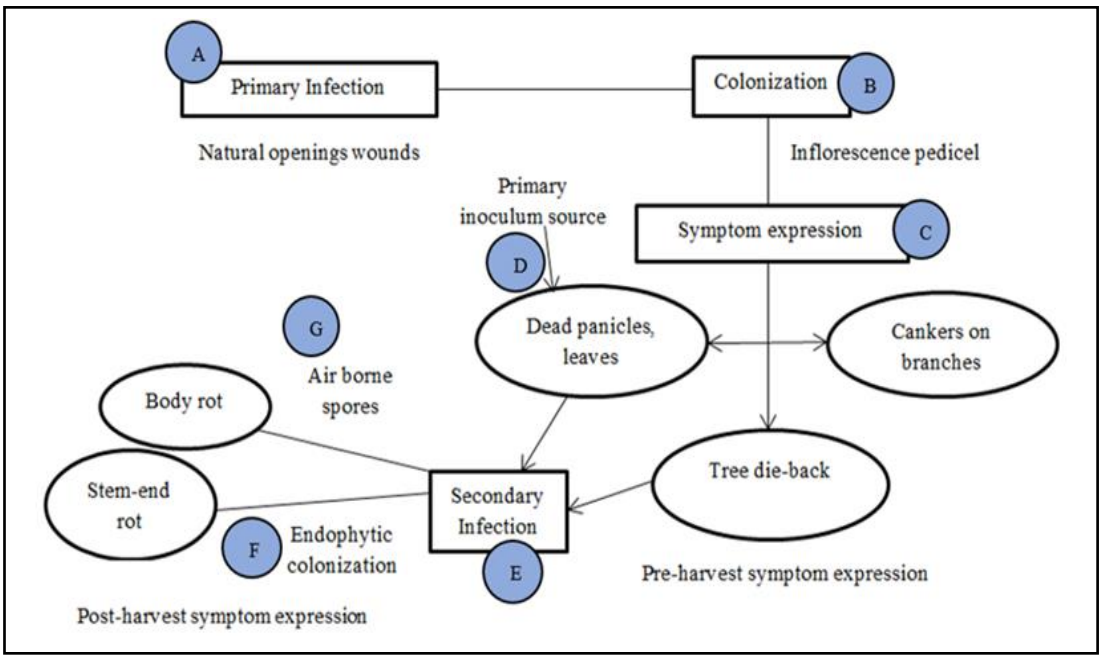

Figure 2.1: Disease cycle of the stem-end rot of mango. Source: Govender (2004)

In the case of post-harvest stem-end rot, the hyphae of the fungi colonize the pedicel will remain quiescent or dormant. The capability of the fungi in breaching the abscission zone barriers of the pedicel is the main factor causing stem-end rot development in ripening mango fruit (Johnson et al., 1992). The ripening of fruits results in the increase of sugar content and favoured the growth of the pathogen. The fungi change its lifestyle from endophyte to necrophyte (Davidzon et al., 2010). It becomes pathogenic and incites a soft rot by invade the fruit pedicel through cell wall degradation using the combination of several pectinolytic and cellulolytic enzymes (Prakash & Srivastava, 1987). The pathogens then infect the seed initially through funiculus, endocarp, testa then into the embryo where it started to decay, causing necrosis of fruits tissues and post-harvest symptom such as rotting start to emerge on the fruit stem end (Figure 2.1F) (Gosbee et al., 1996; Govender, 2004; Prusky et al., 2009).

The epiphytic growth of stem-end rot fungi is also possible when the air-borne conidia or spores landed on the fruits surface (Figure 2.1G) (Govender, 2004). Infection that occurred in this way is usually developed earlier than endophytic infections. However, infection by the epiphytic fungi did not occur through uninjured epicarp even when the fruits are fully ripe. Compared to epiphytes, the endophytic fungi grow through the hyphae, and has the advantage of infection and colonization as the endophyte growth did not hinder by other organisms or microbes (Johnson et al., 1992).

