CHARACTERISATION AND PATHOGENICITY OF FUNGI CAUSING SOOTY BLOTCH AND FLYSPECK (SBFS) DISEASE OF MANGO (*Mangifera indica* L.) IN PENINSULAR MALAYSIA

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

$	U.S. dollar
%	Percentage
℃	Degree Celsius
cm	Centimetre
E	East
g	Gram
h	Hour
kg	Kilogram
L	Litre
lb	Pound
ml^{-1}	Per millilitre
mA	Milliampere
min	Minute
ml	Millilitre
mm	Millimetre
mt	Metric tons
N	North
pH	Potential of Hydrogen
s	Second
µl	Microlitre
µm	Micrometre
V	Volt

>	Greater than
/	Per

LIST OF ABBREVIATIONS

ACT	Actin
ANOVA	One-way analysis of variance
AWA	Acidified water agar
bp	Base pair
CBS	Centraalbureau voor Schimmelcultures
CPA	Colonies per apple
CPM	Colonies per mango
DNA	Deoxyribonucleic acid
et al.	And others
FS	Flyspeck
HSD	Honestly significant difference
ITS	Internal transcribed spacer
KH_2PO_4	Potassium dihydrogen phosphate
KNO3	Potassium Nitrate
LSU	Large subunit
LWD	Leaf wetness duration
MA	Mango clone
MEA	Malt extract agar
MEGA 7.0	Molecular Evolutionary Genetics Analysis version 7.0
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
ML	Maximum likelihood
MLBS	Maximum likelihood bootstrap
MP	Maximum parsimony
MPBS	Maximum parsimony bootstrap

mtSSU	Mitochondrial small subunit
mrSSU	Mitochondrial ribosomal small subunit
nrSSU	Nuclear ribosomal small subunit
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
pcs	Pieces
PDA	Potato dextrose agar
PPP	Plant-penetrating parasite
P-value	Probability value
rDNA	Recombinant DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RM	Ringgit Malaysia
RPB2	RNA polymerase II
SB	Sooty blotch
SBFS	Sooty blotch and flyspeck
SEM	Scanning electron microscopy
SNA	Synthetic nutrient agar
sp.	Species
spp.	Species (Plural)
SPSS	Statistical package for the social sciences
TBE	Tris-borate EDTA
TBS	*Trichothecium* black spot
TEF	Translation elongation factor
TUB2	β-tubulin

UPGMA	Unweighted pair group method with arithmetic
U.S.	United States of America
Var.	Variety
WA	Water agar

LIST OF APPENDICES

PENCIRIAN DAN KEPATOGENAN KULAT PENYEBAB PENYAKIT 'SOOTY BLOTCH' DAN 'FLYSPECK' (SBFS) PADA MANGGA (*Mangifera indica* L.) DI SEMENANJUNG MALAYSIA

ABSTRAK

Kompleks penyakit 'sooty blotch' dan 'flyspeck' (SBFS) yang disebabkan oleh spesies kulat berbilang membawa kepada tompok hitam dan gugusan bintik hitam pada pelbagai buah, termasuk mangga yang menjejaskan kebolehpasaran sebagai buah segar. Walaupun tidak pernah dilaporkan di Malaysia, lawatan lapangan pada 2018 menjumpai simptom penyakit SBFS yang sama pada mangga di pelbagai kebun di Perak. Oleh itu, tujuan penyelidikan ini adalah untuk: 1. Memencilkan dan mengenal pasti kulat SBFS pada mangga yang dikumpul dari Perak, Pahang dan Perlis berdasarkan kepada pendekatan morfologi dan molekul; 2. Menaksir hubungan filogenetik antara pencilan kulat SBFS; 3. Menentukan kepatogenan kulat SBFS yang dipencilkan daripada mangga. Sebanyak 87 buah mangga yang menunjukkan simptom penyakit SBFS telah dikumpulkan, dan bilangan koloni SBFS pada setiap mangga (CPM) dan peratusan kawasan jangkitan SBFS telah ditentukan. Kemudian, 2106 sampel kulit mangga telah diperoleh dan menjalani pemencilan. Pencirian morfologi menghasilkan pengenalpastian 33 kultur tulen terkelompok dalam genera kulat SBFS, seperti *Zasmidum*, *Peltaster*, *Exophiala*, *Pseudocercospora* dan *Ochroconis*. Penjujukan DNA bagi jujukan penjarak transkripsi dalaman (ITS) dan subunit besar (LSU) mengesahkan 18 pencilan wakil sebagai spesies yang berbeza termasuk *Z. citrigriseum*, *Z. aporosae*, spesies seperti *Peltaster*, '*Pseudopeltaster*', *E. bergeri*, *E. spinifera*, spesies *Pseudocercospora* dan *O. cordanae*. Analisis filogenetik menggunakan pendekatan parsimoni maksimum (MP) dan kebolehjadian maksimum

(ML) menunjukkan bahawa kompleks kulat SBFS pada mangga adalah berkait dengan kedua-dua ahli SBFS yang diketahui secara global dan taksa bukan SBFS. Postulat Koch yang diubah suai telah berjaya digunakan secara *in situ* di kebun mangga dan pada buah yang dipotong menggunakan varieti Harumanis dan Chok Anan, mengesahkan kesemua 18 pencilan wakil sebagai patogen SBFS pada mangga, mempamerkan pelbagai jenis miselium. Di samping itu, kajian ini juga menunjukkan interaksi yang signifikan antara negeri dan peratusan kawasan jangkitan SBFS pada mangga. Kebun di Perak menghasilkan CPM dan keterukan penyakit SBFS yang lebih tinggi berbanding dengan kebun mangga di Perlis dan Pahang. Kajian ini mewakili laporan pertama penyakit SBFS pada mangga di Malaysia.

