CHARACTERIZATION, PATHOGENICITY AND CHEMICAL CONTROL OF *Colletotrichum* spp. ASSOCIATED WITH CHILLI (*Capsicum annuum* AND *C. frutescens*) ANTHRACNOSE IN PENINSULAR MALAYSIA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABREVIATIONS

ACT	Actin
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
β-tubulin	Beta tubulin
CAL	Calmodulin
CHS-1	Chitin Synthase 1
CYA	Czapek Yeast Extract Agar
dNTP	Deoxyribonucleotide Triphosphate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GS	Glutamine Synthetase
ISSR	Inter Simple Sequence Repeat
HIS3	Histone 3
ITS	Internal Transcribed Spacer
$MgCl_2$	Magnesium chloride
ML	Maximum Likelihood
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
PDA	Potato Dextrose Agar
ppm	Parts Per Million
RAPD	Random Amplified Polymorphism DNA
rRNA	Ribosomal Ribonucleic Acid
RFLP	Restriction Fragment Length Polymorphism
TBE	Tris-Borate-EDTA
WA	Water Agar

LIST OF SYMBOLS

°C	Degree Celsius

PENCIRIAN, KEPATOGENAN DAN KAWALAN KIMIA *Colletotrichum* spp. YANG BERASOSIASI DENGAN PENYAKIT ANTRAKNOS CILI (*Capsicum annuum* DAN *C. frutescens*) DI SEMENANJUNG MALAYSIA

ABSTRAK

Penyakit reput buah antraknos disebabkan oleh *Colletotrichum* spp. merupakan penyakit lepas tuai yang serius menyebabkan kerugian cili di Semenanjung Malaysia. Untuk mengenalpasti patogen penyebab, banyak kajian hanya berdasarkan ciri-ciri morfologi dimana tidak mencukupi kerana banyak spesies *Colletotrichum* tergolong dalam kompleks spesies. Pencirian secara molekul berdasarkan satu gen sahaja juga tidak mencukupi terutamanya untuk membezakan spesies yang mempunyai hubungan yang rapat. Pengenalpastian yang betul spesies *Colletotrichum* yang berasosiasi dengan antraknos adalah penting untuk menyusun strategi kaedah kawalan penyakit yang sesuai dan untuk tujuan kuarantin. Dalam kajian ini, pencirian secara morfologi dan molekul, serta kepatogenan pencilan *Colletotrichum* dari antraknos cili telah dinilai. Keberkesanan racun kulat sistemik, iaitu benomil dan difenoconazol, dan racun kulat sentuh, mankozeb dan propineb dinilai menggunakan teknik racun makanan dan resapan agar disk untuk merencat pertumbuhan miselium pencilan *Colletotrichum*. Sejumlah 130 pencilan *Colletotrichum* diperoleh daripada antraknos cili hijau dan cili merah *C. annuum* (cili besar) dan *C. frutescens* (cili padi). Berdasarkan ciri ciri morfologi yang utama seperti warna koloni dan bentuk konidia, pencilan diperoleh dikenal pasti sebagai *C. gloeosporioides* (14 pencilan), *C. acutatum* (62 pencilan) and *C. truncatum* (54 pencilan). Pengenalpastian secara molekul dan analisis filogenetik adalah berdasarkan

kawasan Penjarak Transkripsi Dalaman, gen β-tubulin, gen Aktin dan gen gliseraldehid 3-fosfat dehidrogenase. Berdasarkan pencarian BLAST, pencilan yang dikenal pasti secara morfologi sebagai *C. gloeosporioides* telah dikenal pasti semula sebagai *C. fructicola* (tiga pencilan) dan *C. siamense* (11 pencilan), dan pencilan yang dikenal pasti secara morfologi sebagai *C. acutatum* dikenal pasti semula sebagai *C. scovillei* (58 pencilan) dan *C. fioriniae* (empat pencilan). Identiti *C. truncatum* yang dikenal pasti secara morfologi telah disahkan sebagai *C. truncatum*. Pohon filogenetik yang dijana berdasarkan pohon Kebolehjadian Maksimum dan pohon Penyambung- Jiran menunjukkan bahawa pencilan yang dikenal pasti secara molekul bagi spesies yang sama berkelompok dengan strain epitip setiap spesies, mengesahkan lebih lanjut identiti pencilan antraknos cili. Ujian kepatogenan menunjukkan 50 pencilan yang dipilih dari setiap spesies adalah patogenik terhadap *C. annuum* dan C. *frutescens*. Hanya beberapa pencilan *C. truncatum* dan *C. scovillei* didapati patogenik menggunakan rawatan tanpa luka. Ujian racun kulat menunjukkan benomil dan difenoconazol paling efektif untuk merencat pertumbuhan miselia *C. siamense*, *C. fructicola*, *C. scovillei* dan *C. fioriniae.* Difenoconazol hanya efektif terhadap *C. truncatum*. Kajian ini menunjukkan lima species *Colletotrichum*; *C. siamense*, *C. fructicola*, *C. scovillei*, *C. fioriniae* dan *C. truncatum* berasosiasi dengan antraknos cili. Kejadian lima *Colletotrichum* spp. berasosiasi dengan antraknos cili menunjukkan bahawa nama spesies yang betul adalah penting bukan sahaja untuk merangka pengurusan penyakit yang efektif tetapi juga untuk merangka dasar kuarantin yang berkesan.