2.4.1(b) Causal pathogens of stem-end rot of mango

Various ascomycetes fungi from the family Botryosphaeriaceae and Diaporthaceae are known to be important causal agents of stem-end rot of mango (Ploetz, 2003; Krishnapillai & Wijeratnam, 2015). Different species of the pathogenic fungi from these two families may predominant in certain areas or locations due to the climate, weather or seasons and cause variable symptoms at the stem end (Diskin et al., 2017).

2.4.1(b)(i) Family Botryosphaeriaceae

Members of the family Botryosphaeriaceae are known to be cosmopolitan and exist as endophytes in many woody plants. They can become pathogenic and caused serious diseases ranging from dieback, canker, shoot blights, leaf spots, fruit and seed rot when their hosts are stressed (Phillips et al., 2013). Species from different genera of the family Botryosphaeriaceae which includes *Lasiodiplodia theobromae*, *Neofusicoccum parvum*, *Botryosphaeria dothidea*, and *Neoscytalidium*

dimidiatum have been reported as the causal agents of mango stem-end rot (Lim & Khoo, 1985; Slippers et al., 2005).

Cultures of Botryosphaeriaceae are usually simple to be recognized as the colonies is grey to black in colour on both sides of Petri dishes which are distinct from most of other fungi (Slippers et al., 2005; Phillips et al., 2013). Although genera characterization has mostly relied on the morphological features of the ascospores, which are large, ovoid to oblong, usually hyaline and aseptate, the conidial states are mainly being studied due to the asexual state is more common. Conidia from this family could be separate into two groups, namely those that are thin-walled, narrow and fusicoccum-like, and the thick-walled, wider, diplodia-like. The combination of morphological characteristics with sequence-based molecular data through phylogenetic analysis has contributed significantly to genera and species recognition. Thus, 17 genera have been recognized phylogenetically in the family Botryosphaeriaceae (Phillips et al., 2013).

2.4.1(b)(ii) Family Diaporthaceae

The family Diaporthaceae consists of many genera with the species of the genus *Diaporthe* are considered the most widespread endophyte present in both tropical and temperate woody plants (Mostert et al., 2001; Udayanga et al., 2012). Species of *Diaporthe* are mainly endophyte but can also exist as opportunistic pathogens on stressed hosts (Gomes et al., 2013). Pathogenic *Diaporthe* spp. have been reported as causal agents of branch canker, shoot blight, die-back, leaf spot, fruit rot and stem-end rot in some agricultural plants such as kiwi (Lee et al., 2001),

mango (Davidzon et al., 2010), fig (Ploetz, 2003), and olive (Úrbez-Torres et al., 2013).

The most common characteristics of this genus is the white mycelium with yellowish pigmentation developing in cultures and the production of fusiform α- as well as filiform β-conidia or the isolate may produce only one type of conidia (either α- or β-conidia) provided the species are not sterile. However, these characters are not useful in delineate species within *Diaporthe* (Gomes et al. 2013).

Due to the intrinsic sterile conditions in most of the species and highly similar morphological characteristics among different species, *Diaporthe* spp. have been previously identified based on host association which is inaccurate as many species turned out to have wide host range and one or more species or regarded as species complex could occur on a single host (Gomes et al. 2013). Several groups of important *Diaporthe* species complexes that associated with major field crops including soybean (*Glycine max*) (Santos et al., 2011), sunflower (*Helianthus annuus*) (Thompson et al., 2011), citrus (Huang et al. 2015), and grapes (*Vitis* spp.) (Baumgartner et al. 2013) have been studied. These studies showed that *Diaporthe* represents a highly complex genus containing numerous cryptic species, which could only be resolved with the application of combination of several markers such as ITS, CAL, HIS, TEF1-α, β-tubulin, Apn2 and FG1093 (Santos et al., 2017).

2.4.2 Anthracnose

Anthracnose is the most common rotting disease of mango fruit, mainly caused by *Colletotrichum* species in the *C. gloeosporioides* and *C. acutatum* species complexes. For *C. gloeosporioides* species complex, several species including *C. asianum, C. fructicola,* and *C. siamense* were reported as the causal agents of mango anthracnose in India (Sharma et al., 2013) and China (Mo et al., 2018). Anthracnose of mango in Brazil was associated with *C. asianum*, *C. fructicola*, *C. tropicale*, and *C. siamense* (Lima et al., 2013). *Colletorichum asianum* and *C. fructicola* were reported causing mango anthracnose in South Africa (Sharma et al., 2015) and Korea (Joa et al., 2016), respectively. Fitzell (1979) reported that *C. acutatum* causes anthracnose of mango in New South Wales, which was isolated from leaves, panicles and fruits.