CHARACTERISATION AND PATHOGENICITY OF FUNGI CAUSING SOOTY BLOTCH AND FLYSPECK (SBFS) DISEASE OF MANGO (*Mangifera indica* L.) IN PENINSULAR MALAYSIA

ABSTRACT

The sooty blotch and flyspeck (SBFS) disease complex, caused by multiple fungal species, leads to black smudges and clusters of black spots on various fruits, including mango, impacting their marketability as fresh fruit. Although not previously reported in Malaysia, field visits in 2018 found similar SBFS disease symptoms on mango in various orchards in Perak. Therefore, this research aimed to: 1. Isolate and identify SBFS fungi on mango collected from Perak, Pahang, and Perlis based on morphological and molecular approaches; 2. Assess the phylogenetic relationship among the SBFS fungal isolates; 3. Determine the pathogenicity of the SBFS fungi isolated from mango. A total of 87 mango fruit showing SBFS disease symptoms were collected, and the number of SBFS colonies on each mango (CPM) and the percentage area of SBFS infection were determined. Subsequently, 2106 mango peel samples were obtained and subjected to isolation. Morphological characterization resulted in the identification of 33 pure cultures belonging to SBFS fungal genera, such as *Zasmidum*, *Peltaster*, *Exophiala*, *Pseudocercospora*, and *Ochroconis*. The DNA sequencing of the internal transcribed spacer (ITS) and large subunit (LSU) confirmed 18 representative isolates as distinct species, including *Z. citrigriseum*, *Z. aporosae*, *Peltaster*-like species, '*Pseudopeltaster*', *E. bergeri*, *E. spinifera*, *Pseudocercospora* species, and *O. cordanae*. Phylogenetic analyses using maximum parsimony (MP) and maximum likelihood (ML) approaches indicated that the SBFS fungal complex on mango was related to both known SBFS members globally and non-SBFS taxa.

Modified Koch's postulates were successfully applied *in situ* in mango orchards and on detached fruit using Harumanis and Chok Anan varieties, confirming all 18 representative isolates as SBFS pathogens on mango, exhibiting various mycelial types. In addition, the present study also showed a significant interaction between different states and the percentage area of SBFS infection on mango. Orchard in Perak resulted higher CPM and SBFS disease severity compared to mango orchards in Perlis and Pahang. This study represents the first report of SBFS disease on mango in Malaysia.

CHAPTER 1

INTRODUCTION

Mango (*Mangifera indica* L.) belongs to the family Anacardiaceae, a family of flowering plants. Mango is one of the commonly cultivated tropical fruits in many Asian countries, including Malaysia. In Malaysia, mango is sought-after, due to the differences in fruit appearance and taste in every mango variety available in local markets. Sabah, Perak, and Sarawak are the main mango-producing states in Malaysia, contributing both local and export markets with the production of 2,151 metric tons (mt), 1,751 mt, and 1,642 mt, respectively recorded in 2022 (Jabatan Pertanian Malaysia, 2023).

In Malaysia, the hot and humid climate provides a conducive environment for various fungal diseases to infect mango crop, causing significant losses in mango production (Crane and Campbell, 1994; Khalid et al., 2018). Some of the common fungal diseases of mango include anthracnose, mango scab, powdery mildew, and mango malformation, affecting different plant parts, such as branch, twig, stem, root, leaf, petiole, flower, and fruit (Misra and Prakash, 2000).

Sooty blotch and flyspeck (SBFS) is a disease complex that produces visible dark blemishes on the surface of the infected fruits (Gleason et al., 2019). The disease symptoms include black blemishes on the fruit surface, reducing the aesthetic value of the fruits and lowering their quality for fresh-market sale (Gleason et al., 2019). Previously, SBFS has been widely reported on apple, orange, and pear (Gleason et al., 2011). In 2017, Ajitomi and co-workers reported the first onset of SBFS disease on mango in Japan. In Malaysia, SBFS on mango has never been reported. However, during field visits to several mango orchards in Perak conducted in 2018, the observed

mango fruit showed similar symptoms with SBFS. Subsequent analyses are required to confirm the occurrence of the SBFS disease on mango in Malaysia.

The identification of fungal pathogens is one of the essential steps before determining the appropriate control measures against disease infection by the pathogen. The general approach commonly used to identify fungi is by distinguishing the observable morphology characters, such as colony appearance, colony growth diameter, the shape of conidiogenous cells, the shape, size, and arrangement of conidia (Kidd et al., 2016). In addition to the general structures possessed by fungi, SBFS fungi have a unique appearance upon colonization on the surface of the host—mycelial types used for preliminary characterization of SBFS fungi (Gleason et al., 2019).

Morphology characters of fungi provide a general insight into the group of fungal genera to which they belong. However, findings from the morphological observations coupled with analyses at the molecular level, such as DNA sequencing and phylogenetic analysis are reliable for fungal species determination (Raja et al., 2017). The internal transcribed spacer (ITS) has been regarded as the fungal DNA barcode marker (Schoch et al., 2012). However, due to the complexity among SBFS fungal species, it is recommended to couple the ITS gene with a different gene marker for a proper SBFS species identification, such as large subunit (LSU) (Batzer et al., 2005; Gleason et al., 2011).

The role as pathogen causing a disease can only be determined by completing all four principles of the Koch's postulates. The third and fourth postulates involve establishing the disease on the healthy host: third postulate requires that the disease must be reproduced by inoculating a healthy host with the cultured microorganism and fourth postulate stipulates that the microorganism must be isolated and matched the

initial microorganism from the inoculated, diseased host. Failing to fulfil Koch's postulates indicates that the isolated fungi were not pathogenic but had different lifestyles, such as saprotroph or commensal.

Confirming the role of SBFS fungi as pathogens of the disease through pathogenicity tests has been conducted on both attached fruit in the field and detached fruit (Batzer et al., 2005; Rosli et al., 2020; Gao et al., 2014). Koch's postulates for confirming the SBFS pathogen involve using a spore suspension of SBFS, inoculated on the fruit either by hand-spray or swab (Batzer et al., 2005; Rosli et al., 2020). The SBFS colonies that appear on the inoculated fruit are then compared to the original isolate using morphological characteristics or ITS sequences (Gleason et al., 2011; Gleason et al., 2019). Pathogenicity tests of SBFS fungi have been conducted in various countries, such as the U.S. (Rosli et al., 2020), Bulgaria (Piperkova and Yonkova, 2014), and Korea (Kwon et al., 2012).

