CHARACTERISATION OF FUNGI ASSOCIATED WITH DISEASED COCOA (*Theobroma cacao*) IN WEST COAST OF PENINSULAR MALAYSIA

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by

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LIST OF SYMBOLS

%	Percentage
®	Registered
™	Trademark
°C	Degree celcius
µl	Microlitre
µm	Micrometre
cm	Centimetre
g	Gram
ha	hectare
kg	Kilogram
km	kilometer
L	Litre
L	Length
ml	Millilitre
mm	Millimetre
t	Tonne
W	Width

LIST OF ABBREVIATIONS

act	Actin
ANOVA	One-Way Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C_2H_5OH	Ethanol
caM	Calmodulin
CDC	Commonwealth Development Corporation
CLA	Carnation leaf agar
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate polymerase
DOA	Ministry of Agriculture
gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Gross Domestic Product
gs	Glutamine synthetase
hrs	Hours
ITS	Internal transcribed spacer
KM	Koko Mardi
LSU	Large subunit ribosomal DNA
MCB	Malaysian Cocoa Board
MEGA	Molecular Evolutionary Genetic Analysis
$MgCl_2$	Magnesium chloride
min	Minute
ML	Maximum likelihood
NaOCl	Sodium hypochlorite

NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rpb1	RNA polymerase subunit I
rpb2	RNA polymerase subunit II
rpm	Revolutions per minute
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
SSU	Small subunit ribosomal DNA
TBE	Tris-Borate-EDTA
tef1-α	Translation elongation factor 1-alpha
tub2	β-tubulin
USM	Universiti Sains Malaysia
WA	Water agar

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PENCIRIAN KULAT YANG BERASOSIASI DENGAN PENYAKIT KOKO (*Theobroma cacao*) DI PANTAI BARAT SEMENANJUNG MALAYSIA

ABSTRAK

Koko (*Theobroma cacao*) ialah pokok malar hijau yang tergolong dalam famili Malvaceae. Tanaman koko telah banyak menyumbang secara signifikan kepada ekonomi negara dan juga menjadi sumber pendapatan kepada petani kecil. Walau bagaimanapun, penyakit kulat merupakan salah satu masalah utama dalam penanaman koko, di mana penyebaran jangkitan yang tidak terkawal boleh menyebabkan kerugian hasil yang ketara. Objektif kajian ini adalah untuk mengenal pasti dan mencirikan pencilan kulat yang berasosiasi dengan *T. cacao* yang berpenyakit di Malaysia melalui analisis morfologi, molekul, dan kepatogenan. Sampel daun, batang, dan buah *T. cacao* yang berpenyakit telah dikumpul dari pelbagai ladang di Malaysia. Analisis morfologi dan molekul dijalankan untuk mengenal pasti pencilan kulat. Analisis filogenetik telah dilakukan untuk mengenal pasti dan mencirikan pencilan kulat dengan tepat menggunakan beberapa gen seperti penjarak transkripsi dalaman (ITS), translasi pemanjangan faktor 1-alfa (*tef1-α*), β-tubulin (*tub2*), gliseraldehid-3-fosfat dehidrogenase (*gapdh*), dan RNA polimerase subunit II (*rpb2*). Untuk menilai keupayaan patogenik pencilan kulat, ujian kepatogenan telah dijalankan pada daun, batang, dan buah *T. cacao* yang sihat menggunakan kepingan miselia dengan rawatan luka. Sebanyak 116 pencilan kulat telah dipencilkan daripada daun, batang, dan buah *T. cacao* yang berpenyakit dari ladang yang berlainan di negeri Perlis, Kedah, Pulau Pinang, Perak, Selangor, Negeri Sembilan, Melaka, dan Johor, Malaysia. Berdasarkan analisis morfologi, molekul, dan filogenetik, enam spesies kulat telah dikenalpasti:

Lasiodiplodia theobromae (57 pencilan, 49%), *Colletotrichum siamense* (19 pencilan, 16%), *Diaporthe tulliensis* (17 pencilan, 15%), *Neopestalotiopsis clavispora* (10 pencilan, 9%), *Fusarium solani* (tujuh pencilan, 6%), dan *Fusarium proliferatum* (enam pencilan, 5%). Keputusan ujian kepatogenan menunjukkan pencilan spesies kulat yang ditemui bertanggungjawab menyebabkan penyakit hawar daun (*C. siamense*, *D. tulliensis*, *L. theobromae*, dan *N. clavispora*), kanker batang (*D. tulliensis*, *F. proliferatum*, *F. solani*, dan *L. theobromae*), dan reput buah *T. cacao* (*C. siamense* dan *L. theobromae*). Kajian ini menyimpulkan bahawa pelbagai spesies kulat berasosiasi dengan penyakit tumbuhan koko. Penemuan kajian ini akan menjadi asas kepada pembangunan kawalan penyakit yang berkesan dan rancangan kuarantin untuk tanaman koko.

CHARACTERISATION OF FUNGI ASSOCIATED WITH DISEASED COCOA (*Theobroma cacao*) IN WEST COAST OF PENINSULAR MALAYSIA

ABSTRACT

Cocoa (*Theobroma cacao*) is an evergreen tree belonging to the Malvaceae family. The cacao plant has contributed significantly to the country's economy and has also become a source of income for smallholder farmers. However, fungal diseases are one of the major problems in cocoa plantations, where the spread of uncontrollable infections can lead to significant yield losses. The objective of this study was to identify and characterise the fungal isolates associated with diseased *T. cacao* in Malaysia through morphological, molecular, and pathogenicity analyses. Diseased samples of leaves, stems, and pods of *T. cacao* were collected from different plantations in Malaysia. Morphological and molecular analyses were carried out to identify the fungal isolates. Phylogenetic analysis was carried out to accurately identify and characterise the fungal isolates using various genes such as internal transcribed spacer (ITS), translational elongation factor 1-alpha (*tef1-α*), β-tubulin (*tub2*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and RNA polymerase subunit II (*rpb2*). To assess the pathogenic capabilities of the fungal isolates, pathogenicity tests were carried out on healthy leaves, stems, and pods of *T. cacao* using mycelial plugs with wound treatment. A total of 116 fungal isolates were isolated from diseased leaves, stems, and pods of *T. cacao* from different plantations in the states of Perlis, Kedah, Penang, Perak, Selangor, Negeri Sembilan, Melaka, and Johor in Malaysia. Based on morphological, molecular, and phylogenetic analyses, six fungal species were identified: *Lasiodiplodia theobromae* (57 isolates, 49%), *Colletotrichum*