CHARACTERIZATION, PATHOGENICITY AND CHEMICAL CONTROL OF *Colletotrichum* spp. ASSOCIATED WITH CHILLI (*Capsicum annuum* AND *C. frutescens*) ANTHRACNOSE IN PENINSULAR MALAYSIA

ABSTRACT

Anthracnose fruit rot caused by *Colletotrichum* spp. is a serious post-harvest disease causing losses of chilli in Peninsular Malaysia. For identification of the causal pathogen, many studies were only based on morphological characteristics which are not sufficient as many *Colletotrichum* spp. belongs to a species complex. Molecular characterization based on a single gene is also not sufficient especially to differentiate closely related species. Correct identification of *Colletotrichum* spp. associated with anthracnose is important to strategize suitable disease control methods and for quarantine purposes. In this study, morphological and molecular characteristics as well as pathogenicity of *Colletotrichum* isolates from chilli anthracnose were evaluated. The effectiveness of systemic fungicides, namely benomyl and difenoconazole and contact fungicides, mancozeb and propineb were assessed using poison food and agar disc diffusion techniques to inhibit mycelial growth of *Colletotrichum* isolates. One hundred and thirty isolates of *Colletotrichum* spp. were isolated from anthracnose of green and red cayenne pepper (*C. annuum*) and green and red bird's eye chillies (*C. frutescens*). Based on the main morphological characters such as colony colour and conidial shape, the isolates were identified as *C. gloeosporioides* (14 isolates), *C. acutatum* (62 isolates) and *C. truncatum* (54 isolates). Molecular identification and phylogenetic analysis were based on Internal Transcribed Spacer regions, β-tubulin, Actin and Glyceraldehyde-3-

Phosphate Dehydrogenase genes. Based on BLAST search, the morphologically identified *C. gloeosporioides* isolates were re-identified as *C. fructicola* (three isolates) and *C. siamense* (11 isolates), and morphologically identified *C. acutatum* isolates as *C. scovillei* (58 isolates) and *C. fioriniae* (four isolates). The identity of morphologically identified *C. truncatum* was confirmed as *C. truncatum*. Phylogenetic trees generated based on Maximum Likelihood and Neighbour Joining methods showed that molecularly identified isolates of the same species were grouped with the epitype strains of each species which further confirm the identity of the isolates from chilli anthracnose. Pathogenicity test showed that 50 selected isolates from each species were pathogenic towards *C. annuum* and *C. frutescens* on wounded treatment. Only a few isolates of *C. truncatum* and *C. scovillei* were found to be pathogenic using unwounded treatment. Fungicide testing showed that benomyl and difenoconazole were the most effective fungicides to inhibit mycelial growth of *C. siamense*, *C. fructicola*, *C. scovillei* and *C. fioriniae.* Difenoconazole was only effective against *C. truncatum*. The present study showed that five species of *Colletotrichum*; *C. siamense*, *C. fructicola*, *C. scovillei*, *C. fioriniae* and *C. truncatum* were associated with chilli anthracnose. The occurrence of five *Colletotrichum* spp. associated with chilli anthracnose indicates that correct species name is important not only to formulate effective disease management but also to formulate effective quarantine policy.

CHAPTER 1

GENERAL INTRODUCTION

Chilli (*Capsicum* spp.) is one of the most valuable cash crops cultivated mostly in tropical and sub-tropical countries such as China, Indonesia, Thailand and Malaysia (Hussain and Abid, 2011). Chilli fruit which is valued for its aroma, taste, colour and pungency play an important role in culinary preparations, food industry and formulation of pharmaceutical products. In Malaysia, the demand for fresh chillies can reach up to 50,000 tonnes per year which is exceeding the production (Awang et al., 2013). Chilli production in Malaysia can be considered as very low and to meet the demand, Malaysia needs to import chilli fruits from other countries (Jalili and Jinap, 2012). One of the major constrains of chilli production in Malaysia and other producing countries is anthracnose disease that can reduced more than 50% of total yield (Pandey and Gupta, 2015).

Anthracnose disease is widely occurring in chilli growing areas affecting the crop at pre and postharvest stages. Infection commonly occurs on mature and ripe fruits (Saini et al., 2016). Typical anthracnose symptoms on chilli fruits are blackened and sunken lesions with concentric rings of acervuli and severe infection will lead to fruit rot or decay (Susheela, 2013). Small lesions or blemishes on chilli fruits can deteriorate the market value and thus reducing profit.

Chilli anthracnose or fruit rot is caused by fungal species in the genus *Colletotrichum*. Three *Colletotrichum* species namely, *C. gloeosporioides*, *C. truncatum* and *C. acutatum* are commonly associated with chilli anthracnose (Than et al., 2008b). In Malaysia, the most common species associated with chilli anthracnose are *C. truncatum* and *C. gloeosporioides* (Mahmodi et al., 2014; Yun et al., 2009). Recently, a few new species of *Colletotrichum* have been found to be the causal agent of chilli anthracnose in Australia (De Silva et al., 2016) and China (Diao et al., 2016b). As more than one species of *Colletotrichum* are associated with chilli anthracnose, correct identification of the causal pathogen is important particularly to formulate effective disease management to control the disease.

Morphological identification based on macroscopic and microscopic characteristics is still widely used to identify and characterize *Colletotrichum* spp. However, several morphological characters such as cultural characters and conidia are not always adequate for reliable species differentiation due to environmental conditions which can lead to inconsistent or variable characteristics (Hyde et al., 2009a). Moreover, for *Colletotrichum* spp., a few species complexes have been reported (Cai et al., 2009). Species in a species complex can only accurately identify using molecular method particularly DNA sequencing of specific gene or region. To overcome the inadequacies of morphological identification, a combination of morphological and molecular methods is a good and reliable approaches to study *Colletotrichum* spp. especially species in a species complex.