Studies on SBFS have been conducted in many countries involving various fruit crops (Batzer et al., 2016; Hao et al., 2013; Hassan et al., 2021; Kwon et al, 2012). Based on previous findings on SBFS diversity, some SBFS species appear to be globally distributed, while others are restricted to specific geographic locations (Batzer et al., 2005; Díaz Arias et al., 2010; Ivanović et al., 2010; Mayfield et al., 2013). A study conducted by Díaz Arias et al. (2010) found that higher SBFS species diversity was found in apple orchards located in the western United States of America (U.S.) compared to that of eastern U.S. The SBFS fungi were also highly diverse in unsprayed orchards compared to conventionally sprayed orchards (Díaz Arias et al., 2010).

Sooty blotch and flyspeck disease has caused significant losses to various fruit crops in several countries, such as the U.S. and China (Gao et al., 2014; Gleason et al.,

2011). The SBFS disease on mango has been reported elsewhere, but never been reported in Malaysia. However, the disease was currently observed in the field. Therefore, the present study was conducted to gain a better insight into SBFS disease of mango in Malaysia based on the following objectives:

1. To isolate and identify SBFS fungi on mango collected from Perak, Pahang, and Perlis based on morphological and molecular approaches.
2. To assess the phylogenetic relationship among the SBFS fungal isolates.
3. To determine the pathogenicity of the SBFS fungi isolated from mango.

CHAPTER 2

LITERATURE REVIEW

2.1 Mango

Mango, along with other economically important crops, such as cashew, sumac, and pistachio belong to the Anacardiaceae family and is grown worldwide (Shah et al., 2010). Native to southern Asia, mango originated from Indo-Burma region and the Andaman Islands over 4000 years ago (Crane and Campbell, 1994; Yadav and Singh, 2017). Mango, known as "manga", "mempelam" or "ampelam" among locals, has different common names in various countries (Table 2.1).

Table 2.1. The common names of mango among different countries. (Bally, 2006)

Country/ Language	**Common Name**
Cambodia	Svaay
Dutch	Bobbie manja, kanjanna manja, maggo, manggaboom, manja
German	Mangobaum
Hindi	Aam, am, amb
Laos	Mwàngx
Indochina	Ma muang
Indonesia	Manga, mempelam
Malaysia	Manga, mempelam, ampelam
Myanmar	Tharyetthi
New Guinea	Mango
Philippines	Paho

Table 2.1. Continued

Country/ Language	Common Name
Portuguese	Manga
Spanish	Manga, mango
Tagalog	Mangga
Tamil	Ampleam
Thailand	Mamung
Vietnam	Xoài

To date, nearly hundreds of countries cultivate mango, with Asia being the largest continent for mango production, followed by South America and Africa (Rekhapriyadharshini, 2015). Mango thrives under specific growth conditions, including temperature ranging between 24 to 27 ℃, yearly rainfall of approximately 250 to 3000 mm, and soil with a pH ranging from 5.5 to 7.5 (Orwa et al., 2009; Rajan, 2012). Mature mango trees can withstand air temperature as low as -3.9 ℃ (Crane and Campbell, 1994; Nagao and Nishina, 1993). As a result, mango cultivation is thriving in countries with climates suitable for mango growth, such as Thailand, China, India, and Malaysia (Altendorf, 2017).

Mango is rich in vitamin A and C, calcium, phosphorus, and iron (Lebaka et al., 2021). Mango can be consumed fresh, frozen, dried, or processed into jams (Bally, 2006; Shah et al., 2010). In Malaysia, mangoes are cultivated mostly in Sabah, Perak, and Sarawak (Jabatan Pertanian Malaysia, 2023) and come in various varieties, including Golek (MA 162), Masmuda (MA 204), Maha 65 (MA 165), Chok Anan (MA 224), Nam Dok Mai (MA 223), Sala (MA 249), and Harumanis (MA 128) (Abdullah et al., 2011; Sani et al., 2018).

The most popular variety is Harumanis, which is oblong in shape with a distinct beak (Figure 2.1). The skin is green in colour with a bit of glossy, changing to yellowish green when ripe. The fruit size ranges from 300 to 650 gram, and the flesh is orange and flavourful (Sani et al., 2018). Another well-known mango variety commercially planted in Malaysia is Chok Anan, with a golden-yellow external appearance, medium fruit size (250-350g), orange-yellow flesh, sweet taste, and a pleasant aroma (Figure 2.1) (Abdullah, 2018; Mohd Azhar et al., 2012). Mango prices vary depending on factors, such as season, location, fruit quality, and demand.

Figure 2.1. Common mango varieties in Malaysia. A. Harumanis. B. Chok Anan.

Chok Anan mango is commonly sold for around RM 4 to RM 10 per kg, while Harumanis is more expensive, generally costing between RM 15 to RM 40 per kg (local mango growers, personal communication).

2.2 Fungal diseases of mango

Like many other crops, mango is susceptible to various fungal diseases that can infect the fruit at any stage of development (Misra and Prakash, 2000). Some common diseases reported in Malaysia include mango malformation (Mohamed Nor et al., 2013), anthracnose (Zakaria et al., 2015), and stem-end rot of mango (Li et al., 2021). Each of these diseases exhibits different disease symptoms: mango malformation result in abnormal flower, leaf, and shoot growth, severely affecting mango production (Joshi et al., 2014); anthracnose initially appears as water-soaked lesions of the fruit surface, later becoming soft and slightly sunken, sometimes with the presence of conidial masses (Zakaria et al., 2015); stem-end rot causes a brown ring to form at the stem-end of the fruit, with rot developing throughout the fruit (Figure 2.2) (Galsurker et al., 2020).

