



**BIOCONTROL POTENTIAL OF INDIGENOUS FUNGI AGAINST
MYCOTOXIGENIC FUNGI AND MYCOTOXIN CONTAMINATION IN GRAIN
MAIZE AGRO-ECOSYSTEMS IN TERENGGANU, MALAYSIA**

By

SITI NUR EZZATI BINTI YAZID

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Doctor of Philosophy**

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Biological control against mycotoxigenic fungi and the subsequent mycotoxins have been widely studied, and some are also commercially available in many countries, but the same cannot be said in Malaysia. Therefore, the present study aimed to identify novel indigenous fungal biocontrol agents against mycotoxigenic fungi and their subsequent mycotoxin production in pioneer grain maize farms in Malaysia. The objectives of the present study were: (1) to establish the mycoidiversity of indigenous mycotoxigenic fungi and potential biocontrol agents from grain maize agro-ecosystem, (2) to molecularly and toxigenically differentiate the indigenous *Aspergillus flavus* isolates from grain maize agro-ecosystem, (3) to screen the antagonism of potential biocontrol agents against isolated indigenous mycotoxigenic fungi from grain maize agro-ecosystem, and (4) to determine effective biocontrol agents in controlling multi-mycotoxin production by indigenous mycotoxigenic fungi from grain maize agro-ecosystem using different pathogen:antagonist inoculum ratios. Fungi

isolated from two pioneer grain maize farms in Terengganu were morphologically and molecularly identified. Mycotoxigenic potential of all mycotoxigenic isolates were also examined using high performance liquid chromatography (HPLC). Next, seven *A. flavus* isolates obtained from the farms were characterised molecularly and chemically by examining for the presence of aflatoxin biosynthesis genes, and confirmed their aflatoxigenicity using HPLC. Phylogenetic relationships of all *A. flavus* isolates were validated. Thereafter, dual-culture assay was conducted on grain maize agar (GMA) with 12 isolates of potential fungal antagonists against mycotoxigenic isolates producing aflatoxins, ochratoxin A, and fumonisins, respectively. Five pathogen:antagonist inoculum ratios – 100:0, 75:25, 50:50, 25:75, and 0:100, were then employed to examine mycotoxin reduction by selected fungal antagonists on GMA and grain maize kernels. Fungal isolation from two pioneer grain maize farms revealed among 30 species isolated, the mycotoxigenic *Fusarium verticillioides* was predominant. Other fungal isolates that could have potentials as biocontrol agents (e.g., *Trichoderma* spp.) were also isolated. The selected *A. flavus*, *A. niger*, *F. verticillioides*, and *F. proliferatum* isolates that could produce aflatoxins (AFBs), fumonisins (FBs), and ochratoxins A (OTA), respectively, were used for the subsequent experiments. Next, the identity of seven *A. flavus* isolates were taxonomically resolved. Among these isolates, five were aflatoxigenic and two were non-aflatoxigenic. Two non-aflatoxigenic isolates were examined for AFB₁ reduction against the aflatoxigenic counterparts. Although both non-aflatoxigenic showed weak antagonistic activity against growth of the aflatoxigenic isolates, all could significantly ($p < 0.05$) reduce AFB₁. Among 12

potential fungal antagonists, *Trichoderma* spp. exhibited the highest inhibitory activity against growth of the tested mycotoxigenic isolates. Against OTA and fumonisins, the efficacy of *T. asperelloides* Ta39KR was demonstrated for the first time. Against AFB₁, the efficacy of *Penicillium janthinellum* Pj46SD was also demonstrated for the first time. The pathogen:antagonist inoculum ratios assay showed non-aflatoxigenic *A. flavus* Af1KD as the most effective against AFBs. *T. asperelloides* Ta39KR also effective against FBs. The indigenous *P. janthinellum* Pj46SD and *T. asperelloides* Ta39KR have potential to be developed as novel biocontrol agents against mycotoxigenic fungal growth and their subsequent aflatoxins, ochratoxin A, and fumonisins, respectively.

Keywords: biocontrol, grain maize, indigenous fungi, mycotoxigenic fungi, mycotoxin

SDG: GOAL 2: Zero Hunger, GOAL 3: Good Health and Well-being, GOAL 12: Responsible Consumption and Production

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**POTENSI KAWALAN BIOLOGI KULAT TEMPATAN TERHADAP KULAT
BERMIKOTOKSIN DAN PENCEMARAN MIKOTOKSIN DALAM
EKOSISTEM-TANI JAGUNG BIJIAN DI TERENGGANU, MALAYSIA**

Oleh

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Kawalan biologi terhadap kulat bermikotoksin dan mikotoksin yang terhasil telah banyak dikaji, malah ada yang dikomersialkan di banyak negara, namun keadaan yang sama tidak berlaku di Malaysia. Oleh itu, kajian ini bertujuan untuk mengenal pasti agen kawalan biologi kulat tempatan yang baharu terhadap kulat bermikotoksin dan penghasilan mikotoksinnya di ladang perintis jagung bijian Malaysia. Objektif kajian ini adalah: (1) menetapkan mikodiversiti kulat bermikotoksin tempatan dan agen kawalan biologi berpotensi dari ekosistem-tani jagung bijian, (2) membezakan secara molekul dan toksigenik isolat *Aspergillus flavus* tempatan dari ekosistem-tani jagung bijian, (3) menyaring antagonisme agen kawalan biologi berpotensi terhadap kulat bermikotoksin tempatan yang diasingkan dari ekosistem-tani jagung bijian, dan (4) menentukan agen kawalan biologi yang berkesan dalam mengawal penghasilan multi-mikotoksin oleh kulat bermikotoksin tempatan dari ekosistem-tani jagung bijian menggunakan nisbah inokulum

patogen:antagonis yang berbeza. Kulat yang diasingkan dari dua ladang perintis jagung bijian di Terengganu telah dikenal pasti secara morfologi dan molekul. Potensi mikotoksigenik semua isolat bermikotoksin juga diuji menggunakan kromatografi cecair berprestasi tinggi (HPLC). Seterusnya, tujuh isolat *A. flavus* yang diperoleh dari ladang-ladang tersebut telah dicirikan secara molekul dan kimia dengan mengkaji kehadiran gen biosintesis aflatoksin, dan keupayaan penghasilan aflatoksin mereka disahkan menggunakan HPLC. Hubungan filogenetik semua isolat *A. flavus* telah disahkan. Selepas itu, ujian dwi-kultur dijalankan pada agar jagung (GMA) dengan 12 isolat kulat antagonis tempatan yang berpotensi terhadap isolat bermikotoksin yang menghasilkan aflatoksin, okratoksin A, dan fumonisin. Lima nisbah inokulum patogen:antagonis – 100:0, 75:25, 50:50, 25:75, dan 0:100, telah digunakan untuk menilai pengurangan mikotoksin oleh kulat antagonis yang terpilih pada GMA dan biji jagung. Pengasingan kulat dari dua ladang perintis jagung bijian menunjukkan bahawa daripada 30 spesis yang diasingkan, kulat bermikotoksin *Fusarium verticillioides* adalah yang paling dominan. Kulat-kulat lain yang berpotensi sebagai agen kawalan biologi (contohnya, *Trichoderma* spp.) juga telah diasingkan. Sebahagian isolat *A. flavus*, *A. niger*, *F. verticillioides*, dan *F. proliferatum*, yang masing-masing dapat menghasilkan aflatoksin (AFBs), fumonisin (FBs), dan okratoksin A (OTA) digunakan untuk eksperimen seterusnya. Identiti tujuh isolat *A. flavus* telah diselesaikan secara taksonomi. Daripada isolat tersebut, lima adalah aflatoksigenik manakala dua lagi bukan-aflatoksigenik. Dua isolat aflatoksigenik telah dikaji lebih lanjut bagi menentukan pengurangan AFB₁ oleh isolat bukan-aflatoksigenik. Walaupun kedua-dua isolat bukan-