siamense (19 isolates, 16%), *Diaporthe tulliensis* (17 isolates, 15%), *Neopestalotiopsis clavispora* (10 isolates, 9%), *Fusarium solani* (seven isolates, 6%), and *Fusarium proliferatum* (six isolates, 5%). The results of the pathogenicity tests showed that the isolates of the fungal species found were responsible for causing leaf blight (*C. siamense*, *D. tulliensis*, *L. theobromae*, and *N. clavispora*), stem canker (*D. tulliensis*, *F. proliferatum*, *F. solani*, and *L. theobromae*), and pod rot (*C. siamense* and *L. theobromae*) of *T. cacao*. The study concluded that a variety of fungal species were associated with diseased cocoa plants. The results of the present study will serve as a basis for the development of an effective disease control and quarantine plan for cocoa plants.

CHAPTER 1

INTRODUCTION

The cocoa tree (*Theobroma cacao*) is one of 22 recognised species of the genus *Theobroma*, which belongs to the family Malvaceae. In general, the cocoa tree is a perennial evergreen that grows in the uppermost layer of the rainforest in the shade of large trees. It has a taproot, dark green, glossy, ovate leaves, small yellowish white to pale pink flowers, and ovoid pods with seeds surrounded by a white pulp that is sun-dried and fermented into cocoa beans (Kew Royal Botanic Gardens, 2022). The plant is grown and harvested all over the world because of the value and importance of the seeds as a raw material for chocolate.

In Malaysia, cocoa has been widely cultivated since the 1980s. However, its cultivation and cocoa bean production keep decreasing by years until 2021 according to Malaysian Cocoa Board (MCB) (MCB 2022a, b). Cocoa bean production has declined from 1995 (131,475 t) to 2002 (47,661 t), and by 2021, a dramatic drop of only 361 t was recorded compared to the peak production in 1990 (247,000 t) (MCB, 2022b). Furthermore, the total cocoa acreage in Malaysia was 414,236 ha in 1989 but then began to decline to 190,127 ha in 1995 and 48,632 ha in 2002, until a decline to 5,955 ha was recorded in 2021 (MPIC, 2021). The significant decrease in cocoa production in Malaysia is caused by a decline in local cocoa bean production, the conversion of many plantations to grow palm oil and rubber plants, rising production costs, labour shortages, diseases, and pest infestations (Chizari et al., 2017; Fadzim et al., 2017).

The *T. cacao* tree, like other Malvaceae plants, is susceptible to fungal attack, causing a variety of diseases and posing a potential threat of significant crop loss. High

humidity, unmanageable shade, and inadequate ventilation are some of the environmental factors that contributed to the development of fungal-caused plant diseases. Black pod and canker are two important diseases affecting cocoa growers worldwide, and *Phytophthora* species namely *P. palmivora*, *P. capsici*, *P. citrophthora*, and *P. megakarya* have been identified as the causal pathogens (Drenth & Guest, 2004; Bailey & Meinhardt, 2016). *Lasiodiplodia theobromae* and *L. pseudotheobromae* have also been linked to pod rot and canker, potentially posing a new threat to the cocoa crop (Asman et al., 2019; Puig et al., 2021). Furthermore, other pathogens of cocoa pod rot disease have also been identified, namely *C. gloeosporioides* (Nair, 2021), *C. siamense* (Serrato-Diaz et al., 2020), *C. tropicale* (Serrato-Diaz et al., 2020), and *Neofusicoccum parvum* (Puig et al., 2021). In addition to pod rot and canker, cocoa is also infected with leaf blight caused by *C. gloeosporioides* (Suryanto et al., 2014). Besides that, other fungal pathogens have also been identified responsible of causing diseases on *T. cacao* including *Rosellinia pepo* and *R. bunodes* (black root rot), *Oncobasidium theobromae* (dieback), *Moniliophthora roreri* (frosty pod rot), *Erythricium salmonicolor* (pink disease), *Marasmiellus crinisequi*, *M. cyphella*, *M. neosessilis*, and *M. scandens*, (thread blight), and *Moniliophthora perniciosa* (witches' broom) (Adedeji, 2006; Guest & Keane, 2007; Azhar et al., 2009; Phillips-Mora & Rolando Cerda, 2009; Akrofi et al., 2014).

Identification of the fungal plant pathogen is crucial for the development of effective disease control strategies (Manawasinghe et al., 2021). The traditional identification method based on morphology has often been used which commonly involved the use of a microscope to assist the observation of the pathogen’s structures such as conidia, conidiophores, and fruiting bodies (Levetin, 2002). However, conflicting morphological features between fungal species tend to result in

misidentification. To address this problem, many studies have used a molecular and phylogenetic approach based on DNA sequences to delineate the identity of fungi down to the species level and provide information on phylogenetic relationships.

The internal transcribed spacer (ITS) region is commonly used for molecular identification and has been proposed as a universal DNA barcode marker because it offers the best chance for successful identification of fungi with the most clearly delineated barcode gap between inter- and intraspecific variations (Schoch et al., 2012). However, for some genera, ITS alone is unreliable for species identification (Schoch et al., 2012). Protein-coding genes such as translation elongation factor 1-alpha (*tef1-α*), β-tubulin (*tub2*), and the second largest subunit of RNA polymerase II (*rpb2*) are used as alternative markers for species differentiation. The use of a multigene phylogenetic analysis is also essential to accurately define the identity of fungal species.

Pathogenicity tests are used to determine the ability of the isolated fungi to cause diseases on cocoa plants by ensuring that the four principles of Koch's postulates are met. For a disease to develop successfully, three important components must work together: a susceptible host, a virulent pathogen, and a favourable environment, referred to as the disease triangle (Agrios, 2005). On the other hand, time has also been added to the disease triangle (Scholthof, 2007).