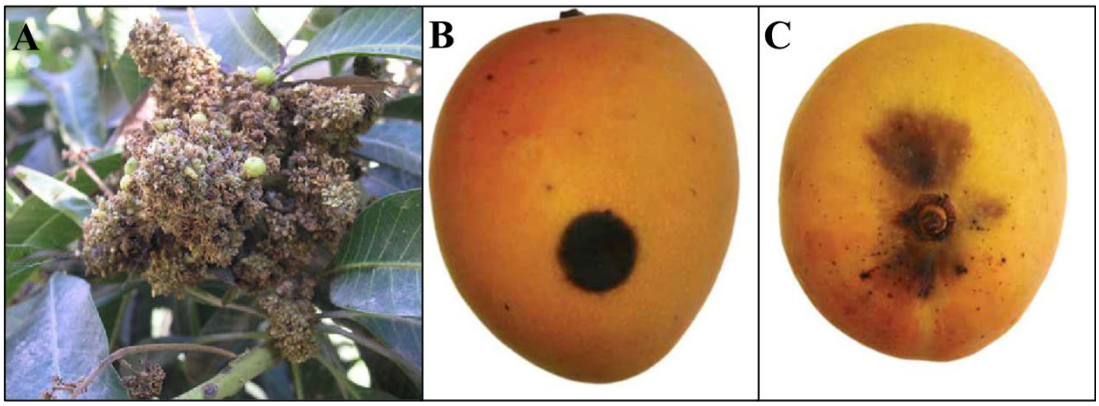

Figure 2.2. Different disease symptoms caused by various fungal diseases. A. Mango malformation. B. Anthracnose. C. Stem-end rot. (Kvas et al., 2008; Tavassoli-Kafrani et al., 2022)

Sooty blotch and flyspeck is a disease complex, caused by multiple fungi commonly infecting pome fruits and many wild plants, leading to disease symptoms of dark blotches and clusters of black dots on the fruit surface (Figure 2.3) (Gleason et al., 2019).

Figure 2.3. Sooty blotch and flyspeck disease symptoms on apples. (North Carolina State University, 2018).

The black blotches and blemishes on the surface are also signs of the pathogens, as these blotches represent the fungal colonies of SBFS fungi after the establishment of infection (Gleason et al., 2011). Similar SBFS symptoms observed on apple have also been reported previously on mango in the U.S. (Martinez et al., 2009) and Japan (Ajitomi et al., 2017). These SBFS symptoms reduce the aesthetic value of the crop, significantly impacting the quality and price of fresh fruit. Economic losses due to SBFS infection have been recorded in many countries, including the U.S. (Gleason et al., 2019), Turkey (Mayfield et al., 2013), Serbia (Ivanović et al., 2010), Germany (Batzer et al., 2016), China (Gao et al., 2015), Iran (Heidari et al., 2015), Poland (Mirzwa-Mróz and Winska-Krysiak, 2011), Norway (Batzer et al., 2015), Japan (Ajitomi et al., 2017), and Korea (Kwon et al., 2012), with varying economic impacts on apple production by country and region.

Infected fruits with SBFS can easily be confused with another disease called sooty mold. Sooty mold typically causes the leaf lamina (flat and broad part of a leaf that is responsible for most of the leaf's photosynthesis) to develop a thin, black, velvety membrane (Misra et al., 2012). The black membrane covers the entire leaf or appears as flakes on the leaf due to the presence of honeydew-secreting insects, such as mango hopper, scales, coccids, and mealy bugs. Sooty mold is a more serious issue in older, dense orchards with minimal sunlight and can lead to reduced fruit set and early fruit drop (Figure 2.4) (Misra et al., 2012). In dry conditions, affected leaves become curled and shrivelled, and spores are typically dispersed by wind or rain droplets (Chomnunti et al., 2014; Nelson, 2008).

Figure 2.4. Sooty mold disease. A. Mango leaves. B. Mango fruits. (Misra et al., 2012)

Unlike SBFS disease, sooty mold disease can be easily diagnosed by removing the black fungal growth on the leaves on plants using fingers. Therefore, sooty mold can be gently cleaned off using soapy water or diluted home bleach solution (Nelson, 2008).

2.3 Sooty blotch and flyspeck fungi

Sooty blotch and flyspeck disease complex was initially reported as a disease of apples caused by a single pathogen, *Dothidea pomigena* Schwein in Pennsylvania, U.S. (Batzer et al., 2005; Schweintz, 1832). The pathogen was later renamed by Saccardo (1883) as *Phyllachora pomigena* Schwein (Gleason et al., 2019). In the early 19th century, another fungus, *Leptothyrium pomi* (Mont. & Fr.) Sacc. was reported by Selby (1900) as the fungal pathogen of the disease (Gleason et al., 2019). The understanding of sooty blotch (SB) and flyspeck (FS) as diseases caused by two different pathogens was confirmed by Colby (1920), describing, SB, a disease caused by *Gloeodes pomigena* Schwein, leading to colonies with mycelial mats and sclerotium-like bodies, whereas FS colonies characterized by sclerotium-like bodies without mycelial mat (Gleason et al., 2019). Hesler and Whetzel (1971) determined that the fungal pathogens, *P. pomigena* and *L. pomi* are responsible for two diseases, SB and FS (Gleason et al., 2019).

The understanding of SB and FS were caused by only two different pathogens revised after Johnson et al. (1997) reported three fungal pathogens associated with SB disease in North Carolina, U.S.: *Leptodontium elatius*, *Peltaster fructicola* Eric M. Johnson, T.B. Sutton & Hodges, and *Geastrumia polystigmatis* Bat. & M.L. Farr. Flyspeck was further supported to be caused by a sole pathogen that was later renamed and transferred into a different genus, *Schizothyrium pomi* (Mont. & Fr.) by von Arx (1959) with the anamorphic stage described as *Zygophiala jamaicensis* E. Mason (Durbin 1953; Gleason et al., 2019).

For more than a hundred years, SB and FS had been regarded as two distinct diseases. However, with the complementary approach of DNA-based molecular works

to support the conventional morphology-based studies, the understanding of SBFS as a disease complex that is caused by multiple fungal species has been accepted by subsequent workers (Díaz Arias et al., 2010; Gleason et al., 2019). This disease complex model is associated with a wide spectrum of mycelial types on the surface of the host upon disease infection, with hundreds of described and putative species reported worldwide (Batzer et al., 2005).

Upon infection, SBFS fungal mycelia will colonize the fruit's surface and exhibit various colony appearances. Batzer et al. (2005) further characterized the different colonies observed on the apple fruits and grouped the colony appearances into different mycelial types, such as flyspeck, ramose, ridged honeycomb, punctate, and fuliginous having different characteristics (Figure 2.5).