For molecular identification of *Colletotrichum* spp., more than one gene is usually applied as many species of *Colletotrichum* belong to a species complex. Internal Transcribed Spacer regions (ITS) is widely used and very useful for preliminary identification of *Colletotrichum* species as well as to place a species in a species complex (Than et al., 2008a). Common species complex of *Colletotrichum* are *C. gloeosporioides* species complex and *C. acutatum* species complex. In addition to ITS regions, protein coding genes, namely β-tubulin, Actin (ACT) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) are employed for further confirmation of the species (Damm et al., 2012; Weir et al., 2012). After molecular identification using several genes, phylogenetic analysis is applied to differentiate species in a species complex and to study the relationship among the isolates. Currently, phylogenetic analysis based on multiple genes is recommended to understand and to determine phylogeny and systematics of *Colletotrichum* species (Cai et al., 2009; Hyde et al., 2009a).

After identification, pathogenicity test is conducted to verify the identified fungal isolate as causal pathogen of chilli anthracnose as well as to determine pathogenic variability of the isolates. Pathogenicity test is also important to determine the level of virulence of each isolate of the *Colletotrichum* species.

Chemical control method using fungicide is considered as the most practical method for fast and effective control of anthracnose (Oo and Oh, 2016). Fungicide can destroy the fungal pathogen or inhibit the growth of mycelium and spores. Selection of appropriate fungicide is important to maximize the effectiveness of the products. Therefore, *in vitro* fungicide testing is carried out to determine the

efficacy of systemic and contact fungicides to inhibit mycelial growth of *Colletotrichum* spp. causing chilli anthracnose.

In Malaysia, although there are reports on chilli anthracnose, most of the work was done in late 1980's (Mah, 1987; Nik et al., 1988; Sariah, 1989) which relied heavily on morphological characteristics for species identification and only used ITS sequences for molecular identification which is not sufficient to distinguish closely related species especially species in a species complex. Currently, more species within *C. gloeosporioides* species complex and *C. acutatum* species complex have been identified to be associated with chilli anthracnose in Australia and China (De Silva et al., 2016; Diao et al., 2016b). Hence, in Malaysia, there could be other *Colletotrichum* species besides *C. truncatum* and *C. gloeosporioides* associated with chilli anthracnose. Moreover, many species within *C. gloeosporioides* species complex and *C. acutatum* species complex have been discovered or re-identified using multiple genes. Correct identification of *Colletotrichum* spp. causing chilli anthracnose is important to formulate effective disease management strategy as many *Colletotrichum* spp. can cause infection on a wide host range. Therefore, the objectives of the present study were:

1) To identify *Colletotrichum* spp. isolated from chilli anthracnose (*Capsicum annuum* and *C. frutescens*) in Peninsular Malaysia using morphological and molecular characteristics.
2) To determine phylogenetic relationship of the *Colletotrichum* species using ITS region, β-tubulin, ACT and GAPDH genes.
3) To determine the pathogenicity and level of virulence of *Colletotrichum* isolates on *C. annuum* and *C. frutescens*.

4) To assess the effectiveness of systemic fungicide (benomyl and difenoconazole) and contact fungicide (mancozeb and propineb) *in vitro* to control mycelial growth of *Colletotrichum* spp. causing chilli anthracnose.

CHAPTER 2

LITERATURE REVIEW

2.1 Chilli

2.1.1 Origin and Taxonomy

Chilli or pepper is native to South and Central America, including Southern Mexico and was introduced in South Asia in the late 15th century (Huq and Arshad, 2010). Christopher Columbus introduced chilli to Europe and subsequently to Africa and Asia, and by the end of 17th century, chili has become the most important vegetable fruit and spice worldwide (Bosland et al., 2012).

Chilli is annual or perennial herbs, belongs to the highly diverse family "Solanaceae" and genus "*Capsicum*" (Jagtap et al., 2012). The family Solanaceae consist of at least 98 genera including *Solanum* (potato and tomato), *Nicotian*a (tobacco) and *Petunia* (Bosland et al., 2012). *Capsicum* differed from other genus by the production of capsaicin, the active compounds that cause the heat sensation in chilli fruit (Eshbaugh, 2012).

The taxonomic classification of chilli is listed below (National Center for Biotechnology Information, 2017a):

Kingdom: Plantae

Subkingdom : Viridiplantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Solanales

Family: Solanaceae.

Genus: *Capsicum*

2.1.2 Domesticated chilli

There are approximately 25 to 27 species of *Capsicum* but only five species are considered as domesticated plants which are *Capsicum annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L. and *C. pubescens* Ruiz. & Pav. (Makari et al., 2009). Among the five species of domesticated chillies, *C. annuum* and *C. frutescens* are the most widely cultivated all over the world with majority of the productions in tropical and sub-tropical developing countries (Walsh, 2001). There are many varieties of *C. annuum* including cayenne pepper, bell pepper and jalapeno (Boning, 2010).

Cayenne pepper is considered as medium hot pepper and extensively used as main spices in Indian and Southeast Asian cuisines. The fruits of cayenne pepper are long, tapered and the colours varied from green to red at ripening stage (**Plate 2.1A**) (Bosland et al., 2012). Bell pepper or sweet pepper is considered as pepper with subtle hotness (less pungency). The bell pepper fruits can turn from green to red, purple, yellow and orange when they are fully ripen (**Plate 2.1B**) (Marin et al.,

2004). Jalapeno pepper fruit is cylindrical, medium size with mild to medium pungency and usually consumed while it is still green (**Plate 2.1C**) (Boning, 2010).

Plate 2.1: Different cultivars of *C. annuum*. (A) Cayenne pepper. (B) Bell pepper. (C) Jalapeno. (Source: Feiertag, 2014).