aflatoksigenik menunjukkan aktiviti antagonis yang lemah terhadap pertumbuhan isolat aflatoksigenik, kesemuanya berjaya ($p < 0.05$) mengurangkan AFB₁ secara ketara. Daripada 12 kulat antagonis yang berpotensi, *Trichoderma* spp. menunjukkan perencatan tertinggi terhadap pertumbuhan isolat bermikotoksin yang diuji. Terhadap OTA dan fumonisin, keberkesanan *T. asperelloides* Ta39KR ditunjukkan buat kali pertama. Terhadap AFB₁, keberkesanan *P. janthinellum* Pj46SD juga ditunjukkan buat kali pertama. Ujian nisbah inokulum patogen:antagonis menunjukkan bahawa *A. flavus* Af1KD bukan-aflatoksigenik adalah antagonis yang paling berkesan terhadap AFBs. *T. asperelloides* Ta39KR juga paling berkesan terhadap FBs. Isolat tempatan *P. janthinellum* Pj46SD dan *T. asperelloides* Ta39KR berpotensi untuk dibangunkan sebagai agen kawalan biologi yang baharu terhadap pertumbuhan kulat bermikotoksin serta aflatoksin, okratoksin A, dan fumonisin.

Kata kunci: jagung bijian, kawalan biologi, kulat bermikotoksin, kulat tempatan, mikotoksin

SDG: MATLAMAT 2: Kelaparan Sifar, MATLAMAT 3: Kesihatan Baik dan Kesejahteraan, MATLAMAT 12: Penggunaan dan Pengeluaran Bertanggungjawab

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*“...no man is an island, entire of itself; every man is a piece of the continent,
a part of the main...”*

John Donne, Meditation 17 of Devotions upon Emergent Occasions, 1624

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LIST OF ABBREVIATIONS

A.D.	Anno Domini (in the year of the Lord)
AFB ₁	Aflatoxin B ₁
AFB ₂	Aflatoxin B ₂
AFG ₁	Aflatoxin G ₁
AFG ₂	Aflatoxin G ₂
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
B.C.	Before Christ
BLAST	Basic Local Alignment Search Tool
BRIS	Beach Ridges Interspersed with Swales
CCA	Coconut Cream Agar
CFU	Colony Forming Unit
DG-18	Dichloran 18% Glycerol
DDGS	Distillers' Dried Grains with Soluble
DON	Deoxynivalenol
DRBC	Dichloran Rose Bengal Chloramphenicol
<i>et al.</i>	<i>et alia</i> (and others)
<i>e.g.</i>	<i>exemplia gratia</i> (for example)
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
GMA	Grain Maize Agar
GAP	Good Agricultural Practices
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
<i>i.e.</i>	<i>id est</i> (that is)
IPM	Integrated Pest Management
ITS	Internal Transcribed Spacer
LOD	Limit of Detection
LOQ	Limit of Quantification
MEA	Malt Extract Agar
NCBI	National Centre for Biotechnology Information

OPA	<i>ortho</i> -phthalaldehyde
OTA	Ochratoxin A
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PHRED	Photochemical Reactor for Enhanced Detection
PIRG	Percentage Inhibition of Radial Growth
RSD	Relative Standard Deviation
SNP	Single Nucleotide Polymorphism
UN	United Nations
USDA	United States Department of Agriculture
VCG	Vegetative Compatibility Group
WHO	World Health Organisation
ZEN	Zearalenone

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Feeding the global population of nearly eight billion people is indeed the most daunting challenge facing mankind in the modern world. Food security, which is defined as “...when all people, at all times, have physical, social and economic access to sufficient, safe, and nutritious food which meets their dietary needs and food preferences to support an active and healthy life...”, is the primary concern in subduing that challenge (Food and Agriculture Organisation, 2009). Since time immemorial, agriculture and aquaculture have been, and still are, the primary nutritional supports to mankind, and acting as the first pillar of food security which is food availability. The other three pillars are food accessibility, food utilisation, and food stability. However, the continual expansion of human population and competition for land, water, and energy will eventually and inevitably impair our agricultural ability and capacity to produce sufficient food to feed the increasing global demand (Godfray *et al.*, 2010). What is more, the global agricultural sector is constantly left at the mercy of its old archenemy– the pests! It was recently estimated that up to 40% crop yields globally and approximately 40 to 50% crop yields in the developing world are wastefully lost to pests and crop diseases during pre-harvest as well as post-harvest (National Institute of Food and Agriculture, 2015).

Pest is a broad term frequently used and defined as any species or strain of living organism (e.g., animals, plants, microorganisms), usually occurring in high density at a specific niche, which brings upon detrimental and injurious effects towards humans, animals or plants. In agriculture, pests are regarded as the key biotic component which can adversely impact on the agricultural yields and productivity along the supply chain, with the major abiotic component being the climate. Pests, particularly phytopathogenic fungi, have contributed to the most devastating and largest crop losses, with an estimated value of 70 to 80% worldwide (Peng *et al.*, 2021). Phytopathogenic fungi invade and infect plant hosts by killing them (necrotrophs) and feed on the dead material, or by colonising the living tissue (biotrophs). Phytopathogenic fungi have caused, among others, anthracnose, blight, canker, damping-off, dieback, mildew, rust, and wilt in root, seed, seedling, leaf, stem, and ear of many economically-relevant agricultural crops (Doehlemann *et al.*, 2017).