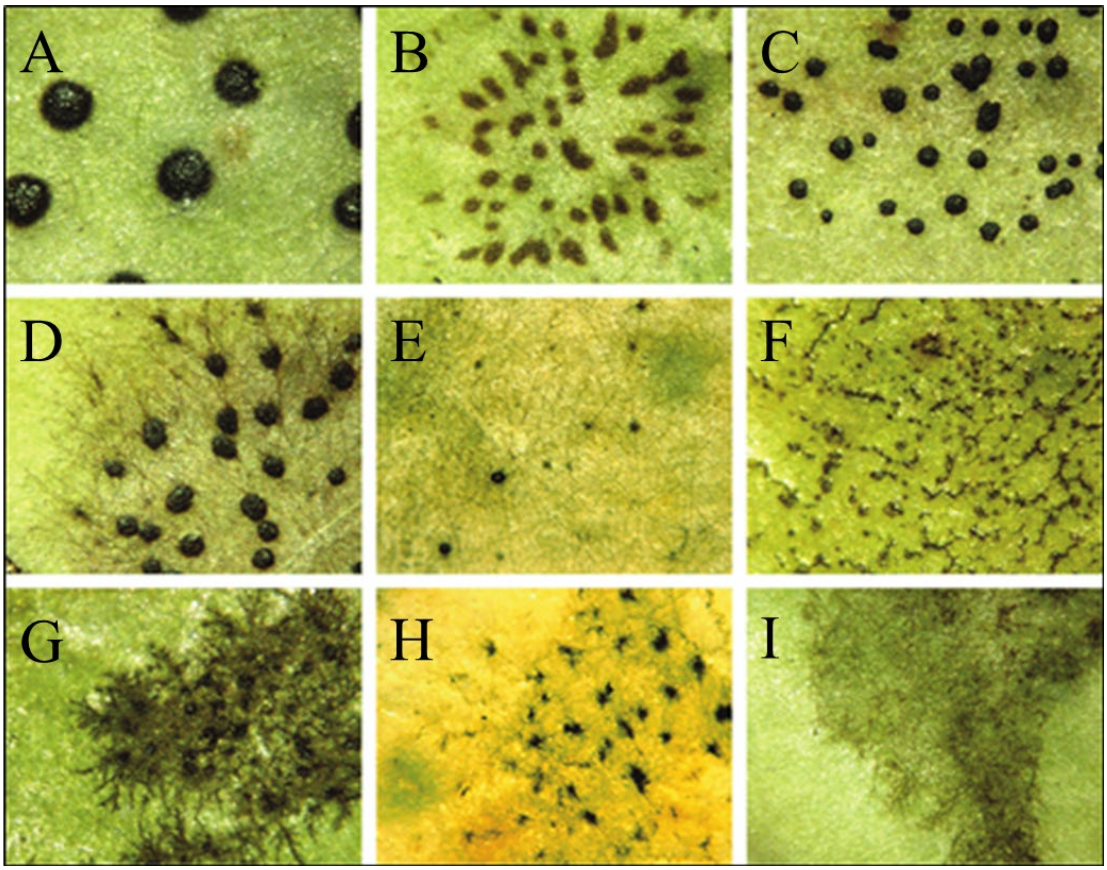

Figure 2.5. Different mycelial types of SBFS on apple peel. A. Flyspeck. B. Compact speck. C. Discrete speck. D. Ramose. E. Sparse ramose. F. Ridged honeycomb. G. Arborescent punctate. H. Punctate. I. Fuliginous. (Gleason et al., 2019)

Flyspeck mycelial type possess sclerotium-like bodies that are black, shiny, circular to oval, and flattened. These sclerotium-like bodies are clustered mycelia, which aid in attachment to the fruit surface (Xu et al., 2016). The sclerotium-like bodies of ramose colonies are bigger, shinier, and more convex towards the centre of the colony than at the margins. Ramose exhibits interconnecting cells in their sclerotium-like bodies. On the other hand, sclerotium-like bodies of punctate mycelial type are dull brown colour with many overlapping hyphal threads that is consistent across the mycelial mat. Rigid honeycomb is characterized by clumps and ridges of mycelia. Fuliginous exhibits uniform mats of mycelia, but edges of the colonies are varied from abrupt to feathery (Batzer et al., 2005). The mycelial types provide preliminary identification of the SBFS fungal species, although it is essential to note that multiple SBFS fungal species can share one mycelial type (Díaz Arias et al., 2010; Mayfield et al., 2013).

2.4 Sooty blotch and flyspeck infection

The disease cycle of SBFS species in the field has not been clearly outlined, as it appears to be species-specific and affected by various factors (Gleason et al., 2019). Generally, SBFS fungi overwinter on leaves, stems, twigs, infected fruit, and various other reservoir plant species (Gleason et al., 2011). In spring and early summer, conidia are dispersed by wind and rain to nearby fields with developing fruits, such as banana, mango, squash, and plum (Weber et al., 2016; Williamson & Sutton, 2000). Baines and Gardner (1932) observed the presence of mature pycnothyria, containing conidia of the SBFS fungus *G. pomigena*, on twigs of 10 different plant species in late May and early June. In contrast, Johnson et al. (1997) reported that mature pycnothyria of

P. fructicola were observed March to May, and continuously on apple fruit and blackberry stems throughout the growing season.

The timing of SBFS infection is also influenced by biographical regions. For example, fruits in the southeastern U.S. typically exhibit SBFS infection from late April to mid-May (Brown & Sutton, 1993, Latham & Hollingsworth, 1973 Williamson & Sutton, 2000). In contrast, the disease symptoms appear much later in northern regions, usually in June (Williamson & Sutton, 2000). Following infection, symptoms can manifest within 8 to 12 days (Sutton, 1990; Williamson & Sutton, 2000); however, some species may take 20 to 25 days for symptoms to appear. The initial signs of SBFS fungi on apple were reported in mid-June in Alabama (Latham & Hollingsworth,1973) and from early June to late July in North Carolina (Brown & Sutton, 1993; Brown & Sutton, 1993; Williamson & Sutton, 2000). Secondary infections are also common in SBFS. Conidia produced in sclerotium-like bodies on the infected plants can be dispersed by wind and wind-driven rain onto neighbouring hosts, initiating new infections during the growing season. These conidia then overwinter, perpetuating the infection cycle in the following growing season (Gleason et al., 2011).