Capsicum frutescens has a small size fruit with a very high pungency (Chatterjee, 2012). Some of well-known cultivar of *C. frutescens* are piri piri (African pepper) (**Plate 2.2A**), bird's eye chili (Thai pepper) (**Plate 2.2B**) and tabasco (Mexican pepper) (**Plate 2.2C**) (Russo, 2012).

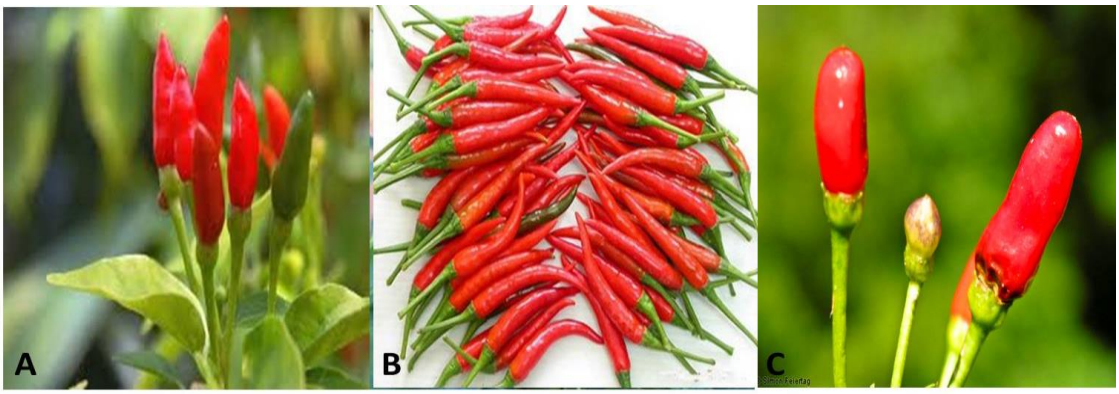

Plate 2.2: Different cultivars of *C. frutescens*. (A) Piri piri (African pepper). (B) Bird's eye chilli (Thai pepper). (C) Tabasco (Mexican pepper). (Sources: Feiertag, 2014; Ross, 2015).

Capsicum chinense or habanero pepper has been recorded as the hottest chili in the world (Canto-Flick, 2008). Habanero pepper fruits varied in shape with elongated bonnet or bell shape, but some are small and rounded (**Plate 2.3A**). *Capsicum baccatum* or aji pepper is one of the most cultivated species of chilli in Peru. The fruits of aji pepper are usually elongated and globose and the colour varied from brown, red, orange or yellow (**Plate 2.3B**) (Jarret, 2007). *Capsicum pubescens* or manzano pepper is hot pepper commonly used as salsa sauce or eating raw as vegetable condiment. This species has unique fruit characteristics in which the fruits resembling apple or pear shapes (**Plate 2.3C**) (Perez-Grajales et al., 2004).

Plate 2.3: Various types of fruit produced by different species of chilli. (A) *Capsicum chinense*; Habanero pepper. (B) *Capsicum baccatum*; Aji pepper. (C*) Capsicum pubescens*; Manzano pepper. (Source: Feiertag, 2014).

2.1.3 Uses and nutritional values

Chilli fruits are important vegetables consumed as fresh or as a main spice in food preparations. The fruits are also processes into a paste or flavouring agent and colorant. Capsaicin is important chemical compounds belong to capsaicinoids that responsible for heat sensation or pungent flavour of chilli fruits (Gurung et al., 2011). Consumption of capsaicin provides many benefits to human and this

substance has widespread application in pharmaceutical. According to Ludy et al. (2011), capsaicin is useful as natural herbal supplements to assist in weight loss since consumption of this substance will increase energy expenditure in human body and enhance fat oxidation, a process to break down lipid molecules into energy. Capsaicin extracts has been extensively used by many pharmaceutical company to produce topical cream that effective for pain relief of arthritis, peripheral neuropathies (artery diseases) and muscle strain (Singletary, 2011).

Chilli contain high levels of phenolic compounds or flavonoids that rich in antioxidant activities (Loizzo et al., 2015). The antioxidant properties of this compound can protect human bodies against free radical and thus, reduced risk of cancer (Carvalho et al., 2014). Flavonoids in chilli were also found to have anti-inflammatory activities in which the compound can reduce histamine release, a chemical that cause irritation and inflammation during allergic reactions (Rathee et al., 2009).

Different colours of chilli fruits especially during ripening stage are influence by different pigments produced by carotenoids. Lutein is the main carotenoid in green and yellow chillies, and β-carotene is abundant in orange chilli. Capsanthin and capsorubin are the most important carotenoid that produced red pigment in chilli (Schweiggert et al., 2007). Carotenoids, rich in antioxidant activity and provitamin A properties have many benefits to human health such as prevention of cardiovascular diseases, cancer, aging and eye disorders (Giuffrida et al., 2013). Chilli has high Vitamin C or Ascorbic acid content which is very important in human diet, that

promote growth and tissues repairing, as well as maintaining collagen production and act as antioxidant (Yahia et al., 2001).

2.1.4 Production and cultivation

Approximately 1.7 million hectares of land worldwide is cultivated with chilli (Hussain and Abid, 2011). The world fresh chilli productions in 2014 were 33.1 million tonnes with major producers are China (16 million), Mexico (2.7 million), Indonesia (1.8 million) and Japan (1.4 million) (FAOSTAT, 2014). ASEAN countries contributed 75% of world chilli production (Ali, 2006). In Malaysia, *C. annuum* (cayenne pepper) and *C. frutescens* (bird's eye chilli) are planted intensively. In 2015, total fruits production of *C. annuum* and *C. frutescens* are 47015 tonnes and 1979 tonnes, respectively (Department of Agriculture, 2015). There are several types of chilli varieties of *C. annuum* preferred by growers in Malaysia, namely Kulai 469, 151, 223, 461 and 568 while the popular varieties for *C. frutescens* are 'Centel' and 'Bara' (Mohd et al., 2016).