Apart from causing plant diseases, some fungi namely mycotoxigenic fungi can also produce toxic secondary metabolites known as mycotoxins. These mycotoxigenic fungal species infect damaged (as opportunistic pathogen, such as *Aspergillus* spp.) or healthy (as endophyte, such as *Fusarium* spp.) plant parts, before proliferating and producing mycotoxins under suitable environmental conditions, which leads to primary infection to the plants. Some mycotoxigenic fungi that could withstand storage conditions post-harvest, could survive as spores in the tissue of agricultural crop commodities, before germinating, proliferating, and further producing mycotoxins. Since mycotoxins are thermally- and chemically-stable, and can persist during many industrial

processing and temperatures along the production chain, there is risk of them contaminating the final food and feed products. This further exposes humans and animals to the threats of mycotoxins otherwise known as secondary exposure to the final consumers. Mycotoxins are also carcinogenic, hepatotoxic, nephrotoxic, mutagenic, teratogenic, and in some cases, can even cause death to both humans and animals at wide-ranging concentrations (Bennett & Klich, 2003).

Grain maize is a feed crop, and has been cultivated worldwide. In Malaysia, unlike sweet maize intended for human consumption and have establish its own market niche, the mass-cultivation and commercialisation of grain maize particularly for local commercialisation and consumption for the ruminant and poultry industries, has just begun (Anonymous, 2016). This cultivation effort, which was initiated by the Malaysian government, is aimed to reduce the importation of grain maize and grain maize-based products, with the final objective to improve local food security. To strengthen this effort, the government lays Strategy 5 (*i.e.*, Reduce Dependency on Imported Feed), under the strategies for livestock subsector in National Agrofood Policy 2.0 (Department of Agriculture, 2021). Besides, in the Malaysian 2018 budget, the government gazetted grain maize as one of the five new wealth-generating commodities alongside coconut, durian, dairy, and culinary industries (Bank Negara Malaysia, 2017).

The pioneer project for grain maize cultivation began in year 2016 at the states of Terengganu, Kedah, and Perak, with Terengganu having the highest

cultivated area (*i.e.*, 18.54 hectares). From 2017 until 2023, this cultivation was expanded to the other nine states of Malaysia, namely Perlis, Kelantan, Pahang, Selangor, Melaka, Negeri Sembilan, Johor, Sabah, and Sarawak. The largest cultivated area during the period was in the year 2018, of approximately 387.25 hectares. During and post- COVID-19 pandemic era showed decline in cultivation area of approximately less than 100 hectares. During those periods, there were also decline in the annual grain maize production, however the production maintained at above 70 tonnes. It was estimated that the total domestic production of grain maize in 2017 – 2023 reached approximately 1,829.99 tonnes (Department of Agriculture, 2023; 2020). Although not as big as the sweet maize, the domestic production and market size of grain maize seems promising. Under the Grain Maize Development Master Plan 2018 – 2032, the production has been targeted to reach 30% of the grain maize domestic requirement (of approximately 1.4 million tonnes) for animal feed (Rosali *et al.*, 2019).

However, like other cereals which are good sources of carbohydrates and energy, grain maize is naturally susceptible to fungal contamination and the subsequent mycotoxin contamination. Furthermore, Malaysian tropical weather of hot and humid will constantly expose grain maize to the threat of fungal infestation and mycotoxin contamination, particularly mycotoxigenic species from *Aspergillus* section *Flavi* (*e.g.*, *A. flavus* and *A. parasiticus*) and *Fusarium* section *Liseola* (*e.g.*, *F. proliferatum* and *F. verticillioides*) (Uka *et al.*, 2019; Alberts & Gelderblom, 2016). The constant hot and humid weather and compounded by the longer planting and maturation period of grain maize

than the sweet maize (approximately 150 days for the former, and 70 days for the latter) (Zhao *et al.*, 2012), provide a longer window of time for the development of outbreak and disease severity within the grain maize farms (Singh *et al.*, 2023). Damages and diseases caused by mycotoxigenic fungi as well as the threats from mycotoxins will certainly hamper the government's effort in achieving food security, especially in the ruminant and poultry industries.

As if the ever-presence of fungal contaminants and the production of their dangerous mycotoxins on grain maize and its products is not severe enough, the population especially the agricultural community is faced by the imminent threat of climate change scenarios which positively affect fungal proliferation and mycotoxin production in habitats otherwise unfavourable for the fungal contaminants (Paterson & Lima, 2010). Therefore, a proper control method is not only timely, but urgently warranted. These control measures are generally divided into physical, chemical, and biological, and have been employed either individually or collectively as part of Integrated Pest Management (IPM) and Good Agricultural Practices (GAP).

In controlling mycotoxigenic fungi and mycotoxin production, however, physical control is often ineffective at large-scale (too time- and energy-consuming), or during which the fungal contamination is already rampant (Sweany, 2019). So, farmers often turn to chemical control (use of pesticides). Although chemical control is generally effective and rapid, it has been associated with many flaws and drawbacks. Many compounds in chemical

pesticides are toxic to humans, animals, and the environment, and their presence in agricultural commodities intended as foods and feeds are rigorously monitored by existing legislations. The excessive usage of pesticides could also stimulate pesticide resistance among the targeted pathogens which will exacerbate the problem of pests and pathogens (Bommarco *et al.*, 2011). Therefore, biological control, or simply biocontrol, provides an alternative against the harmful usage of chemical pesticides and laborious physical methods. Against mycotoxigenic fungi and mycotoxins, biocontrol has been studied, patented, and applied worldwide. The most promising and effective biocontrol agent currently in use is the non-aflatoxigenic *Aspergillus flavus* which is often applied to mitigate the infection of its aflatoxigenic counterpart and its subsequent aflatoxin production. This type of biocontrol agents has been applied in many parts of the world, particularly in the United States of America, and many African countries (Moore *et al.*, 2022).

1.2 Problem statement

Mycotoxigenic fungi and mycotoxins are ever-present, and their contamination of crop commodities intended for food and feed products will threaten the food safety and food security in Malaysia. At present, the laborious physical control and hazardous chemical control are insufficient in mitigating mycotoxigenic fungal proliferation and mycotoxin contamination. Although biological control could be an excellent solution, and the fact that it has been successfully applied outside of Malaysia, its research and development in Malaysia is,

unfortunately, still in infancy. Since the first introduction of biocontrol in Malaysia in 1970s (Simmonds, 1980), only a handful of publications (less than ten) are available on controlling fungal growth and mycotoxin production as compared to controlling other plant pathogens and diseases. Additionally, as earlier mentioned, Malaysia's hot and humid tropical climate will certainly exacerbate fungal infestation in economically-relevant agricultural crops. This then provides a favourable condition for mycotoxin production during pre- and post-harvest. This is evidenced by the breadth of literature which reports the incidence of mycotoxins in various Malaysian agricultural commodities and their products.