Ascospores mature from the overwintering dormant stage and are released during a discrete period in spring, initiating primary infections on apples and reservoir hosts (Baker et al., 1977, Williamson & Sutton, 2000). The timing of ascospore maturity varies across seasons and geographical locations (Baines, 1940; Williamson & Sutton, 2000). Conidia trapping studies in North Carolina revealed that conidia were present from late May through mid-September, with the number of colonies and the percentage of affected fruits increasing until mid-September (Sutton, 1990). These studies also found that spore concentrations were positively correlated with temperature and wind

speed, and negatively correlated with relative humidity and leaf wetness. However, no significant correlation observed with rainfall (Sutton, 1990; Williamson & Sutton, 2000).

2.5 Colonization of SBFS

Research by Ismail et al. (2016) indicated that SBFS fungal lineages originated from plant-parasitic ancestors with common features, including the ability to produce cell-wall degrading enzymes. Unlike their ancestors, SBFS fungi adhere to surfaces of the host plants, either above or within the wax crystals of the epicuticular wax layer (Belding et al., 2000; Gleason et al., 2011). Due to this, SBFS complexes were labelled as 'epiphytic' for many years (Belding et al., 2000). These fungi do not primarily utilize epicuticular wax for energy but instead absorb sugar-rich fruit leachates (Belding et al., 2000; Xu et al., 2016). Generally, SBFS infection is more apparent near harvest due to the increase of sugar exudates, such as glucose and fructose that leak through the exterior peel as the fruits mature (Wrona and Grabowski, 2004). Batzer et al. (2010) reported the effects of different concentrations of nutrient leachates and SBFS fungal growth *in vitro*.

Previous confirmation of SBFS fungi's epiphytic niche was based on scanning electron microscopy (SEM) studies (Belding et al., 2000). However, Xu et al. (2016) expanded on these findings by examining pathogen-host interactions in greater detail. The study discovered that SBFS fungal colonies degrade the epicuticular wax layer of apple fruits more extensively than previously described. Importantly, these SBFS fungi do not penetrate the epidermal cell wall. These observations led to the introduction of the term 'ectophytic' to describe the parasitic lifestyle of SBFS fungi, which is distinct form mere epiphytic surface colonization (Xu et al., 2016) (Figure 2.6).

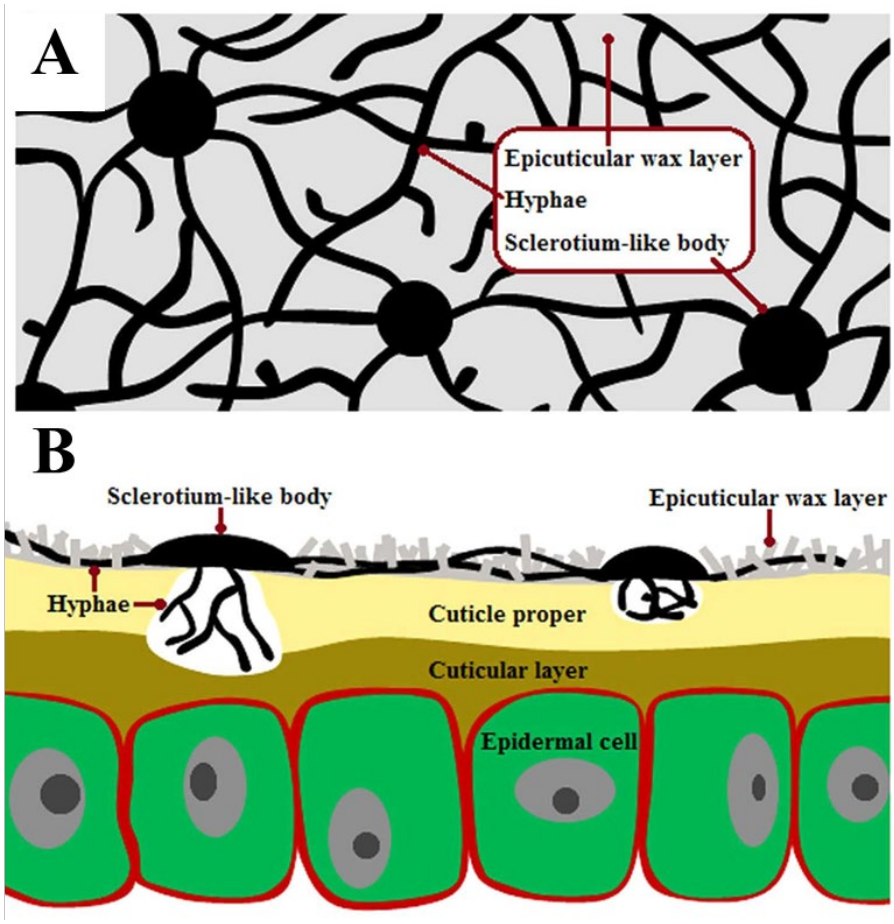

Figure 2.6. Schematic diagram of the SBFS-infected apple fruit surface. A. Top view of *Peltaster fruticola* development on the surface of apple. B. Cross-section view of *P. fruticola* on the surface of apple. (Xu et al., 2016)

Xu et al. (2016) conducted comparative genomic studies, focusing on *P. fructicola*, the most predominant SBFS fungus. The study revealed that *P. fructicola* exhibited specific genetic adaptations to its ectophytic lifestyle. Notably, genes responsible for melanin biosynthesis and appressorium formation were upregulated. This adaptation enhances fungal attachment to the fruit surface and improves access to fruit leachates. However, Xu et al. (2016) also found a significant reduction in the genes encoding enzymes responsible for typical pathogenicity traits, such as plant cell wall degradation, synthesis and transport of several biomolecules, and secondary metabolism. Instead, the gene encoding the cutinase enzyme was upregulated, indicating the ability of *P. fructicola* to penetrate the cuticle proper layer of the host plant but not the cell wall (Figure 2.6).

The research by Xu et al. (2016) also shed light on the genomic characteristics of *P. fructicola*. This SBFS fungal species had smaller genomes with decreased repeat content and a reduced number of genes compared to related plant-penetrating parasite (PPP) fungal species. These genomic adaptations suggest convergent evolution tailored to its ectophytic niche, enabling *P. fructicola* to thrive and survive on various hosts.