1.1 Problem statement

According to preliminary research, various disease symptoms were observed on cocoa trees in Malaysia, including leaf blight, stem canker, and pod rot. The lack of information on the aetiology of cocoa diseases, especially in Malaysia, complicates the disease management efforts. This has led to research focusing on the aetiology and

symptomatology of the three diseases of *T. cacao* in Malaysia and the determination of their causal pathogens.

1.2 Objectives

Therefore, the objectives of the present study were:

(i) to explicate the causal pathogens of leaf blight, stem canker, and pod rot of *T. cacao* based on morphology and DNA sequences.

(ii) to delineate the phylogenetic relationships of fungal isolates using multigene phylogenetic approach.

(iii) to determine the pathogenicity of the fungal isolates towards *T. cacao*.

1.3 Significance study

(i) the present study provides knowledge on disease aetiology and symptomatology that may assist in strategising effective disease management of the host plant.

(ii) the molecular phylogeny of fungal isolates recovered from disease cocoa (*T. cacao*) will describe their evolutionary history and elucidate species boundaries.

(iii) the findings may also be useful in disease monitoring and quarantine purposes.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Theobroma cacao*

The cocoa tree, scientifically known as *Theobroma cacao*, is a small evergreen tree known by various names including kakaw, pokok coklat, koko, chocolate, cacao, koko, criollo, cacaoyer, and kakao (Azhar et al., 2009). The cocoa tree is thought to originate in the Neotropical rainforest, particularly in the Amazon Basin and the Guyana Plateau (Lachenaud et al., 2007). *Theobroma* is derived from the Greek term for 'food of the gods' (Nair, 2021). The Maya, Olmec, Toltec, and Aztec used cocoa beans as the basis for a tasty beverage (Afoakwa, 2019; Nair, 2021). In addition, the Aztecs and Incas used cocoa beans as currency for trade or to make a drink known as chocolatl (Afoakwa, 2019). The drink was prepared by roasting and grinding cocoa nibs, which were then pureed with water, often with the addition of vanilla, spices, or honey (Afoakwa, 2019). After adopting the techniques for making the drink from the Aztec Empire, the Spanish was the first Europeans to drink cocoa in the sixteenth century (Nair, 2021). The word *cacao* comes from the Mayan and Aztec languages, Kakaw and Cacahuatl respectively, and was once reserved for royal use only (Dillinger et al., 2000; Portal Koko Duniaku, 2010a; Nair, 2021).

Cocoa has a long history in Malaysia, dating back to 1778 when the Portuguese attempted to establish a plantation in Malacca but failed (Ismail, 1987; Azhar et al., 2009). In 1931, the Ministry of Agriculture (DOA) of Peninsular Malaysia initially imported many varieties of cocoa from Forastero and Nicaragua, but they did not grow well in experimental fields in Negeri Sembilan and Serdang, Selangor (Azhar et al.,

2009). As a result, the DOA planted Trinitario cocoa in experimental fields in Cheras, Selangor; Kuala Lipis, and Temerloh, Pahang in 1934 (Azhar et al., 2009). After numerous efforts to cultivate cocoa by the DOA, Trinitario cocoa bore fruit successfully in 1937 (Ismail, 1987; Azhar et al., 2009). In 1950, the West Africa Cocoa Research Institute's Amelonado seedlings were distributed in Peninsular Malaysia, Sabah, and Sarawak (Ismail, 1987; Azhar et al., 2009). The first large-scale cocoa cultivation project using Amelonado cocoa varieties in Malaysia then began in 1953 in Jerangau, Terengganu (Azhar et al., 2009). According to Ismail (1987), Malayan Koko Limited collaborated with Harrisons and Crossfield, Cadbury of London, and the Commonwealth Development Corporation (CDC) in this project. In Sabah, Borneo Abaca Limited (BAL Plantation Sdn. Bhd.) commercially planted Trinitario and Amelonado cocoa varieties (Azhar et al., 2009). Subsequently, cocoa cultivation became a flourishing crop in 1956, prompting the Sabah Ministry of Agriculture to establish the Quoin Hill Agricultural Research Station in Tawau, Sabah in 1957 (Ismail, 1987; Portal Koko Duniaku, 2010b).

Due to the rapid development of the cocoa industry in the late 1970s and early 1980s, Act of Parliament 343 established the Malaysian Cocoa Board (MCB) in 1988, which became operational in 1989. The objective was to promote the Malaysian cocoa industry to be well integrated and competitive in the world market, as well as to increase the productivity and efficiency of cocoa bean production and increase downstream activities (MCB, 2022c). The organisation focuses on the following areas: research development, market development and techno-economics, regulation and quality control, target group development, technology extension and transfer, and technical and advisory services (MCB, 2022c). Cocoa trees were planted in plantations and by smallholders in several states in Peninsular Malaysia in 1996, and by the

twentieth century cocoa plantations were common throughout Malaysia (Portal Koko Duniaku, 2010b).

2.2 Taxonomic classification of *T. cacao* and its varieties

The following is the taxonomic classification of *T. cacao* according to the Integrated Taxonomic Information System (ITIS) (2021):

Kingdom: Plantae – plants

Subkingdom: Viridiplantae – green plants

Infrakingdom: Streptophyta – land plants

Superdivision: Embryophyta

Division: Tracheophyta – vascular plants

Subdivision: Spermatophytina – spermatophytes, seed plants

Class: Magnoliopsida

Superorder: Rosanae

Order: Malvales

Family: Malvaceae – mallows, mauves

Genus: *Theobroma* L.

Species: *Theobroma cacao* L. – cacao

Theobroma cacao is a prominent tropical rainforest tree, formerly classified as a member of the family Sterculiaceae before being reclassified as a member of the family Malvaceae, which originated in tropical South America (Wood & Lass, 2001; Bayer & Kubitzki, 2003; Bartley, 2005; Bailey & Meinhardt, 2016). Among the 22 recognised species of *Theobroma* genus, only *T. cacao* has economic value and is commonly cultivated outside its natural distribution zone (Azhar et al., 2009; Hebbar et al., 2011; Nair, 2021). Several other species including *T. bicolor*, *T. grandiflorum,*

T. speciosum, and *T. subincanum* are cultivated modestly or harvested wild for human consumption (Hebbar et al., 2011).

Based on pod shape, *T. cacao* can be categorised into three main varieties namely Criollo, Forastero, and Trinitario (Bartley, 2005; Hebbar et al., 2011). Criollo, according to Hebbar et al. (2011), refers to a group of genetically identical trees that produce weakly coloured seeds and share several other physical characteristics. The pods range from green to dark red and produce the best quality of cocoa beans (Azhar et al., 2009; Afoakwa, 2019). Nevertheless, this variety is currently very rare, less cultivated, and only found in old plantations in Venezuela, Central America, Madagascar, Sri Lanka, and Samoa. This is due to indicators of inbreeding depression, low vigour and yield, stress attacks, and susceptibility to diseases and pests (Hebbar et al., 2011; Beckett et al., 2017; Afoakwa, 2019).