Due to the imminent threat of climate change scenario which will allow the propagation and infestation of fungal pathogens in otherwise unfavourable habitats, the application of biocontrol agents thus seems plausible. However, one question remains, *"...if Malaysia has not developed a suitable local biocontrol agent, can we just export it from abroad, since success stories on this application over there are abound?..."*. The answer, unfortunately, must be "no". Although there is a possibility of using exotic/foreign biocontrol agents in our local climate, habitat, and niche, these agents have been associated with many issues that will raise concerns over their usage locally. Firstly, as the name implies, exotic/foreign agents are isolated from different climates, regions, and ecosystems from Malaysia; therefore, they may either take too long to adapt, or altogether fail to be established when applied under Malaysia's locale (Moore *et al.*, 2022). Secondly, there is high risk that these exotic agents turn invasive (*i.e.*, aggressive or uncontrollable spread or

dispersion), and attack on the non-target organisms instead, therefore disrupting the local ecological balance, thus defeating the purpose of their introduction on Malaysian ecosystems (Hajek & Eilenberg, 2018). Therefore, more research on locally-isolated biocontrol agents is unequivocally required to mitigate the risk of introducing foreign agents.

Grain maize is the commodity of choice for isolation of the potential indigenous biocontrol agents against the indigenous mycotoxigenic fungi and mycotoxin contamination because it has been highly implicated with the said pathogens and their subsequent mycotoxins in many parts of the world (Atnafu *et al.*, 2024; García-Díaz *et al.*, 2020; Rodrigues & Naehrer, 2012). Since the mass-cultivation and commercialisation of grain maize in Malaysia which started in the year 2016 are still growing and the future market opportunity seems promising, opportunity thus exist to fully utilise this grain commodity to screen, isolate, and develop potential indigenous/local biocontrol agents to mitigate the propagation of mycotoxigenic fungi and their subsequent production of mycotoxins.

1.3 Research aim and objectives

The present study ultimately aimed to isolate novel indigenous fungal biocontrol agents against mycotoxigenic fungi and mycotoxin contamination.

Two pioneer grain maize farms located in Terengganu, Malaysia were selected as the sampling area. These pioneer farms were also referred to as grain maize agro-ecosystems in the context of the present study, primarily because

the isolation and assessment for antagonism of the fungal biocontrol agents was made considering its potential to be apply and release onto the same, or at least similar agro-ecosystem.

The overall aim was further divided into four objectives that were arranged into four major Chapters (**Chapter 3 – 6**) of the thesis. These were:

- (a) To establish the mycodiversity of indigenous mycotoxigenic fungi and potential biocontrol agents from grain maize agro-ecosystem,
- (b) To molecularly and toxigenically differentiate the indigenous *Aspergillus flavus* isolates from grain maize agro-ecosystem,
- (c) To screen the antagonism of potential biocontrol agents against isolated indigenous mycotoxigenic fungi from grain maize agro-ecosystem, and
- (d) To determine the effective biocontrol agents in controlling multi-mycotoxin production by indigenous mycotoxigenic fungi from grain maize agro-ecosystem using different pathogen:antagonist inoculum ratios.

The present study only covered the isolation and screening for potential fungal biocontrol agents against mycotoxigenic fungi and their subsequent mycotoxin production *in vitro* (i.e., maize agar) and on maize kernels with various relevant assays and parameters, and not *in planta* (i.e., on maize plant). However, this was included in Chapter 7 as recommendation for future work. **Chapter 3** was designed to examine the diversity of indigenous fungi, including the mycotoxigenic species and potential fungal biocontrol agents from two pioneer

farms in Terengganu, Malaysia. This was performed by the isolation and identification of fungal isolates using culture-dependent method. Particular attention was made to the mycotoxigenic isolates, specifically from *Aspergillus* section *Flavi* and *Fusarium* section *Liseola*, as both genera consist of mycotoxigenic fungi and their mycotoxins (*i.e.*, aflatoxins, fumonisins) frequently occurred and contaminating maize in tropical countries including Malaysia. This Chapter lays the foundation for antagonistic study in Chapters 4 and 5. **Chapter 4** was designed to explore the possibility of using the indigenous non-aflatoxigenic *A. flavus* as potential biocontrol agents against the indigenous aflatoxigenic counterparts. This includes characterisation of all the indigenous *A. flavus* isolates obtained from two pioneer farms in Terengganu, using molecular and chemical identification. This is to ensure that the non-aflatoxigenic strain, is indeed, non-aflatoxigenic. The antagonism of these non-aflatoxigenic isolates against growth and aflatoxin production was confirmed *in vitro* using dual-culture assay. This Chapter lays the foundation for inoculum ratio assay in Chapter 6. **Chapter 5** was designed to identify potential indigenous fungal antagonist candidates from the other fungal species excluding the non-aflatoxigenic *A. flavus*. This was done by examining mycotoxigenic fungal growth and mycotoxin reduction *in vitro* by dual-culture assay, which also lays the foundation for Chapter 6. **Chapter 6** was designed to examine the efficacy of the selected potential antagonists from Chapters 4 and 5, to reduce mycotoxins under different inoculum ratios on grain maize agar and grain maize kernels. Figure 1.1 summarises the research objectives, research methodology used to achieve each objective, and their arrangements in the thesis Chapters.