2.6 Characterizing SBFS fungi

The isolation of SBFS fungi is often a challenging task, primarily because the standard surface sterilization technique commonly used for fungal isolation can reduce the viability of SBFS fungi (Gleason et al., 2019). Additionally, many SBFS fungi have slow growth in culture, and some are difficult to sporulate. Therefore, SBFS fungal isolation is commonly carried out using water agar (WA) amended with lactic acid. Lactic acid serves the purpose of inhibiting the sporulation of bacteria and slowing down the growth of some non-SBFS fungi (Batzer et al., 2005; Batzer et al., 2022).

Colony morphology, such as mycelial types on the surface of the host, plays a crucial role in categorizing SBFS fungi into distinct groups of species associated with specific mycelial types. For example, *Zasmidium*, *Dissoconium*, and *Devresia* species share the same discrete speck mycelial type (Batzer et al., 2015; Li et al., 2013; Zhao et al., 2016). In contrast, *Peltaster* and *Ramularia* species exhibit a punctate mycelial type (Díaz Arias et al., 2010). However, some controversy arises when different species within the same fungal genus have various mycelial types, such as *Dissoconium* species can have both discrete speck and fuliginous mycelial types (Batzer et al., 2005; Díaz Arias et al., 2010). In the U.S. and China, *Uwebraunia*

commune Crous & Mansilla is documented as having a ramose mycelial type, whereas *Uwebraunia dekkeri* de Hoog & Hijwegen displays a punctate mycelial type (Li et al., 2012).

The morphology of SBFS fungi can vary significantly depending on the growth media and cultural conditions, such as carbon source and concentration, pH, light source, and temperature (Gleason et al., 2011). According to Madeiras (2014), the growth of the colony diameter was greater on PDA, where readily available monosaccharides are more abundant compared to malt extract agar (MEA) and WA. The *G. polystigmatis* colonies exhibited dense growth and melanisation on 50% PDA and MEA but grew sparsely and less melanisation on WA. Wrona and Grabowski (2004) highlighted that when initial signs of sooty blotch appeared, there was a noticeable increased in the levels of glucose and fructose found in both the skin of apples and their juice. Batzer et al. (2010) suggested that SBFS fungi utilize the sugars in fruit exudates and an increase in apple juice concentration is associated with enhanced melanisation of some SBFS colonies.

Gleason et al. (2019) reported that pH also exerts an impact on the growth of SBFS. For example, SBFS species *P. fructicola* thrived in culture media with a pH range of 4 to 6. However, the growth became significantly lower at pH levels below 4 or above 6. In contrast, *Ramichloridium luteum* G.Y. Sun, H.Y. Li & Crous exhibited a much wider pH tolerance, surviving in media with a pH range from 1 to 8 (Gleason et al., 2019). However, both *P. fructicola* and *R. luteum* were found to optimally grow at pH 4, a condition similar to the surface pH of apples (Glass et al., 2015; Gleason et al., 2019).

The optimal temperature for the mycelial growth varies among the SBFS species (Batzer et al., 2010; Johnson and Sutton, 2000). A study conducted by Batzer et al. (2010) found that different temperature ranges had varying impacts on the growth response of different SBFS species: *Dissoconium aciculare* de Hoog, Oorschot & Hijwegen was able to grow at 10 and 15 °C, whereas the growth of *P. fruticola* was inhibited at 10 °C (Batzer et al., 2010). These differences in environmental requirements for optimal growth contribute to the observed species diversity among various geographical locations. For example, a survey carried out in 14 states in the U.S. in 2000 and 2005 revealed that *D. aciculare* was only found in the northern regions of the U.S., with temperatures are cooler at approximately 10 °C, while *P. fructicola* was prevalent in the warmer southern states (Díaz Arias, 2007; Díaz Arias et al., 2010). Other species, such as *L. elatius* and *Zasmidium angulare* Batzer & Crous were frequently found in temperate regions with high humidity (Li et al., 2012; Johnson, 1994). In China, *Zygophiala* species and *Devriesia strelitziae* Arzanlou & Crous were predominant in some regions but not in others (Gao et al., 2014; Li et al., 2013). As a result, different climates favour the growth and distribution of specific SBFS species, which contribute to the biogeographic patterns of SBFS species (Batzer et al., 2010; Gleason et al., 2011).

Identifying SBFS fungal species solely on morphological characters is challenging due to the presence of cryptic species among SBFS fungi (Gleason et al., 2019). Therefore, many studies have incorporated molecular studies to support the identification made through morphological observations. Duttweiler et al. (2008) employed the use of the restriction fragment length polymorphism (RFLP) method to rapidly identify SBFS fungi directly from mycelium scraped from the apple surface. The RFLP assay used a reverse primer of ITS gene that was designed to bind

specifically to the SBFS fungi that are most frequently encountered, coupled with a common forward primer for fungi. The resulting DNA fragment was then digested using restriction enzymes, such as *Hae*III or *Alu*I (Duttweiler et al., 2008). However, RFLP assays have been reported to be inconsistent in delineating the species (Hashim and Al-Shuhaib, 2019), and it is recommended to use additional molecular markers to accurately characterize SBFS fungi into distinct species. The PCR-RFLP technique has limitations in detecting nucleotide variations, as each restriction enzyme only recognizes specific sequences. Consequently, the PCR-RFLP may overlook or miss other sequences that contain variations, which explains why such variations are often not detected by this method (Hashim and Al-Shuhaib, 2019).

Some of the molecular markers commonly used for DNA-sequencing of SBFS fungi include the LSU and ITS regions of rDNA (Batzer et al., 2005). Newly described species of SBFS fungi that have been described using ITS and LSU genes, such as *P. cerophilus* Jana Frank & Schroers, *Microcyclosporella mali* J. Frank, Schroers & Crous, and *Zygophiala wisconsinensis* Batzer & Crous (Batzer et al., 2005; Díaz Arias et al., 2010; Mayfield et al., 2013). Partial translation elongation factor 1-alpha (TEF), β-tubulin (TUB2) actin (ACT) and mitochondrial small subunit (mtSSU) are some of additional genes that were previously used by various studied describing several SBFS species, such as *Devriesia* species. (Li et al., 2013), *P. gemmifer* H. Rosli & J.C. Batzer (Rosli et al., 2018), *Scolecobasidium musae* G.Y. Sun & Lu Hao (Hao et al., 2013), and *Zasmidium litsease* G.Y. Sun & Wanyu Zhao (Zhao et al., 2016).