Chilli requires warm and humid conditions during growing period and optimum temperatures of 24 - 30°C during daytime and 15–17°C during night time for good fruit development (Burt, 2005). Temperature below 15°C or exceeds 30°C will reduce the yield and fruit quality. Chilli grows best in well-drained loam or silt loam soil with optimum pH of 5.5–6.8 (Berke et al., 2005). Mulching is important to conserve soil moisture and to prevent weed infestation and soil compaction.

2.1.5 Pests and diseases

There are many types of pests and diseases that attack chilli plant which can reduce the yield and fruit quality and become a major constrain in chilli fruits production. Major pests causing severe infestations on chilli are thrips, mites, and whitefly (Mariyono and Bhattarai, 2009). Chilli thrips or *Scirtothrips dorsalis* is an economically important pest of vegetable fruits, and widely distributed in southern and eastern Asia (Seal et al., 2006). The thrips species caused severe damage on chilli plant through its feeding activities and the species is also abundant on citrus, cocoa, mango and tomato (Venette and Davis, 2004). In chilli, adults and larvae of the thrips prefer to feed young leaves, buds and fruits which cause distortion and discoloration and severe damage which can lead to stunted growth of chilli plant (Arthurs et al., 2013).

Polyphagotarsonemus latus is a mite that can also caused severe infestations on chilli fruits (Mikunthan and Manjunatha, 2006). Mites prefer to suck sap from growing leaves and shoots causing leaf curl upwards and downwards and uncontrolled infestations will kill the plant (Varghese and Mathew, 2013). The whitefly, *Bemisia tabaci* is a tiny insect with broad wings, feeds on chilli plant and has been recognized as a main vector of plant virus diseases (Khalid et al., 2009). Besides transmitted plant viruses, the whitefly feeding activities can cause direct plant injury and thus reducing the quality of chilli fruits (Naser et al., 2015).

Chilli plants are susceptible to various diseases caused by infectious disease agents such as fungi, bacteria and virus. Among the diseases that cause substantial losses in chilli production are anthracnose (fruit rot), powdery mildew, bacteria wilt

and mosaic viruses (Hussain and Abid, 2011). Anthracnose caused by *Colletotrichum* spp. is a serious disease that leads to a substantial decline in chilli production worldwide, especially in tropical and subtropical regions (Than et al., 2008b). Infection on mature fruit causing pre and postharvest fruit decay is a major problem caused by anthracnose (Bosland et al., 2003).

Powdery mildew is also a serious fungal disease caused by *Oidiopsis* spp. (Monkhung et al., 2011). The disease affects lower leaf surface through production of white to grey powdery mycelia (Anand et al., 2010). Severe infection will lead to leaf defoliation and reduce the fruit sizes.

Bacteria wilt is another major disease infecting chilli and other Solanaceae plants including tomato and potato (Boukaew et al., 2011). *Ralstonia solanacearum* has been reported to cause wilting which begins on the younger leaves during hot temperature. At the early stage of infection, the wilted leaves may recover under cool temperatures, however severe infection will lead to sudden and permanent wilt (Kim et al., 2016).

Various mosaic viruses infected chilli plant including Alfalfa Mosaic Virus, Tobacco Mosaic Virus and Cucumber Mosaic Virus which mostly transmitted by aphids and whiteflies vectors (Hussain and Abid, 2011). The most common mosaic symptoms are formation of chlorotic and necrotic ring spots on the leaves and infected leaves may drop prematurely. Severe infection will caused distortions which affect fruit quality and reduce yield (Arogundade et al., 2012).

2.2 Chilli anthracnose

Anthracnose fruit rot of chilli is a serious threat to the production of high yield and quality chilli fruits especially in tropical developing countries such as, India, Thailand, Malaysia and Indonesia (Than et al., 2008b). Typical anthracnose symptoms are formation of black and sunken necrotic lesion with orange conidial masses and concentric ring of acervuli in the middle of the lesion (Ali et al., 2016). Anthracnose can infect leaves, stems and other parts of chilli plant however, infection on mature fruits is the main concern. Anthracnose infected chilli fruits both at pre and postharvest stages in which infection on ripe fruits will lead to fruit decay while lesions or blemishes on chilli fruit can tremendously reduce their marketable value (Raj et al., 2015).

Colletotrichum spp, the causal agent of chilli anthracnose can produced latent infection in which the infected chilli remain symptomless and will only develop the symptoms after ripens (Ramdial and Rampersad, 2015). The anthracnose symptoms on the fruits usually do not visible in the field but start to develop after the fruits are harvested. The infection is facilitated with warmer temperature and high humidity during handling and storage of the chilli fruits (Sivakumar and Banos, 2014; Ali et al., 2016).

2.2.1 Causal agent of chilli anthracnose

Colletotrichum was first reported as *Vermicularia* by Tode in 1970 and later, the genus '*Colletotrichum*' consist of Coelomycetes with *Glomerella* as teleomorph stage was introduced by Corda in 1831 (Hyde et al., 2009a). *Colletotrichum* is asexual stage that produced conidia and conidiophores within fruiting body

structures known as pycnidia and acervuli while *Glomerella* or teleomorph is the sexual stage (Figtree et al., 2013). The taxonomic classification of *Colletotrichum* is listed below (National Center for Biotechnology Information, 2017b):

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Order: Glomerellales

Family: Glomerellaceae

Colletotrichum is a filamentous fungi distributed widely in tropical and subtropical regions, causing devastating disease of high value or economical crops such as chilli, mango, citrus, banana, strawberry, maize and coffee berries (Cannon et al., 2012; Dean et al., 2012). *Colletotrichum* can infect all plant parts including stems, leaves, flowers and fruits (Hyde et al., 2009a).