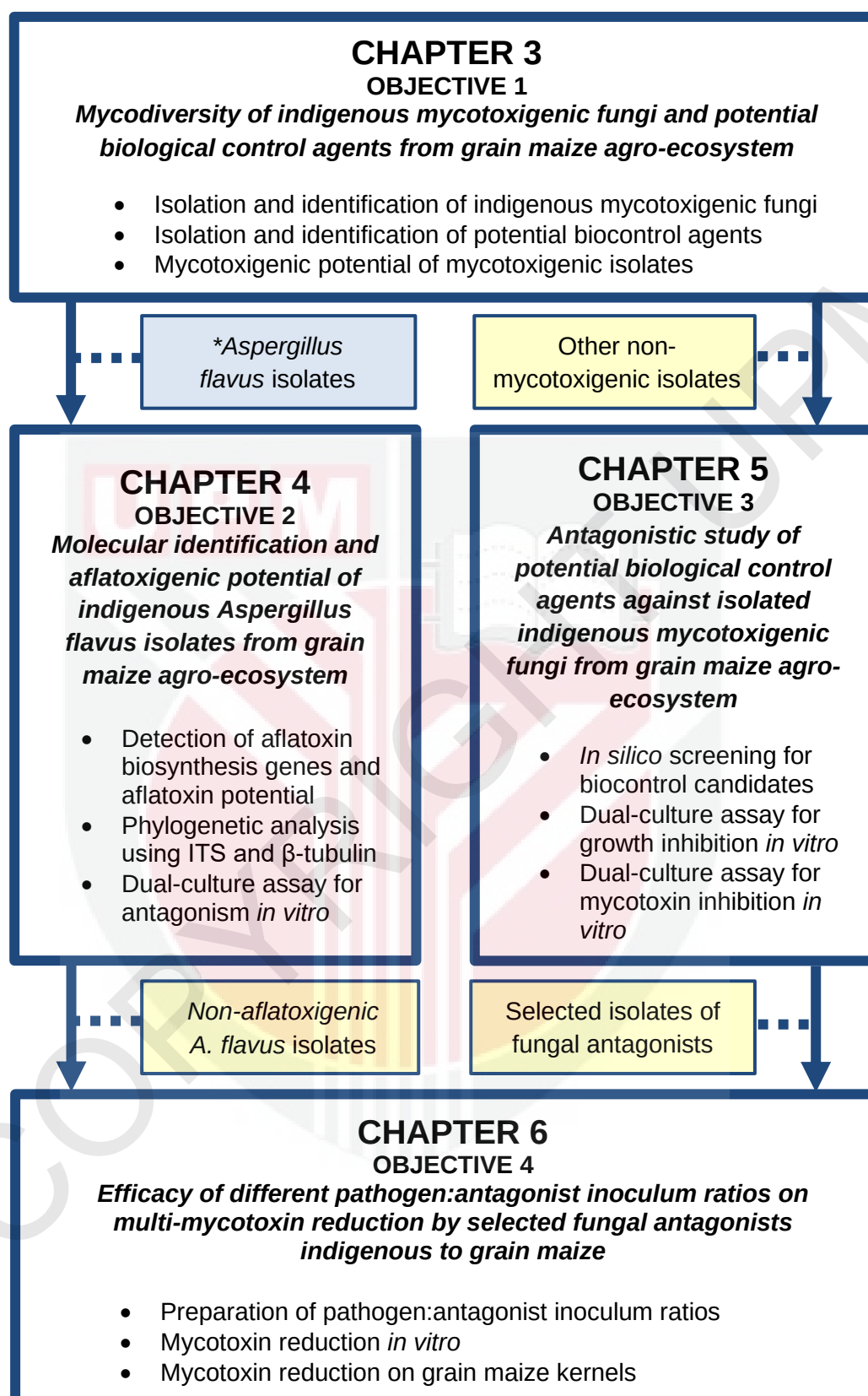


Figure 1.1: Flow diagram of research objectives, their methodology, and the thesis arrangement. Yellow box – fungi that were selected as potential antagonist for the following Chapter. *Light blue box – all *Aspergillus flavus* isolates were characterised before the selection of non-aflatoxigenic *A. flavus*

CHAPTER 2

LITERATURE REVIEW

2.1 Grain maize cultivation

Maize (*Zea mays* L.) is a plant belonging to the grass family Poaceae, and was domesticated from wild grass *teosinte*. Through selective breeding, the wild plant was gradually transformed into the modern-day maize (Ranum *et al.*, 2014). Maize cultivation originated from Mexico before the indigenous tribes and explorers disseminated maize to many parts of the world including the Caribbean, the United States of America, Canada, Europe, Asia, and Africa. Today, this cereal crop, which is also dubbed as “*Queen of Cereals*” (Jaidka *et al.*, 2019), ranks second globally in terms of yield and acreage, with their production surpasses one billion tonnes, and is widely grown in both developing and developed countries (Food and Agriculture Organisation, 2023; Erenstein *et al.*, 2021).

Being a versatile and multi-purpose crop, maize has been used for various applications, primarily as food and feed. In countries such as Latin America, Sub-Saharan Africa, and some parts of Asia, maize has been a staple human diet, and is consumed either raw, or as ingredients in the making of various foods such as tortillas, tamales, pupusas, breakfast mealie meals, and gorditas (Ranum *et al.*, 2014). In other countries where the livestock meat consumption is high, such as in the United States of America and a large part of Asia, maize

is primarily cultivated and utilised for animal feed, with a small percentage being used for biofuel and industrial production (Erenstein *et al.*, 2022).

Maize can be classified into many classification systems, with the kernel type being one of the most common, namely: dent, flint, flour, pod, pop, sweet, and waxy. For maize that is grown mainly for human consumption, they are usually from the flour, pop, or sweet type, with sweet being the predominant type. This type of maize is also known as sweet maize, and contains high residual sugar. Meanwhile, for those grown mainly for animal feed and other products, the dent and flint types are often used, with dent being predominant. Maize grown for this purpose is known commercially as grain maize (or field maize). As compared to sweet maize, grain maize has lower residual sugar content and harder kernels (Ranum *et al.*, 2014).

2.1.1 Anatomy and nutritional composition

Maize plant is shallow-rooted, and held by the aboveground brace roots that emerge into main stalk, leaf sheaths wrapping the stalk, leaf blades, ears that consist of kernels and silk, and tassels (Ritchie *et al.*, 1993). Figure 2.1 depicts the structural anatomy of maize plant and ear. Maize plant developing stages are divided into two: vegetative stage (V-stage) and reproductive stage (R-stage). The V-stage is the primary developmental stage, starting from the maize seed germination (VE stage, also known as emergence), followed by development of leaves where stages are determined by the number of leaves

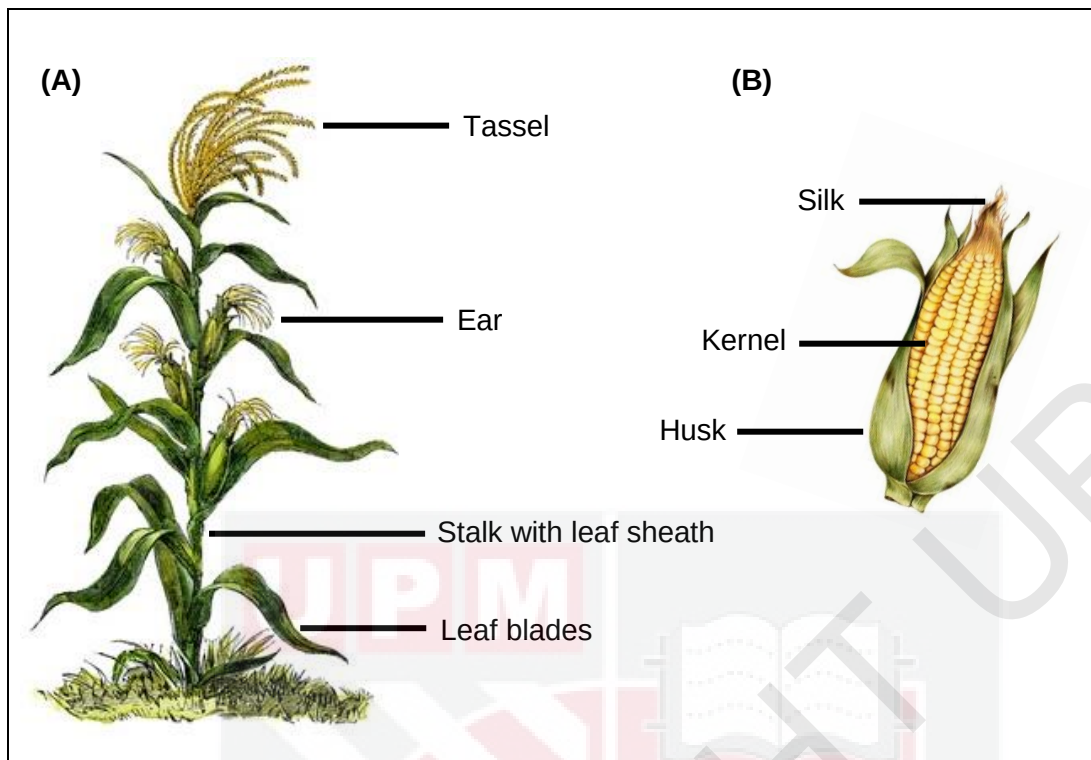


Figure 2.1: Structural anatomy of (A) maize plant and (B) maize ear
(Adapted from Ritchie *et al.*, 1993)

visible (Vn , n is the number of leaves), and VT (tasselling). Next, the R-stage is the secondary developmental stage when kernel development occurs. The stage starts with R1 (silking, the emergence of silk outside ear husk), followed by five stages of grain kernel development, which are R2 (blister), R3 (milk), R4 (dough), R5 (dent), and R6 (physiological maturity) (Zhao *et al.*, 2012).