Most Criollo trees have been replaced by hybrids or Forastero trees, which are stronger and hardier (Azhar et al., 2009; Hebbar et al., 2011). The pods of Forastero are green or red and the seeds are deep purple (Azhar et al., 2009). This variety has greater variability in tree and fruit shape, is less susceptible to diseases and pests, and produces higher yields than Criollo variety (Hebbar et al., 2011; Afoakwa, 2019). However, the quality of beans of this variety is lower than that of the Criollo variety (Azhar et al., 2009).

In addition, the Amelonado types are a subset of the Forastero varieties that have been grown regularly in West African countries since the nineteenth century (Nair, 2021). Amelonado pods have a melon shape with a relatively smooth pod surface and are often self-compatible (Nair, 2021). The Trinitario variety, on the other hand, is a natural cross between the Criollo and Amelonado-type Forastero varieties,

differing in shape, size, colour, and pod surface (Azhar et al., 2009; Hebbar et al., 2011; Beckett et al., 2017; Afoakwa, 2019; Nair, 2021). Compared to Criollo, this combination delivers significantly higher quality and yield and disease resistance than Forastero (Afoakwa, 2019).

2.3 Cultivation, propagation, and morphology of *T. cacao* plant

The cocoa tree can grow at high temperatures with a maximum annual average of 30°C to 32°C and a minimum annual average of 18°C to 21°C (Azhar et al., 2009; Beckett et al., 2017; Afoakwa, 2019; International Cocoa Organization (ICCO), 2020a). Rainfall is also preferred for optimal growth of the cocoa tree, with annual rainfall ranging from 1,250 mm to 3,000 mm, preferably between 1,500 mm and 2,000 mm (Azhar et al., 2009; Afoakwa, 2019; ICCO, 2020a). The cacao tree also requires high humidity, which can be between 70% and 80% during the day and up to 100% at night. If the dry season lasts longer than 3 months, irrigation may be required (Beckett et al., 2017; Afoakwa, 2019).

Apart from that, cocoa trees require shade at all stages of their growth in the field, with immature trees requiring about 25% full sunlight and light requirements increasing as the plant matures (Azhar et al., 2009). Afoakwa (2019) also opined that lack of shade causes severe ecological stress to cocoa trees and makes them vulnerable to pest attacks. There are three types of shade strategies, namely permanent, semi-permanent, and temporary shades (Azhar et al., 2009; Afoakwa, 2019). Permanent shade can be provided by remnants of thinned forests or economically valuable species such as coconut, and legumes (Azhar et al., 2009; Afoakwa, 2019). According to Azhar et al. (2009), tall fruit trees with a sparse canopy such as *Durio zibethinus* (Durian) and *Parkia speciosa* (Petai) are suitable shade trees as they allow sufficient light to fall

on the cocoa trees. Semi-permanent trees such as banana and papaya are needed until the cocoa tree reaches maturity and removed as soon as the cocoa tree starts to bear pods (Azhar et al., 2009). This intercropping can also provide farmers with additional income during the immature stage of the cocoa plant. Meanwhile, temporary shade crops including maize and tapioca can protect cocoa seedlings from sunlight and insect attacks (Azhar et al., 2009).

Besides growth requirements, proper techniques of propagation are important to maintain the characteristics and youthfulness of cocoa plants. The most common type of cocoa propagation is open or hand-pollinated seed, known as hybrid (Azhar et al., 2009; Beckett et al., 2017). Propagation of cocoa from seed produces a tree with a vertical and straight trunk that forms three to five main branches at the jorquette, where the plant produces different yields, pods, bean characteristics and quality, which is undesirable (Azhar et al., 2009; Beckett et al., 2017). In virtually all cocoa-producing countries, clonal seedlings are preferred over hybrid seedlings for commercial cultivation involving vegetative propagation methods such as cuttings, marcotting, grafting, and budding (Azhar et al., 2009; Nair, 2021). The clone tree provides the same tree morphology, pod, and bean characteristics as the parent tree. Clone trees bear more pods due to their more open branch structure, have larger and more uniform beans, higher butter content, are resistant to major pests and diseases, and are adaptable to a wide range of agro-climatic conditions (Azhar et al., 2009; Beckett et al., 2017).

The cocoa tree can be characterised as a small deciduous plant that reaches a height of 4 m to 10 m in the field, depending on spacing, fertilisation, and shade conditions (Azhar et al., 2009; Afoakwa, 2019). Its stem has a straight, thin, and smooth bark, and the tree grows vertically until it reaches a height of 1 m to 2 m, after

which lateral branches, also called jorquettes, are formed (Azhar et al., 2009; Nair, 2021) (Figure 2.1A). Branches that form at the jorquette are called fan branches. Chupons are shoots that protrude from the main stem and extend to form new jorquettes (Azhar et al., 2009; Nair, 2021). The growth of new leaves that are pale green or red in colour is called a flush (Figure 2.1B), which gradually hardens and forms a fan branch (Azhar et al., 2009). Flowers appear on the trunk and branches of the cocoa tree, with only 1 to 5% of the flowers developing into pods (Azhar et al., 2009; Nair, 2021) (Figure 2.1C). The fruit of *T. cacao* is commonly referred to as a pod, and it takes 5 to 6 months from pollination to mature (Azhar et al., 2009) (Figure 2.1D). When immature, the pod may be green or red, but it becomes yellow or orange when mature (Azhar et al., 2009; Nair, 2021). In addition, cherelle development results in immature pods until they become mature (Azhar et al., 2009) (Figure 2.1E). The seeds, also called beans covered by a mucilaginous pulp, are the most important part of this plant (Figure 2.1F). The number of seeds per pod ranges from 30 to 60, and the seeds come in a variety of sizes, shapes, and colours (Lachenaud et al., 2007; Azhar et al., 2009; Nair, 2021).