Typical symptom of anthracnose is sunken dark-brown to black lesions with orange, salmon or pinkish conidial masses of acervuli, often formed in concentric rings on the surface of infected fruits, and the lesions are usually restricted to the peel. Appearance of sunken lesions on the fruit surface not only reduce the quality of fruit but also affect its market value (Lim & Khoo, 1985; Prakash & Srivastava, 1987).

Conidia produced by *Colletotrichum* spp. could remain on dead panicles, leaves, and mummified fruits served as primary inoculum and spread through rain-splashed to other leaves or flowers, causes secondary infection. The pathogens remained quiescent on infected fruit until the onset of ripening with symptom started to appear. The incidence of anthracnose can reach 100% under very humid condition as high relative humidity encourage conidial germination and appressorium formation (Akem, 2006).

2.4.3 Insidious fruit rot

Insidious fruit rot is considered as physiological disorder as no specific causal pathogens have been identified. Although several species of yeast and bacteria were found from the infected tissues, no single microorganism was consistently isolated (Lim & Khoo, 1985). According to Tarmizi et al. (1993), the causal reason of the rot is over fertilization, high nitrogen with low calcium levels of the tree. High concentration of nitrogen may result in excessive enzymes production such as hydrolases, α-amylases, and cellulase, causing starch molecules breakdown, cell wall softening, and deterioration of the fruit (Raymond et al., 1998).

The typical symptom of insidious fruit rot is the disintegration of mesocarp tissues. The tissues become watery, soft, and yellowish brown with yeasty odour (Tarmizi et al., 1993). This physiological disorder is dangerous as the symptoms appeared on the fruit surfaces after a long period of time. The lack of firmness of the fruit may be an indication of insidious fruit rot but can only be confirmed after the fruit are sliced open or by using non-destructive x-ray imaging technique (Hiller & Thornton, 1993). The incidence of insidious fruit rot can reach up to 80% under severe conditions with larger size of fruits are more easily affected (Tarmizi et al., 1993).

2.4.4 Other mango fruit rot diseases

2.4.4(a) Alternaria rot

Alternaria rot is caused by *Alternaria* spp., mainly *Alternaria alternata* and can only become serious when the fruits are kept in storage for a long period (Prakash & Srivastava, 1987; Meurant & Kernot, 1999). The capability of *Alternaria* spp. to produce mycotoxin play an important role of its pathogenicity on plants (Logrieco et al., 2009). This pathogen could infect the fruit during growing season and then remain quiescent or attack the fruit surfaces and remain latent in the fruit peel during fruit development (Li et al., 2007). The symptoms are black spots and rotting appeared on the fruit surface (Prakash & Srivastava, 1987; Meurant & Kernot, 1999).

2.4.4(b) Black mold rot

Black mold rot is caused by *Aspergillus* spp. and the most common causal pathogen is *Aspergillus niger* (Prakash & Srivastava, 1987; Meurant & Kernot, 1999). This species is an important cosmopolitan fungus and can cause rotting of the fruits due to its capability in producing a wide array of hydrolytic and oxidative enzymes. Infection of the pathogen mostly started at the stem end of the fruit (Gautam et al., 2011). The pathogen can also infect the fruit through wounded surface. The symptoms are black spots on the fruits surface with radiated black conidial head. The infected fruits skin may become wrinkle (Sangchote, 1987).

2.4.4(c) Rhizopus rot

Rhizopus rot is mainly caused by *Rhizopus stolonifer* and *R. nigricans* (Rathod, 2010). *Rhizopus* spp. are able to produce mycotoxin and causing serious fruits rot. The fruit appeared soft and water-soaked decay with grey striped mycelia. The pathogens normally infect the fruit in the field and cause latent infection (Zhou et al., 2018). Rhizopus rot can caused considerable losses, as the disease affects fruit quality, shortens fruits shelf life as well as off-flavour which decrease fruits marketability.