Since maize is a monoecious plant that has both flowering structures on the same plant, tassels act as the male flower (also known as male inflorescence), while ears as the female flower (also known as female inflorescence). As a male flower, tassel produces pollens, and sheds them during pollination. These tens of thousands of pollens are dispersed by wind and gravity to the below ears, or to neighbouring maize plants. The pollens will then travel

through the silk channel to fertilise the egg cells (*i.e.*, the kernel germ, also known as maize embryo) that will eventually grow into the mature maize kernels (Thompson & Raizada, 2018). Maize growing cycle usually takes approximately three to 13 months, depending on the hybrids. For grain maize, growing cycle is around three to five months. Maize requires minerals from the soil, frequent water, carbon dioxide and oxygen, and adequate sunlight for their growth (Zhao *et al.*, 2012).

The morphological character of dent maize is different from the other maize kernels by its endosperm. Dent maize has a vitreous and horny endosperm, both at the side and back of the kernel. The central core of dent maize endosperm that is extending to the crown of the kernel, on the other hand, is floury and soft. Upon maize drying, this central part will collapse, which gives the kernel its distinct indentation character. Dent maize is commonly found in yellow colour, and similar to the other maize kernels; endosperm (*i.e.*, the flesh) is the largest components of the kernel, followed by germ (*i.e.*, the seed) and pericarp (the coat) (Figure 2.2). Maize, including dent type, contains macronutrients such as starch, protein, and fat; micronutrients such as minerals and vitamin E; bioactive compounds such as anthocyanins, carotenoids, phenolic compounds, and tocopherols; and dietary fibres such as cellulose and pectin. The kernel starch is made up by two glucose polymers; amylose and amylopectin. In dent maize, the amylose composition in starch is approximately 25 to 30%, while the amylopectin is higher, by approximately 70 to 75% (Darrah *et al.*, 2019).

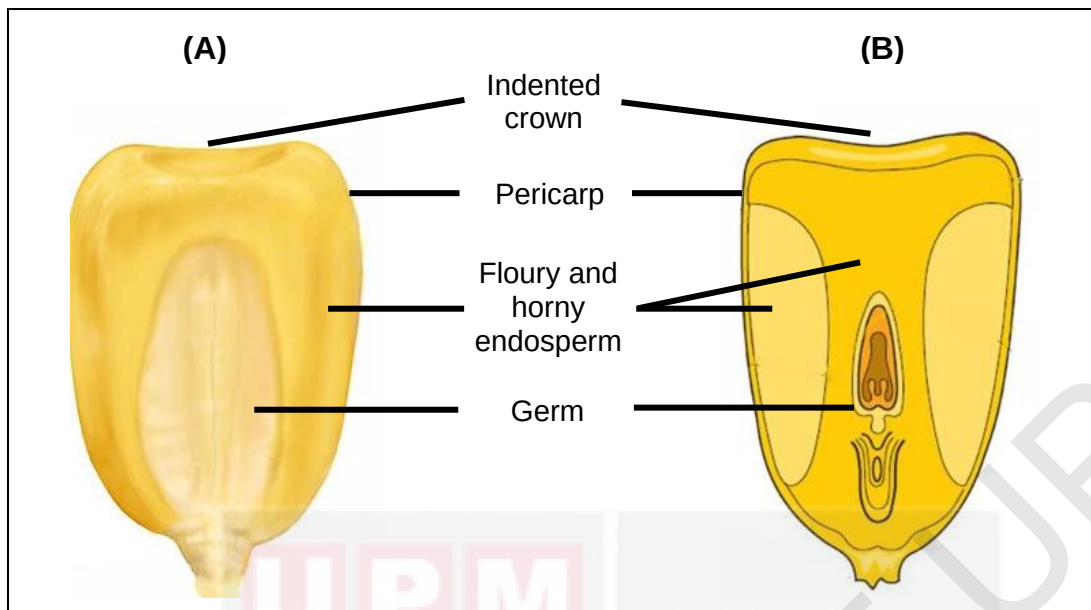


Figure 2.2: Structural anatomy of (A) whole and (B) cross section of dent maize kernel (Adapted from Chaudhary *et al.*, 2014)

2.1.2 Application as animal feed

Maize was first introduced as an ingredient in the livestock feed following World War II when the oversupply of maize in the United States of America encouraged farmers to shift the maize plantation that was predominantly for human consumption to one for livestock feed. This strategy was proven profitable, and had resulted in higher fat and better-quality beef. Grain maize was then widely utilised as the main ingredient in animal feeds, and remains one of the most important ingredients in the livestock and poultry diet (Klopfenstein *et al.*, 2013).

Livestock and poultry diet consists of the grain maize kernels as well as two by-products of the bioethanol industry, namely the distillers' dried grains with solubles (DDGS) and maize gluten meal. Livestock and poultry also consume

forage's residue, or forage processed into silage. Grain maize kernel supplies carbohydrate and protein to enhance animal performances, for example to increase the body weight and milk production of cattle (Wilkinson *et al.*, 2011). In broiler's starter diet, grain maize contributes to approximately > 60% of metabolisable energy and 20% of protein (Sabariah *et al.*, 2019; Cowieson, 2005). However, as compared to the kernel, DDGS and maize gluten meal provide higher feeding value and excellent protein source. Diet based on forage, on the other hand, is often used for lactating dairy cow to enhance milk production, or as feedlot "finishing" diet of cattle (Ranum *et al.*, 2014; Klopfenstein *et al.*, 2013).

2.1.3 Global and local production

Globally, maize including grain maize is the second largest agricultural commodity in terms of production, with the first and the third largest being sugarcane and rice paddy, respectively. Among many countries cultivating maize in 2021, the United States of America is the world's largest producer which accounted to approximately 32% of the total world production, followed by China ($\approx$ 23%), and Brazil ($\approx$ 6%) (Food and Agriculture Organisation, 2023).