Figure 2.1 Morphology of *T. cacao* plant. (A) Cocoa tree; (B) flush; (C) flowers; (D) pods; (E) cherelle; (F) beans covered by a mucilaginous pulp. Scale bars: (A-F) = 5 cm.

2.4 Cocoa production and economic contributions

In 2020, Africa accounted for 68.4% of global cocoa bean production, followed by the Americas (17.3%), Asia (13.5%), and Oceania (0.8%) (FAOSTAT, 2022). Côte d'Ivoire was ranked as one of the top ten cocoa bean producers in 2020 with 2,200,000 t. Ghana (800,000 t) is the second largest producer of cocoa beans in the world, followed by Indonesia (739,483 t), Nigeria (340,163 t), Ecuador (327,903 t), Cameroon (290,000 t), Brazil (269,731 t), Sierra Leone (193,156 t), Peru (160,289 t), and the Dominican Republic (77,681 t) (FAOSTAT, 2022). In 2020, Indonesia is the only Asian country to be among the top ten cocoa bean producers. Previously, Malaysia was among the world's top ten producers from 1990 (ranked fourth) to 2002 (ranked eighth) (FAOSTAT, 2022). In 2021, the total cocoa plantation area declined to 5,955 ha, of which Sabah has the largest plantation area with 3,331 ha, followed by Sarawak with 1,355 and Peninsular Malaysia with 1,269 ha (MPIC, 2021). Despite

this, Malaysia now ranks sixth in the world in cocoa bean grinding, with annual production increasing from 70,000 t in 1990 to 171,318 t in 2021 (MPIC, 2021; MCB, 2022d).

In 2021, there are 53 chocolate and confectionery companies in Malaysia, such as Hershey's, Nestle, Mondelez International, Benns, Beryl's, Network Foods, JB Cocoa, Krishcoco, Guan Chong, and Barry Callebaut (MPIC, 2021). There are also 232 chocolate entrepreneurs enrolled with the Malaysian Cocoa Board (MPIC, 2021). According to MPIC (2021), the cocoa industry contributed RM1.64 billion to the gross domestic product (GDP) in 2020. In the first half of 2021, the cocoa industry's contribution to GDP was lower than the previous year at RM0.85 billion (MPIC, 2021). Furthermore, Malaysia's net exports of cocoa and cocoa-based products amounted to RM6.29 billion in 2020, while net exports of RM3.37 billion were recorded in the first half of 2021 (MPIC, 2021). Cocoa butter accounted for the largest share of total exports at 37.2%, followed by cocoa powder (23%), and re-export of dried cocoa beans (17.3%) (MPIC, 2021).

2.5 Importance of cocoa

Cocoa and cocoa products have diverse nutritional value due to their high content of flavonoids such as flavanol, catechin, epicatechin enantiomer, procyanidin B2, methylxanthines, tannins, saponin, cardiac glycosides, terpenoids, and alkaloids (Kofink et al., 2007; Jalil & Ismail, 2008; Subhashini et al., 2010; Ishaq & Jafri, 2017). Cocoa also has beneficial biological effects, such as high antioxidant, antihypertensive, anticancer, antiplatelet, and anti-inflammatory activities; reduction of stress and depression; reduction of the risk of heart attack and stroke; and control of cholesterol levels (Steinberg et al., 2003; Keen et al., 2005; Buijsse et al., 2006;

Dryden et al., 2006; Selmi et al., 2006; Taubert et al., 2007; Ferrazzano et al., 2009; Schinella et al., 2010; Latif, 2013; Scapagnini et al., 2014).

Dried cocoa beans are the main ingredient used in the production of chocolate, and the raw material for cocoa cake, cocoa liquor, cocoa powder, and cocoa butter (Joel et al., 2013; Beg et al., 2017; Pavlović et al., 2019). However, instead of being discarded and wasted, there are various by-products from the shell of the cocoa bean, the husk of the cocoa pod, and the cocoa mucilage. Among the by-products of the cocoa bean shell are feedstocks, biofuel, adsorbent, colourant, food, cocoa shell tea, used in the bio-recycled packaging industry, in skin care, and in the pharmaceutical industry (Portal Koko Duniaku, 2010c; Sánchez et al., 2010; Nieburg, 2013; Awolu & Oyeyemi, 2015; Munichiello, 2016; Tu, 2016; Fioresi et al., 2017; Okiyama et al., 2017; Figueroa et al., 2019; Jozinović et al., 2019). Additionally, cocoa shell products include livestock feed, soap making, activated carbon, organic fertilisers and organic matter, paper making, biofuels, and chemical production (Taiwo & Osinowo, 2001; Hatta, 2013; Fioresi et al., 2017; Lu et al., 2018a). Cocoa mucilage has also been used to produce cocoa juice, alcoholic cocoa drinks, and cocoa jelly (Fioresi et al., 2017; MCB, 2022e).

2.6 Challenges in cocoa cultivation caused by fungal and fungal-like infection

Cocoa, like many of the mallow family (Malvaceae), is susceptible to fungal infections that have become a potential threat and have led to significant losses in the global cocoa supply, estimated at 30-40% (ICCO, 2020b). Various diseases in cocoa crops include black pod, canker, dieback, leaf spot, thread blight, witches' broom, frosty pod rot, pink disease, and black root rot.

2.6.1 Pod rot and canker

The fungus-like plant pathogen *Phytophthora* species has been identified to infect a wide range of crops. *Phytophthora* species are usually detected in the flowers, cherelle, pods, roots, stems, and leaves of the cocoa plant (Drenth & Guest, 2004). Many studies have identified *Phytophthora* species, particularly *P. palmivora*, *P. capsici*, *P. citrophthora*, and *P. megakarya*, as the pathogens responsible for cocoa pod rot (Drenth & Guest, 2004; Azhar et al., 2009; Akrofi et al., 2015; Vanegtern et al., 2015; Bailey & Meinhardt, 2016; Puig et al., 2021). The genus *Phytophthora* is also reported to be responsible for stem canker, seedling blight, and leaf blight in cocoa plants (Opoku et al., 2005; Nair, 2021).