2.7 Phylogenetic studies of SBFS fungi

Phylogenetic and molecular analysis using MEGA V7.0 (Kumar et al., 2016) has become a common practice for reconstructing phylogenetic trees and determining

evolutionary distances among various fungi (Kumar et al., 2016). Phylogenetic tree construction is employed to differentiate and identify closely related fungal that belong to the same common descent (Gregory, 2008).

The fundamental methods for constructing phylogenetic trees can be categorized into two main groups: distance-based methods and character-based methods (Scott and Gras, 2012). The distance-based methods, including neighbour-joining (Saitou and Nei, 1987; Scott and Gras, 2012) and unweighted pair group method with arithmetic (UPGMA) (Scott and Gras, 2012; Sneath and Sokal, 1973) are based on the estimation of the pairwise distances between each sequence. Maximum parsimony (MP) analysis is one of the common character-based methods in phylogenetics, aiming to construct a tree that requires the fewest number of evolutionary changes to explain the observed differences between taxa. The algorithm assumes that the simplest explanation is the most likely to be correct, thus seeking to minimize the number of evolutionary events required to explain the observed data (Sober, 1987).

Maximum likelihood (ML) is also a character-based method and a well-known technique for determining the most probable evolutionary tree for a set of genetic sequences. It involves constructing a statistical model of how sequences evolve and then calculating the likelihood of observing the sequences given a particular tree structure. ML aims to identify the tree that best describes the data by comparing various tree topologies and branch lengths. Compared to MP, ML is a robust and popular technique capable of handling complex evolutionary models and providing precise estimates of the evolutionary relationships among different species (Cho, 2012; Dhar and Minin, 2015).

Phylogenetic analysis using MP and ML have played a vital role in species description and identification in SBFS research. For example, Medjedović et al. (2014) described *P. cerophilus* as new species after observing distinct morphological characteristics and separate clade groupings of *P. cerophius* from *P. fructiola* species from the U.S., based on the phylogenetic analysis using MP. The recently discovered *P.gemmifer* species was also distinguished using MP analysis, placing it in a separate group from other *Peltaster* species (Rosli et al., 2018). *Microcyclosporella mali* and *Z. jamaicensis* were first reported in Iran, supported by the MP analysis comparing several SBFS species from various countries (Heidari et al., 2015). The use of ML and MP analyses in SBFS diversity studies has been conducted in various countries, such as Turkey (Mayfield et al., 2013), Germany (Batzer et al., 2016), and Spain (Batzer et al., 2022).

2.8 Pathogenicity test of SBFS fungi

In the late 18th century, Koch's postulates were introduced to confirm the pathogenicity of a microorganism towards a disease on a host. Koch's postulates comprise of the following criteria (Segre, 2013):

1. The microorganism must be found in diseased but not healthy ones.
2. The microorganism must be cultured from the diseased individual.
3. Inoculation of a healthy individual with the cultured microorganism must recapitulate the symptoms.
4. The microorganism must be re-isolated from the inoculated diseased individual and similar as the original.

Pathogenicity confirmation of SBFS fungi have been conducted both *in situ* in the orchard and on detached fruit. Several methods for orchard inoculation of SBFS fungi have been documented. Batzer et al. (2005) inoculated apple fruits in the orchard by swabbing fungal inoculum onto the apple surface, while Rosli et al. (2020) used hand spray to apply the fungal inoculum; each hemisphere of the apple was sprayed twice until runoff. In both studies, sterile distilled water was used as control treatment and fruit bags were used to prevent colonization of ambient SBFS inoculum on the inoculated fruits. When preparing conidial suspension for orchard inoculation, it is recommended to use the conidial suspension within 2 hours after preparation to maintain the viability of the conidia (Batzer et al., 2005). Rosli et al. (2020) on the other hand, stored the conidial suspension at 5 °C and used the suspensions for orchard inoculation within 12 hours after preparation.

The *in situ* pathogenicity test of SBFS fungi was previously described by Gao et al. (2014) and Kwon et al. (2012). Conidial suspension was swabbed using Chinese

brush on apple surface before the fruit were transferred to an incubator with the condition of 12 h fluorescent light at 28 ℃ and 85% relative humidity (RH) alternated with 12 h darkness at 25 ℃ with 100% RH for 6 weeks (Gao et al., 2015). Kwon et al. (2012) used cotton swab to inoculate conidial suspension onto apple surface before the inoculated fruits were incubated in a moist chamber, maintained at 22 ℃ for one month. Koch's postulates in both studies were fulfilled by either comparing the morphology characteristic of the re-isolated SBFS isolate on the inoculated fruits with the original inoculated isolate or comparing the ITS sequences between the two isolates (Gleason et al., 2011; Gleason et al., 2019). The pathogenicity tests of SBFS fungi have been conducted in previous studies on several SBFS diversity studies in various countries, such as the U.S. (Rosli et al., 2020), China (Gao et al., 2014), Bulgaria (Piperkova and Yonkova, 2014), and Korea (Kwon et al., 2012). Some of the recently described SBFS fungi confirmed to be pathogens through pathogenicity tests include *Cyphellophora phyllostachysdis*, *C. artocarpi*, *C. musae*, *Dissoconium* species, *Microcyclosporella mali*, *Peltaster gemmifer*, and *Stomiopeltis* species, and *Zygophiala wisconsinenesis* (Gao et al., 2015; Kwon et al., 2021, Rosli et al., 2020).

2.9 Economic impact of SBFS disease

The presence of dark blotches and clusters of black dots on the fruit surface, caused by SBFS infection reduces the fresh-market sales, resulting in a significant economic impact on the revenue of many growers (Gleason et al., 2019; Williamson and Sutton, 2000). In the U.S., diverting SBFS-infected apples to processing, such as converting diseased apples into juice, sauce, and pies serves to salvage some income. However, the revenue losses incurred by diverting fresh fruit from high-value apple varieties to processing can amount to as much as 90% of their earnings (Gleason et al., 2019; Williamson and Sutton, 2000). Apple orchards that employed comprehensive