Many species of *Colletotrichum* have been reported as causal agents of chilli anthracnose in many countries. In Thailand, Indonesia and Malaysia, anthracnose of chilli is caused by *C. acutatum*, *C. truncatum* and *C. gloeosporioides* (Voorrips et al., 2004; Than et al., 2008b; Yun et al., 2009). In China, the disease has been associated with *C. gloeosporioides*, *C. siamense*, *C. fructicola*, *C. truncatum*, *C. scovillei*, and *C. fioriniae* (Lui et al., 2016; Diao et al., 2016b). *Colletotrichum truncatum*, *C. siamense* and *C. fructicola* are among important causal agent of chilli anthracnose in India (Sharma, 2005; Sharma and Shenoy 2014). Recent study in

Australia identified five species of *Colletotrichum* associated with chilli anthracnose, namely *C. siamense, C. simmondsii, C. queenslandicum, C. truncatum* and *C. cairnsense* (De Silva et al., 2016). Among the species, *C. truncatum, C. gloeosporioides* and *C. acutatum* are the most frequent species associated with chilli anthracnose and numerous host plants worldwide (Damm et al., 2009; Damm et al., 2012, Weir et al., 2012).

Previously, *C. truncatum* one of the main causal pathogen of chilli anthracnose was known as *C. capsici*. Due to the economic importance of *C. capsici*, the taxonomic classification of this species was evaluated and the type specimen was re- examined. *Colletotrichum capsici* was originally described from chilli (*Capsicum frutescens*) in India (Sutton, 1992) and the dried specimen of *C. capsici* was obtained from the Swedish Museum of Natural History. However, the dried specimen could not provide a necessary viable culture for DNA analysis. Therefore, an epitype of *C. capsici* with living cultures was designated by Shenoy et al. (2007) to further investigate the taxonomic and phylogenetic placement of the species and also to provide reliable species name.

Based on epitypification by Shenoy, the characters of *C. capsici* that serve as an epitype strain are similar and consistent with the lectotype described by Sydow (1913). The type specimen produced brown to black acervuli on the fruit surface that arrange concentrically on the lesions. The colonies on PDA are white to greyish while the reverse colonies are greyish to green. Conidia are curved or falcate and the setae are abundant with dark brown colour. According to Damm et al. (2009), *C. capsici* was regarded as synonyms of *C. truncatum* as the descriptions of the epitype

strains for both species are similar. The epitype strain of *C. truncatum* was first described by Andrus and Moore (1935) which was obtained from lima bean pods (*Phaseolus lunatus*) in Mississippi, USA. Phylogenetic analysis also showed that all *C. truncatum* isolates are clades together with epitype stain of *C. capsici* which further confirming that they are similar species.

2.2.2 Infection stages and disease cycle

Colletotrichum spp. has many invasion strategies to attack plant host and most anthracnose pathogens exhibits hemibiotrophic lifestyles in which the initial invasion is for nutritional advantage (biotrophic) and subsequently it infects and kill the host tissues (necrotrophic) (Than et al., 2008a). The hemibiotrophic lifestyles of *Colletotrichum* spp. causing anthracnose has been observed in *C. graminicola* in maize (Munch et al., 2008), *C. gloeosporioides* in chilli (O′Connell et al., 2000) and *C. lupini* in lupins (Talhinhas et al., 2016). *Colletotrichum* spp. penetrates and colonizes the host plant through formation of specialized infection structures such as germ tube, appressorium, haustorium and intracellular hyphae (Curry et al., 2002).

The disease cycle of chilli anthracnose involve a series of processes, beginning with inoculation, penetration, invasion, colonization and dissemination (**Figure 2.1**) (Agrios, 2005; Saxena et al., 2016). Infection of *Colletotrichum* spp. starts with initial contact or attachment of conidia (inoculum) on the plant surface either on the leaves, stems or fruits. The conidia germinated and produced adhesive appressoria that will directly penetrate into the plant. The pathogens can also penetrate the plant surfaces through wounds or natural openings. Within the host tissue during the invasion period, the pathogen produce intra and intercellular

hyphae to invade the tissues, obtain nutrients and finally produced symptoms. Successful invasion of *Colletotrichum* spp. can be seen through formation of typical anthracnose symptoms such as blackened and sunken lesions on the plant surface. During colonization period, *Colletotrichum* spp. reproduced at rapid rate and produced abundance of acervuli with masses of conidia on the infected area. Then, the conidia will be disseminated to other healthy plants through water splash or splashing rain (Agrios, 2005; Saxena et al., 2016).