In addition to *Phytophthora* species, the genus *Lasiodiplodia*, particularly *L. theobromae* and *L. pseudotheobromae*, have been reported to cause pod rot and canker, which could be an alarming problem for the cocoa crop (Shamim et al., 2010; Twumasi et al., 2014; Asman et al., 2019; Serrato-Diaz et al., 2020; Nair, 2021; Puig et al., 2021). Several studies have also indicated the association of several fungal genera that act as pathogens of pod rot in cocoa, including *Colletotrichum gloeosporioides*, *C. siamense*, *C. tropicale*, and *Neofusicoccum parvum* (Serrato-Diaz et al., 2020; Nair, 2021; Puig et al., 2021).

The presence of brown or black lesions on the area of infection is a symptom of black pod disease (Figure 2.2A). The mass of white sporangia also spreads rapidly on the outer surface of the pod until the pod is completely covered with fungal mycelia (Figure 2.2B). The sporangia penetrate the pod and contaminate the cocoa beans so that they begin to dry and mummify (Vanegtern et al., 2015) (Figure 2.2C). Symptoms of canker include a reddish-brown discolouration and a reddish fluid that may exude through cracks in the bark (Wood & Lass, 2001; Azhar et al., 2009) (Figure 2.2D and

2.2E). Canker infection leads to wilting of pods, yellowing and defoliation of leaves, and death of branches, possibly resulting in tree death (Mohanan, 1978; Nair, 2021).

Figure 2.2 Signs and symptoms of pod rot and canker of *T. cacao*. (A) Brown or black spot on the infection area; (B) infected pod entirely covered with mycelia; (C) contaminated cocoa beans; (D, E) reddish-brown lesion on cocoa stem. (Location: Durian Tunggal, Alor Gajah, Melaka) Scale bars: (A-E) = 5 cm.

2.6.2 Dieback

Ceratobasidium theobromae belongs to the division Basidiomycota and is the causal agent of vascular streak dieback (VSD) disease on cocoa plants, where it can kill both seedlings and adult trees. The disease was first discovered in Papua New Guinea in the 1960s (Keane et al., 1972; Prior, 1980) and has since become a real problem in cocoa plantations in West Malaysia, Sabah, and Indonesia. The disease has also been reported in other Southeast Asian countries such as Thailand, Burma, Vietnam, and the southern Philippines (Guest & Keane, 2007).

The pathogen exists only in its host environment and can be recognised in the field by the formation of sporophores on infected leaves. The sporophores can remain productive for an average of 10 days and attach to branches (Azhar et al., 2009). Symptoms of VSD include yellowing of leaves, defoliation, swelling of lenticels with a three-point appearance, discolouration of vascular tracks, and vascular streaks on cocoa branches (Keane et al., 1972; Guest & Keane, 2007; Azhar et al., 2009) (Figure 2.3A - 2.3D). According to Guest & Keane (2007), when infected leaves fall in moist conditions, mycelium forms from the leaf scar and the basidiocarp covers the leaf scar and adjacent bark with a white, flat, and silky coating. Basidiospores are released through germination of the basidiocarp at the infected site, and the mycelium infiltrates the leaf by growing directly through the cuticle (Prior, 1980).

Figure 2.3 Symptoms of vascular streak dieback (VSD) of *T. cacao*. (A) Leaf chlorosis; (B) defoliation; (C) three-point appearance on the swollen lenticle; (D) brown stripe on the vascular streak after cutting off. (Location: Durian Tunggal, Alor Gajah, Melaka) Scale bars: (C-D) = 5 cm.

In addition, *Lasiodiplodia* species have also been reported as the causal agents of cocoa dieback. For example, Alvindia & Gallema (2017) reported the occurrence of VSD-like disease caused by *L. theobromae* in Davao, Philippines. *Lasiodiplodia theobromae* is also a vector of cocoa dieback in India, Cameroon, and Western Samoa (Mbenoun et al., 2008; Kannan et al., 2010; Mohali-Castillo & Stewart, 2017; Asman et al., 2019). Furthermore, *L. brasiliensis* and *L. pseudotheobromae* have also been

reported to be associated with cocoa dieback (Mohali-Castillo & Stewart, 2017; Membalik et al., 2021). According to Adu-Acheampong (2009), the symptoms of cocoa dieback caused by *Lasiodiplodia* are the leaves on the outer twigs turning yellow, infected twigs and branches are internally discoloured with brown streaks in the vascular system, white or yellowish gummosis and lead to the death of the tree when the disease spreads throughout the branches.

2.6.3 Leaf spot and anthracnose

Fungi from the *Colletotrichum* species have been reported to cause leaf spot disease or anthracnose on cocoa leaves (Yee & Sariah, 1993; Rojas et al., 2010). In 1975, *Colletotrichum gloeosporioides* was first identified as the causal agent of leaf spot of cocoa in Malaysia (Lin & Liew, 1975; Yee & Sariah, 1993). Besides Malaysia, the disease has also been found in several cocoa-growing countries around the world, including Trinidad, Sri Lanka, Cameroon, Nicaragua, Philippines, Nigeria, and Costa Rica (Yee & Sariah, 1993). Suryanto et al. (2014) characterised brownish lesions and chlorotic halos as symptoms of anthracnose on cocoa leaves caused by *C. gloeosporioides* (Figure 2.4). The brown spot on the lesion is the part where it will coalesce and cause rot (Suryanto et al., 2014). According to Azhar et al. (2009), leaf spot disease occurs mainly on cocoa seedlings in nurseries, with the most common pathogens being *Colletotrichum*, *Gloeosporium*, *Curvularia*, and *Pestalotiopsis*.

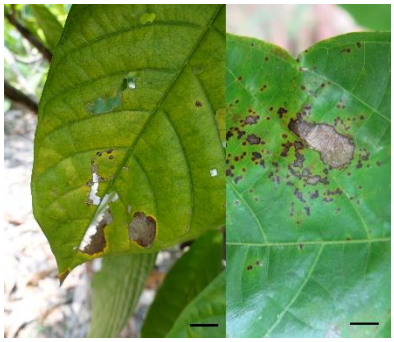

Figure 2.4 Brown spot surrounded by chlorotic halo on leaf of *T. cacao*. (Location: Tanjong Ipoh, Kuala Pilah, Negeri Sembilan) Scale bars = 5 cm.