2.4.4(d) Grey mold

Botrytis cinerea is the causal pathogen of grey mold of fruits. This pathogen is necrotrophy and causing serious fruit rot by inducing host cell death. Symptoms of grey mold are extensive white to grey velvety mycelium growing on the fruits surface and fruit rotting (Aktaruzzaman et al., 2018). The optimal growth, sporulation, spore release, germination and infection of *Botrytis cinerea* are favoured under cool storage condition such as at 13-20ºC. The infection route of the pathogen is through the wounded fruits surface and symptom only developed during fruit storage and transportation (Meurant & Kernot, 1999; Aktaruzzaman et al., 2018).

2.4.4(e) Blue mold

Blue mold is mostly caused by *Penicillium expansum* and *P. italicum.* The infection occur through wounded surface and long storage fruits are more susceptible (Zhang et al., 2018). Symptom of blue mold is blue central sporulation area surrounded by very narrow band of non-sporulation white mycelium on the surface of the infected fruits. The fruits surface will then completely covered with spores and the infection area become soft and water-soaked (Palou, 2014). Secondary spread is possible if the infected fruits are packed together with healthy fruits during storage period (Meurant & Kernot, 1999).

2.5 Disease management of fruit rot disease of mango

Disease management for different types of fruit rot diseases of mango are similar and integrated disease management is usually adopted. Integrated plant disease management involved multiple strategies to maximize the production of fruit crops by minimizing the hazard from potential pathogens (Villa et al., 2017).

First step in integrated plant disease management of mango fruit rot diseases started by selection of planting location. For example, a location that is not infected by fungal pathogens and previously not planted with similar crop. Cultivation of scion wood that free from infection is also important in reducing disease incidence (Prakash & Srivastava, 1987). Cultivars that are more resistance to fruit rot diseases should be selected (Nelson, 2008). For instance, mango cultivars such as Kaew, Chok Anan and Rad are more resistance to anthracnose infection (Dinh, 2002).

Sanitation in mango orchard is important to prevent the accumulation of primary inoculum by removing dead twigs and branches in the orchard. Infected

dead twigs and branches are destroyed using incinerator to eradicate any possible fungal survival structures such as sclerotia which could survive under adverse condition (Johnson et al., 1991a; Akem, 2006). The tools used for pruning are disinfested before moving to new trees to avoid spreading of any pathogens from tree to tree. Pruned trees are then sprayed with fungicide, such as benomyl to protect pruning cuts from becoming infected by fungi (Prakash & Srivastava, 1987).

The flowers and developing mango fruits are protected using protectants fungicides such as copper and mancozed as well as prochloraz or copper oxychloride during rainy season when the trees stay wet for long hours (Silimela & Korsten, 2007; Johnson, 2008). Fungicides spray on fruits is essential in every 10 to 14 days as mango fruit is susceptible to fruit-spotting or fruit-rotting fungi (Johnson, 2008).

Good and careful fruit harvesting, and handling are required to prevent wounded skin. Postharvest loses are mostly occurred during transportation and rough handling of fruits (Barbosa-Cánovas, 2003). Clean and disinfected crates are used for fruit packaging and stored under low temperature (around 10ºC) (Barbosa-Cánovas, 2003; Nelson, 2008). Storage temperature not lower than 10ºC before the fruit ripen to avoid chilling injury (Nelson, 2008).

Different control measures are implemented after the fruits are harvested to extend the fruits shelf life. Post-harvest controls of mango fruits include thermal treatment, chemical treatment, or the combination of both. In thermal treatment, harvested fruits are dip in hot water with the temperature that is not too hot to affect the fruits quality but hot enough to suppress or kill the fungal pathogens on the fruit surface. The hot water temperature applied on mango fruit is around 52-55ºC. This treatment has its own advantage as there is no chemical residues left on the fruits

surface (Alvindia & Acda, 2015). The combination of hot water and fungicides treatments have also been practiced in order to improve the efficacy of each treatment. Hot water with carbendazim are used to control anthracnose and stem-end rot (Johnson, 2008).