In Malaysia, grain maize has been used in feed formulation and mainly intended for poultry (Reddy & Salleh, 2011). However, grain maize production in this country is almost negligible, and the local maize production scene is dominated by sweet maize for human consumption. To meet the increasing

demand of poultry, grain maize supply for the local animal feed industries has been entirely dependent on importation, mainly from Argentina, Brazil, and India (Zahari & Wong, 2009). However, the grain maize import bill reported by the Malaysian Department of Statistics in 2016 was approximately MYR 3.09 billion (Mohd Supaat, 2017), and substantially contributed to the country's economic burden and a deficit in the national agro-food trade. If left unchecked, this may eventually jeopardise the local food security. To reduce the country's dependency on grain maize importation, Malaysia has taken the initiative to locally mass-cultivate grain maize. To further strengthen this effort, grain maize also has been gazetted by the Malaysian Ministry of Agriculture and Food Industries as one of the five new wealth-generating commodities alongside coconut, durian, dairy, and culinary industries, as announced in the Malaysian 2018 budget (Bank Negara Malaysia, 2017). Following this, two pioneer grain maize plantations have been established in Kampong Dadong (13 hectares cultivated area) and Rhu Tapai (3.8 hectares cultivated area) in Terengganu, Malaysia, to maximise land use, and increase farmers' income by intercropping grain maize with rice paddy.

2.2 Mycotoxigenic fungi in grain maize

Mycotoxigenic fungi refer to the filamentous fungal species (*i.e.*, moulds) which are able to synthesise mycotoxins (Afsah-Hejri *et al.*, 2013). Among the various fungal genera, mycotoxigenic fungi are mainly found in the genera *Alternaria*, *Aspergillus*, *Penicillium*, and *Fusarium*, but the most significant species are primarily constituted in the three latter genera. These significant mycotoxigenic

species are those that often contaminate economically relevant food and feed products (Pitt, 2000). Furthermore, their mycotoxins can be carcinogenic for which regulations have been prescribed all over the world to limit their accidental consumption by consumers (Magan *et al.*, 2020; Ostry *et al.* 2017). Table 2.1 summarises the mycotoxigenic fungi frequently contaminating foods and feeds, including maize, and their subsequent major mycotoxins. Often, a single mycotoxin is produced by more than one mycotoxigenic species or genus (e.g., ochratoxin A is produced by both *Aspergillus* and *Penicillium* spp.), and some mycotoxigenic species could also produce more than one mycotoxin (e.g., *F. graminearum* producing trichothecenes type B and zearalenone) (Alshannaq & Yu, 2017; Moretti & Susca, 2017).

Table 2.1: Mycotoxigenic fungi and their major mycotoxins that are frequently found in food and feed products

Fungi	Mycotoxin
Genus <i>Alternaria</i>	
<i>A. alternata</i>	<i>Alternaria</i> toxins e.g. alternariol, alternuene, alternariol monomethyl ether
Genus <i>Aspergillus</i>	
<i>A. flavus</i> , <i>A. parasiticus</i> , and <i>A. nomius</i>	Aflatoxins B ₁ , B ₂ , G ₁ , G ₂
<i>A. niger</i> and <i>A. welwitschiae</i>	Fumonisin B ₂ , B ₄
<i>A. carbonarius</i> , <i>A. niger</i> , <i>A. ochraceus</i> , <i>A. steynii</i> , and <i>A. westerdijkiae</i>	Ochratoxin A
Genus <i>Fusarium</i>	
<i>F. verticillioides</i> and <i>F. proliferatum</i>	Fumonisin B ₁ , B ₂ , B ₃
<i>F. acuminatum</i> , <i>F. langsethiae</i> , and <i>F. poae</i>	Trichothecenes type A e.g. diacetoxyscirpenol [DAS], HT-2, T-2, neosolaniol
<i>F. graminearum</i> , <i>F. pseudograminearum</i> , <i>F. culmorum</i> , <i>F. crookwellense</i>	Trichothecenes type B e.g. deoxynivalenol [DON], nivalenol

Table 2.1: Continued

<i>F. graminearum</i> , <i>F. culmorum</i> , <i>F. cerealis</i> , <i>F. incarnatum</i> , <i>F. verticillioides</i> , and <i>F. equiseti</i>	Zearalenone
Genus <i>Penicillium</i>	
<i>P. citrinum</i> , <i>P. expansum</i> , and <i>P. verrucosum</i>	Citrinin
<i>P. commune</i> , <i>P. camemberti</i> , and <i>P. palitans</i>	Cyclopiazonic acid
<i>P. verrucosum</i> and <i>P. nordicum</i>	Ochratoxin A
<i>P. expansum</i> , <i>P. carneum</i> , <i>P. paneum</i> , and <i>P. clavigerum</i>	Patulin

(Adapted from Moretti & Susca, 2017 and Afsah-Hejri *et al.*, 2013).

In grain maize pre-harvest particularly in tropical countries such as Malaysia, major mycotoxigenic fungi are from the genera *Aspergillus* and *Fusarium*, with *Fusarium* spp. being predominant and regarded as notorious maize contaminant. *Penicillium* spp., however, often contaminate maize-based post-harvest products, and produce mycotoxins thereafter (Munkvold *et al.*, 2019).

2.2.1 Genus *Aspergillus* in grain maize

Aspergillus is a genus comprising nearly 350 species based on morphology, physiology, and phylogeny. To ease infrageneric (*i.e.*, within the same genus) classification, subgenus and section levels are introduced and placed in between genus and species levels. Currently, four subgenera and 20 sections of *Aspergillus* are recognised (Samson *et al.*, 2014). Among subgenera and sections of *Aspergillus*, species from *Aspergillus* subgenus *Circumdati* section *Flavi* and *Aspergillus* subgenus *Circumdati* section *Nigri* are those that are commonly associated with maize (Munkvold *et al.*, 2019; Palencia *et al.*, 2010).

Aspergillus section *Flavi* harbours many closely related species (e.g., *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*) that produce various secondary metabolites, among which the most important are the mycotoxin aflatoxins (Uka *et al.*, 2019). Among the many members of this section, *A. flavus* is the most notable in maize since it can cause the devastating ear rot disease, namely *Aspergillus* ear rot, and produce aflatoxins. *A. flavus* infects maize during pre- and post-harvest (Kapeua-Ndacnou *et al.*, 2023; Jallow *et al.*, 2021; Bailly *et al.*, 2018; Klich, 2002). During pre-harvest, *A. flavus* is an opportunistic pathogen that infects and colonises weakened or wounded kernels caused by mechanical damage, insects, and environmental or plant stresses. Since *A. flavus* is predominantly a saprophytic fungus, it infects maize by dispersing its conidia (singular conidium, meaning asexual spores) via air or rain splash. Insects have also been reported as agents for *A. flavus* conidial transmission. These include *Chlorochroa sayi* (Say's stink bug) and *Lygus hesperus* (Western plant bug) (Amaike & Keller, 2011). During post-harvest, conidia of *A. flavus* from pre-harvest or those transmitted by insects can germinate and contaminate storage maize under favourable conditions particularly during storage temperature of > 17°C, and maize kernel moisture content of > 13% (Ferreira-Castro *et al.*, 2012).