2.6.4 Thread blight disease

Thread blight is caused by Basidiomycota fungi, particularly *Marasmiellus* species. There are two types of thread blight namely white thread blight caused by *M. scandens*, *M. cyphella*, and *M. neosessilis*, and horsehair blight caused by *M. crinisequi* (Azhar et al., 2009). The disease is recognised by a thread-like strand of mycelium on the leaves and branches of cocoa plants. White thread blight disease is characterised by the presence of creamy white and black or brown mycelial threads extending along the leaves, petioles, and twigs (Figure 2.5A). The symptom of horse blight disease, on the other hand, is a web of black mycelial threads that cause the leaves to hang loosely on the twigs (Adedeji, 2006) (Figure 2.5B). Since the dead leaves are attached by strands of mycelium, they remain attached to the branches and do not fall to the ground (Azhar et al., 2009).

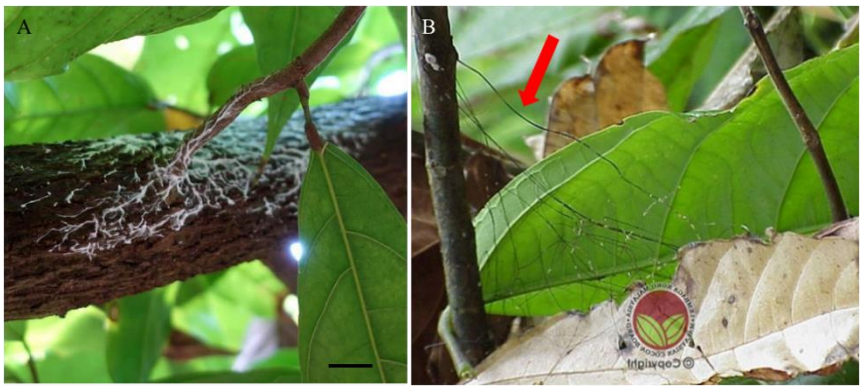

Figure 2.5 Signs and symptoms of thread blight disease of *T. cacao*. (A) White thread blight; (B) horsehair blight (red arrow) (MCB, 2013). (Location: Durian Tunggal, Alor Gajah, Melaka) Scale bars: (A) = 5 cm.

2.6.5 Witches' broom disease

Moniliophthora perniciosa causes witches' broom disease on *T. cacao*. The pathogen belongs to the Basidiomycota and has caused significant losses in cocoa production of 50-90% in cocoa-growing countries such as Guyana, Ecuador, Trinidad, Colombia, and Grenada (Meinhardt et al., 2008). *Moniliophthora perniciosa* has a hemibiotrophic life cycle in which it kills and feeds on dead tissue (necrotrophic phase)

after colonising living cocoa tissue (biotrophic phase) (Evans, 1980). Basidiospores produced by pink basidiocarps from infected plant tissue infect developing shoots, flowers, and pods (Evans, 1980; Meinhardt et al., 2008) (Figure 2.6A and 2.6B). The basidiospore enters the plant through the stromata and triggers the disease, which is accompanied by the appearance of a green-broom structure on the infected area, also known as the biotrophic stage (Meinhardt et al., 2008). The plants then develop symptoms of necrosis leading to a dry broom structure. After a series of rainy periods, the necrotic hyphae transform into basidiomata, and the life cycle of the pathogen continues indefinitely (Teixeira et al., 2015). Green broom on terminal cocoa branches, necrosis on cocoa pod, basidiocarp on dead broom, and pod necrosis are all symptoms of witches' broom disease on *T. cacao* (Teixeira et al., 2015).

Figure 2.6 Signs and symptoms of witches' broom disease on *T. cacao*. (A) Pink basidiocarp on dry branch; (B) pod rot caused by *M. perniciosa* (Meinhardt et al., 2008).

2.6.6 Frosty pod rot

Moniliophthora roreri is a basidiomycete pathogen that causes frosty pod rot on *T. cacao* (Aime & Philips-Mora, 2005). The disease was first observed in 1933 by Ciferri and Parodi in Ecuador (Bailey et al., 2018). According to Bailey et al. (2018), the first outbreak of frosty pod rot caused by *M. roreri* in Ecuador led to a significant reduction in cocoa production. Infection of cocoa with *M. roreri* has been reported to

cause severe economic deprivation, with cocoa yields in growing areas decreasing by 10-100% (Phillips-Mora et al., 2007). It has also been reported to be twice as aggressive as black pod, which is more difficult and riskier to control than witches' broom (Bailey et al., 2018). *Moniliophthora roreri* associated with *T. cacao* has occurred in Bolivia, Jamaica, Colombia, Ecuador, and Mexico (Cuervo-Parra et al., 2011; Phillips-Mora et al., 2015; Maridueña-Zavala et al., 2016; Suárez Contreras, 2016; Johnson et al., 2017). Discolouration, swollen areas on the pods, and dense formation of cream-coloured spores are among the symptoms of frosty pod rot of cocoa (Bowers et al., 2001) (Figure 2.7A and 2.7B).

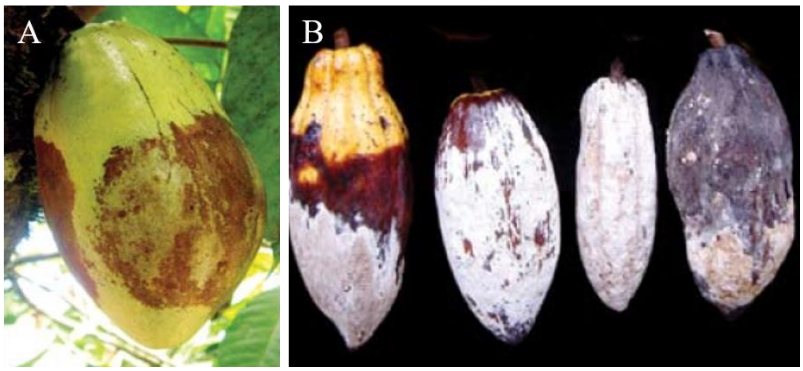

Figure 2.7 Signs and symptoms of frosty pod rot on *T. cacao*. (A) Brown spot and swollen cocoa pod; (B) presence of fungal mycelia on the infected pod (Phillips-Mora & Rolando Cerda, 2009).