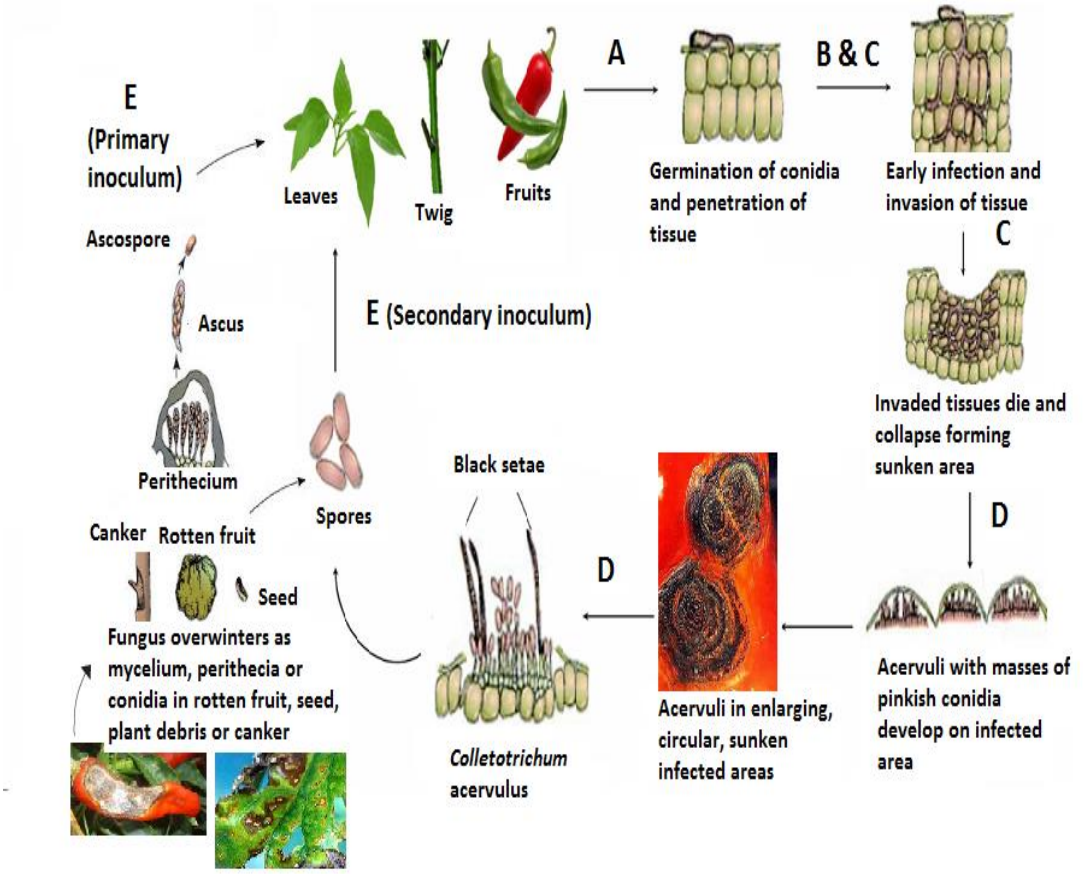

Figure 2.1: Disease cycle of chilli anthracnose as described by Saxena et al. (2016) and Agrios (2005) with some modifications. (A) Inoculation. (B) Penetration. (C) Invasion, (D) Colonization, (E) Dissemination.

Colletotrichum spp. can survive unfavourable or stressful environmental conditions as conidia, mycelium and production of survival structures known as micro-sclerotia (Samuelian et al., 2015). Micro-sclerotia allow *Colletotrichum* to overwinter on alternate hosts, weeds, plant debris and rotten fruits in the field for a long period (Than et al., 2008a). The sexual stage, *Glomerella* can also serve as primary inoculum through production of sexual spores (ascospores). However, the main inoculum of anthracnose disease is the conidia that produced by *Colletotrichum* (Freeman et al., 1998).

2.3 Identification of *Colletotrichum* species

Based on morphological and molecular methods, there are more than 40 accepted *Colletotrichum* species and several new species have been identified (Sutton, 1980; Sutton, 1992; Cai et al., 2009; Hyde et al., 2009b). Cannon et al. (2012) recognized 119 species of *Colletotrichum* that can be separated into nine major clades.

Identification based on morphological characterictics is difficult because lack of reliable characters for differentiation between species (Photita et al., 2005). Previously, *Colletotrichum* species were identified according to the host that they attack which suggests host specificity among species (Cai et al., 2009). However, identification based on host specificity become complicated as most *Colletotrichum* spp. could infect multiple hosts (Freeman et al., 1998; Than et al., 2008a). For instance, *C. truncatum* has been associated with anthracnose of various crops include chilli (Than et al., 2008b), soybean (Chen et al., 2006), mango (Lima et al., 2013) and dragon fruit (Guo et al., 2014).

In previous taxonomic classification of *Colletotrichum,* the term anamorph and teleomorph were used to represent asexual and sexual reproductions of the fungus, whereby, the sexual and asexual stages will have different species names. For instance, *Glomerella cingulata* is the sexual stage while *C. gloeosporioides* is the asexual stage (Abang et al., 2002). The species naming based on anamorph and teleomorph stages is very problematic and unreliable thus, classification of fungi with one name 99only is important (Hyde et al., 2009a). With one fungus, one name concept, separate naming based on asexual and sexual stages has been abolished and only one name, which is '*Colletotrichum'* is adopted for all species representing Glomerella and *Colletotrichum* (Phoulivong et al., 2010; Hawksworth, 2011).

2.3.1 Morphological identification

Colletotrichum spp. were traditionally identified and delimited based on morphological characters. The earliest morphological study was done by von Arx in 1957 and the study had set a new era of *Colletotrichum* taxonomy and classification (Cannon et al., 2012). Based on morphological characteristics, von Arx successfully reduced the number of accepted species within *Colletotrichum* from 750 to 11 species only, and become a foundation in earlier studies of morphological taxonomic of *Colletotrichum* (Hyde et al., 2009a).

After the study by von Arx, subsequent morphological identification of *Colletotrichum* was focused on species group or taxa associated with a particular crop plant (Hyde et al., 2009a). For instance, Simmonds (1965) provided useful morphological characteristics of *C. acutatum* causing ripe fruit rot in Australia. Sutton (1968) discussed in detail morphological characteristics of *C.*

graminicola species complex and the importance of appressorial morphology for *Colletotrichum* identification. Politis (1975) described the perfect stage or sexual stage of *C. graminicola* and revealed that the size and shapes of perithecia, asci, and ascospores of *C. graminicola* were slightly different compared to the other *Colletotrichum* species. Another comprehensive morphological study was done by Sutton (1980) which provided useful information on morphological and cultural characteristics of 22 species of *Colletotrichum*.