2.6 Identification of plant pathogenic fungi

Accurate identification of the causal pathogens of a disease is important to construct an effective strategy of disease management. The common methods used for identification of fungi are morphological and molecular techniques. However, each of the method has its own advantages and limitations (Badotti et al., 2018).

2.6.1 Morphological identification

Morphological identification is an identification method based on fungal phenotypes features mainly their macroscopic and microscopic characteristics. Specific features that constantly produced by certain species especially the presence of essential microscopic characters is usually required to give a general idea about the identity of the species or the genus (Humber, 1997).

Macroscopic characteristics including colony appearance, for example colony colours, reverse colony colours, colony diameter; mycelium texture (fluffy, velvety, downy, and cottony); fruiting body including ascocarp and basidiocarp; and conidiomata such as acervuli, pycnidia, sporodochia, and synnemta (Ownley & Trigiano, 2016). Based on the macroscopic characteristics, some species can be identified from family until genus levels. For example, species from the family Botryosphaeriaceae have grey or black upper and reverse colonies, fully grown on

Petri dishes within several days, cottony (*Lasiodiplodia* spp., *Neofusicoccum* spp. and *Botryosphaeria* spp.) or velvety (*Neosyctalidium* spp.) mycelia texture, with the production of pycnidia (Phillips et al., 2013).

Microscopic features that are used for identification are shape, size, colour, and septation of different spore types such as asexual (conidia) or sexual (ascospores, basidiospores, zygospores) spores, structure that produce or bear the spores such as conidiogenous cells, conidiophore, ascus, basidium, and zygosporangium; and other structures that present among the reproductive organs such as paraphyses that are examined under a compound microscope (Ownley & Trigiano, 2016).

Although sexual stage is giving priority for species characterization and identification, the production of conidia or asexual stage is more common among many species and are studied extensively. Based on the shape of the conidia, the genus or identity of a species would be known. For example, *Lasiodiplodia* spp. and *Neosyctalidium* spp. produced conidia that are diplodia-like, ovoid or sub-ovoid, while *Neofusicoccum* spp. and *Botryosphaeria* spp. produced fusicoccum-like, fusoid or spindle-shaped conidia (Phillips et al., 2013).

Species identification through morphological method is fast, effective and reliable, however there are limitations as the morphological features might change if the fungal isolates are cultured under different environmental conditions (Raja et al., 2017). Moreover, the sizes and shapes of the conidia of some closely related species are highly similar or always overlap. For instance, both *Neofusicoccum* spp. and *Botryosphaeria* spp. produced fusiform or spindle-shaped conidia with sizes that mostly less than 30 µm long which could not be differentiated morphologically (Phillips et al., 2013). For *Diporthe* spp., conidia and other microscopic structures are

rarely produced which make the species in this genus can only be identified to genus level (Udayanga et al., 2012).

The presence of cryptic species and species complex limit the use of morphological identification. A species complex is an individual member that considered as one species but in fact, contain a group of closely related isolates which may represent different species (Fegan & Prior, 2005). The closely related isolates are also known as cryptic species that are morphologically indistinguishable and can only be differentiated using molecular method and phylogenetic analysis (Bickford et al., 2007). For instance, five cryptic species within *N. ribis*/*N. parvum* species complex were previously identified as *N. parvum* (Phillips et al., 2013). Through multiocus phylogenetic analysis, the five cryptic species were described and identified as separate or individual species (Pavlic et al, 2008).

2.6.2 Molecular identification

The DNA data for fungal identification and detection of plant pathogens is regarded as highly specific by using suitable markers. One of the most common method used is sequencing of PCR products which is based on specific regions or genes as markers. The acquired sequences of the region or gene were then compared with other fungal sequences in a database for identification.

Suitable regions or genes used for identification must fulfil several criteria including easy to amplify, adequate species resolution and contains highly conserved area for each specific species or very little variable at intraspecific level while at the same time variable enough to allow discrimination at interspecific level (Badotti et al., 2018). Internal Transcribed Spacer (ITS) region is the most suitable region and is