2.6.7 Pink disease

Erythricium salmonicolor is the causal agent of pink disease of *T. cacao*, which was first reported in Ghana (Akrofi et al., 2014). The disease can cause canker on the tree and eventually kill it by affecting the physiological functions of the plant (Akrofi et al., 2014). According to Kwarteng et al. (2018), pink disease was studied in the eastern region of Ghana as the disease poses a serious threat to the Ghanaian cocoa industry. The symptoms are described as a sparse white silk-like web (mycelium) along the branches and through bark crack and distended lenticel pore, followed by the

appearance of pink or white abscesses (Azhar et al., 2009). The pathogen has been found to be associated with cocoa in Brazil, Colombia, Ghana, Nigeria, Malaysia, Papua New Guinea, Peru, Western Samoa, and Trinidad (Bailey & Meinhardt, 2016).

2.6.8 Black root rot

The fungus *Rosellinia pepo* is the causal agent of black root rot of *T. cacao*, which was first identified in Latin America and the Caribbean (Feitosa & Pimentel, 1991). The disease was reported to cause severe loss of up to 20% of trees in Brazil, and in Colombia more than half of the cocoa plantations have been infected by the disease (Ten hoopen & Kraus, 2006). In addition, pathogens of *R. pepo* and *Rosellinia bunodes* have been identified as causing black root disease in tropical America, the West Indies, West Africa, and South Asia (García et al., 2003). Due to the high content of organic matter, high relative humidity, and severely restricted ventilation, it can favour the infection of cacao trees with *R. pepo* (Phillips-Mora & Rolando Cerda, 2009). The disease affects the root and the base of the trunk, showing signs and symptoms of dry leaves, fan-shaped mycelia under the bark of the roots, and eventually causes the death of the tree (Phillips-Mora & Rolando Cerda, 2009).

2.7 Disease control management

As the occurrence of disease in *T. cacao* has caused a decline in cocoa production, which can have a significant impact on the country's economy, proper disease control management is crucial to control and prevent the spread of disease in the field. Effective preventive measures such as chemical treatment, biological control or good agricultural practices are needed.

To control black pod disease and canker caused by *Phytophthora*, fungicides such as metalaxyl, metalaxyl + mancozeb, fosetyl-aluminium, mefenoxam, Bordeaux

mixture, difolatan, and copper-based fungicides can be used (Azhar et al., 2009; Bailey & Meinhardt, 2016; Nair, 2021). Apart from these, good agricultural practises help in controlling the disease caused by *Phytophthora*, such as reducing the relative humidity of the plantation through shade pruning, regular harvesting of pods, and disposal and destruction of diseased pods (Azhar et al., 2009; Vanegtern et al., 2015; Bailey & Meinhardt, 2016; Nair, 2021). *Colletotrichum tropicale* was reported to play a role as a biocontrol agent capable of reducing the severity of black pod rot in the field (Mejía et al., 2008). Extracts of *Allium sativum*, *Cinnamomum zeylanicum*, *Lawsonia inermis*, and *Adenocalymma allicea* have been studied effectively in inhibiting the development of lesions on detached cocoa pods (Nair, 2021). *Pseudomonas fluorescens* and *Trichoderma virens* were also found to be effective against black pod disease caused by *Phytophthora* (Krauss & Soberanis, 2002; Nair, 2021).

In addition, regular pruning of infected branches and shade helps in controlling vascular dieback, preventing disease spread, and minimising inoculum (Azhar et al., 2009; Bailey & Meinhardt, 2016). Several triazole fungicides, such as triadimenol, triadimefon, and tebuconazole, have also been used to control this disease (Vos et al., 2003; Azhar et al., 2009).

Several studies have reported the difficulties in controlling diseases caused by *Lasiodiplodia* species, as their conidia persist in the soil and leaf litter, and can colonise via cuttings (McDonald & Eskalen, 2011; Adesemoye et al., 2014; Jaiyeola et al., 2014; Bailey & Meinhardt, 2016). Therefore, removing infected plant parts and planting shade trees that do not lure pests can help in reducing the spread of the disease (Azhar et al., 2009; Bailey & Meinhardt, 2016). Subsequently, diseases caused by *Colletotrichum* species such as pod rot, leaf blight, leaf spot, and anthracnose can be

controlled by removing infected parts, shade pruning or applying fungicides such as carbendazim, mancozeb, and copper-based fungicides (Wood & Lass, 2001; Azhar et al., 2009; Bailey & Meinhardt, 2016; Nair, 2021). Biological control with chitinolytic bacteria such as *Enterobacter cloacae* and *Bacillus* sp. has been previously tested to control diseases caused by *Colletotrichum* species (Suryanto et al., 2014).

To avoid re-infection, thread blight disease must be regularly inspected and controlled by cutting off diseased parts and burning them off-farm (Bailey & Meinhardt, 2016). Amoako-Atta et al. (2016) found that a combination of pruning measures and fungicide treatments such as Nordox and Metalm helped in combating thread blight. Moreover, good agricultural strategies have been used to control witches' broom, such as discarding and burning infected parts of the brooms to mitigate the disease (Meinhardt et al., 2008; Nair, 2021). The use of fungicides is not recommended to control the witches' broom disease as it is very expensive and has led to cocoa bean contamination and environmental health problems (Vos et al., 2003; Meinhardt et al., 2008). However, biological control with *Clonostachys rosea*, *Trichoderma longibrachiatum*, *T. stromaticum*, a combination of *T. longibrachiatum* + *T. virens* and *C. rosea* + *T. longibrachiatum* + *T. stromaticum* + *T. virens* has proven helpful in controlling witches' broom disease (Krauss & Soberanis, 2002; Vos et al., 2003).

The frosty pod rot can be controlled by sorting out and destroying diseased pods, maintaining shade, and weeding cocoa plantation regularly (Nair, 2021). Biological control agents such as *Trichoderma* sp., *C. rosea*, and *Clonostachys byssicola* are known to treat the disease (Krauss et al., 2003; Dorado Ore et al., 2017). On the other hand, pink disease can be prevented by proper drainage, shade trimming, pruning, and burning of infected parts (Wood & Lass, 2001; Akrofi et al., 2014; Bailey