In 1980s, most studies on *Colletotrichum* still using morphological characteristics as main criteria for identification. Study by Baxter et al. (1983) on South African isolates of *C. corda* revealed that cultural characteristics such as colony colors and patterns have been successfully used to separate the *Colletotrichum* isolates into 11 species. Meanwhile, microscopics characteristics such as conidiophore structure, setae development and appressoria morphology were less useful to differentiate the *Colletotrichum* spp. In another study, Baxter et al. (1984) published a synoptic key based on morphological and cultural characteristics for all 11 *Colletotrichum* spp. of South African isolates, which is very useful reference for the species identification.

Although in 1990s, many researchers started to use molecular methods for taxonomic studies of *Colletotrichum*, morphological identification is still incorporated in many studies. Among the studies was Smith and Black (1990) who compared morphological characteristics of three *Colletotrichum* species; *C. fragariae*, *C. gloeosporiodes* and *C. acutatum* causing anthracnose disease of strawberry and found that *C. acutatum* could be easily differentiated from *C.*

fragariae and *C. gloeosporiodes* by its significantly slow growth rate. Sutton (1992) discussed in details the teleomorph and anamorph stages of 38 species of *Colletotrichum* which become the main reference for morphological identification of *Colletotrichum* species. Bailey and Jeger (1992) published a book which provided comprehensive information on biology, pathology and control of *Colletotrichum* spp.

Detail description of 20 species of *Colletotrichum* with curved conidia was compiled by Damm et al. (2009) of which most of the species was previously misidentified as *C. dematium*. Each species was comprehensively described and illustrated and become the main reference for preliminary identification of *Colletotrichum* spp. with curved conidia. In later studies, Damm et al. (2012) and Weir et al. (2012) comprehensively described and illustrated the macroscopic and microscopic characteristics of all species within *C. acutatum* and *C. gloeosporioides* species complexes. Damm et al. (2012) stated that conidial shape is a not a uniform feature for identification of *C. acutatum* species complex as not all the species produced fusiform conidia. Meanwhile, Weir et al. (2012) found certain limitations of morphological or cultural features to correctly identify the *Colletotrichum* species within *C. gloeosporioides* species complex.

Primary morphological features used for identification of *Colletotrichum* species are colony color, growth rate, size and shape of conidia, existence of setae and formation of appressoria (Abang et al., 2009; Sangdee et al., 2011). Morphological characteristics are important to sort the isolates into different groups before other identification approaches are conducted. Using morphological

characters such as conidial shape and colony color, Prihastuti et al. (2009) separated *Colletotrichum* isolates causing anthracnose of coffee berries into three groups before further identified using molecular method. In a study by Sutton (1992), *C. acutatum* was morphologically differentiated from *C. gloeosporioides* by its significantly slower growth rate.

Morphological characteristics alone are insufficient for a precise identification of *Colletotrichum* spp. due to overlapping morphological characters produced by different species of *Colletotrichum* which might lead to inaccurate species identification (Sutton, 1992; Adaskaveg and Hartin, 1997; Hyde et al., 2009a). For example, Talhinhas et al. (2002) found that certain isolates of *C. acutatum* causing anthracnose of lupins produced rounded end conidia which were very similar to *C. gloeosporioides*. Prihastuti et al. (2009) in their study found that *C. siamense* produced fusiform conidia with pinkish colonies resembling conidial shape and colony color of *C. acutatum*.

Morphological characters may also vary depending on environmental factors and incubation conditions such as temperature and types of media used. For instance, colonies of *C. truncatum* on Synthetic Nutrient-poor Agar were flat, olivaceous-grey to iron grey with no aerial mycelium while the colonies of *C. truncatum* on Potato Dextrose Agar (PDA) were white to dark grey with cottony mycelium (Than et al., 2008b; Damm et al., 2009). Repeated sub-culturing might also change the colony pigmentation of the *Colletotrichum* isolates (Weir et al., 2012). Therefore it is very important to standardize the incubation conditions of the media used as it is difficult to compare the morphological characters of species that

are grown under different conditions which can lead to taxonomic confusion (Cai et al., 2009). It is also recommended to incubate *Colletotrichum* isolates grow on PDA at 20, 25 and 30°C under constant fluorescent light (Cai et al., 2009).

2.3.2 Molecular identification

To overcome the limitations of morphological identification, molecular method using DNA sequence analysis is applied to characterize and identify taxa within the genus *Colletotrichum* (Moriwaki et al., 2002; Peres et al., 2008; Than et al., 2008a; Damm et al., 2012; Weir et al., 2012). DNA characters are not directly influenced by environment factors, thus the use of DNA analysis is reliable for identification and phylogenetic analysis of *Colletotrichum* species (Cannon et al., 2012). The DNA sequences obtain from single or multiple regions/genes are used to construct a phylogenetic tree in which the isolates that grouped in the same clades are commonly regarded belong to the same species (Peres et al., 2008). Phylogenetic analysis is also important to study the genetic relationship between the species as well as within the isolates of the same species.

The Internal Transcribed Spacer (ITS) regions which lie between the 18S and 5.8S rDNA genes and between 5.8S and 28S rDNA genes (**Figure 2.2**) is the most widely region used to identify and to study the phylogenetic relationship of *Colletotrichum* species. The regions have been recognized as universal DNA barcode marker for fungi (Schoch et al., 2012). For *Colletotrichum*, ITS is the only region in which sequences of all the epitype strains are available for comparison and very useful to give a preliminary identification of *Colletotrichum* species (Cai et al.,