Species of *Aspergillus* section *Nigri* commonly attacking maize are *A. niger*, *A. carbonarius*, and *A. japonicus*, with *A. niger* being the most frequently reported in maize. However, unlike *A. flavus*, *A. niger* often infects maize pre-harvest, and exists as symptomless endophyte, before later develop as pathogen when conditions allow. This species is often associated with the kernel ear rot in

maize with similar mechanism of infection by *A. flavus*. Although the occurrence of this disease in maize by *A. niger* is not as rampant as by *A. flavus*, infection by *A. niger* can still lead to mycotoxin contamination, namely ochratoxin A, in the maize kernels during favourable condition particularly in tropical and subtropical climatic zones, therefore presenting a risk of this mycotoxin contamination in Malaysian foods and feeds (Palencia *et al.*, 2010).

2.2.2 Genus *Fusarium* in grain maize

Among the many species in the genus *Fusarium*, two primary species cause the most notable diseases in maize, namely *Gibberella* ear rot and *Fusarium* ear rot. *Gibberella* ear rot is mainly caused by *F. graminearum*, which usually thrives in colder regions. In tropical climate, such as that of Malaysia, *Fusarium* ear rot which is mainly caused by *F. verticillioides* is most notable. This fungus can infect a large proportion of the maize ear either on random individual kernels, on groups of kernels, or on physically injured kernels, and symptoms of infection include mycelial growth on kernels which is white/light pink/salmon-coloured, or white streaks on pericarps (also called 'star burst', caused by this pathogen growing under the seed coat). In some cases, *F. verticillioides* may infect maize seed without showing any visible symptoms (*i.e.*, asymptomatic, as endophyte). Besides *F. verticillioides*, other *Fusarium* species such as *F. proliferatum*, *F. subglutinans*, and *F. meridionale* were also reported to cause the same disease without showing any visible symptoms (Zhou *et al.*, 2018; Zhang *et al.*, 2014).

Fusarium verticillioides survives in maize agro-ecosystem in the form of sclerotia (singular sclerotium, meaning thickened hyphae). It colonises maize debris and senescent maize tissue or other crops tissues, and may produce huge numbers of micro- and macro-conidia. Their build-up in crop debris, especially in moist soils of reduced tillage or one that practices crop rotation that leaves residues from previous crops and serves as reservoir for the spores, serves as the primary inoculum for the disease. Maize infection by this pathogen may occur through four routes of infection. Firstly, through systemic infection (seedborne), where *F. verticillioides* is transmitted from seed to seedlings to maize plant. In this case, seed carries fungal inoculum inside its tissue or on its surface. Secondly, through silk/tassel infection (airborne), where during pollination, fungal conidia that are dispersed by wind land on silk of flowering ears before germinating and travelling down to infect developing maize kernels and cobs (wind disperses the propagules as both macro-conidia and micro-conidia; the latter being the more predominant since in this form they are relatively lighter, more numerous, and easily dispersed by wind). Fungal propagules may be found within maize fields or at distance location from the field of its primary inoculum (Thompson & Raizada, 2018; Munkvold, 2017). Thirdly, fungal inoculum may also be transmitted through water splash during heavy rain (waterborne). Finally, by insect transmission (insectborne), where insect as vector brings conidia from contaminated area to non-contaminated plants, thus creating wound and providing direct entry for fungal contamination. European corn borer (*Ostrinia nubilalis*) is one of the most important moths related to *Fusarium* ear rot disease (Zhou *et al.*, 2018).

2.2.3 Genus *Penicillium* in grain maize

Not many species in the genus *Penicillium* are associated with maize, especially in tropical climate such as Malaysia. Species from this genus usually thrive, and are more common in colder climatic regions. Many species from this genus infect maize during storage. One species that has been reported infecting maize pre-harvest is *P. oxalicum*. Like *Aspergillus* spp. and *Fusarium* spp., this species usually infects maize through damages caused by insects or other factors (Munkvold *et al.*, 2019).

2.3 Mycotoxins in grain maize

Since the discovery of T-2 toxin that caused alimentary toxic aleukia in Russia in the 1940s (Williams, 1989), and aflatoxins that killed nearly 100,000 turkey poults in England in the 1960s (Blount, 1961; Wannop, 1961), the global scientific community has been alarmed by the disease- and death-causing capability of mycotoxins on both humans and animals (Pitt & Miller, 2017). The term mycotoxin is derived from the Greek “*mykes*” which is fungus, and “*toxikon*”, which is poison. Mycotoxin contamination is in fact the secondary outcome of fungal infestation of crop commodities, with the primary ones being the damages and diseases such as blights, rusts, rots, and wilts.

Mycotoxins are low molecular weight and thermally- and chemically-stable secondary metabolites produced by certain moulds that are capable of causing diseases and death in vertebrates (*i.e.*, humans and animals), even in low

concentrations (Bennett & Klich, 2003). At present, more than 300 moulds have been identified able to produce nearly 500 mycotoxins of diverse structures, and clinical impacts (Nielsen & Smedsgaard, 2003). Although mycotoxins are not necessary for growth of their fungal producers, but because mycotoxins can weaken their competitors (usually other microorganisms competing for the same substrate on crop commodities; competitive exclusion), the fungal producers are thought to use mycotoxins as a strategy to ensure their survival and proliferation (Hussein & Brasel, 2001). In grain maize, common mycotoxins found are aflatoxins, fumonisins, trichothecenes, zearalenone, and ochratoxin A (Munkvold *et al.*, 2019).

2.3.1 Chemical structures and biosyntheses

Of the many identified mycotoxins, aflatoxins are the most toxically potent and naturally abundant. Overall, there are 18 types of aflatoxin analogues occurring in nature, but only four are the most economically important (*i.e.*, they are often found contaminating economically relevant agricultural commodities): aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂). Aflatoxin structures are bifuranocoumarin fused with cyclopentanone for the B-type, and bifuranocoumarin fused with delta-lactone for the G-type. As the main producers of aflatoxins in maize, *A. flavus* only produces the B-type, while *A. parasiticus* produces both B- and G-types (Uka *et al.*, 2020). The B and G get their names from the fact that they fluoresce blue and green under ultraviolet light, respectively.

For fumonisins, currently more than 15 analogues have been characterised, which are divided into types A, B, C, and P. B-type fumonisins (B₁, B₂, and B₃) are the most common and widely studied, with B₁, B₂ being the most toxic. B-type fumonisins' structure contains 20-carbon linear polyketide-derived backbone with terminal amine (-NH₂), one until four hydroxyl (-OH), and two propane-1,2,3-tricarboxylate esters located at various positions (Munkvold *et al.*, 2019; Sun *et al.*, 2019). Fumonisins' major producers in maize are *F. verticillioides* and *F. proliferatum*. Besides, *A. niger*, could also produce FB₂. For ochratoxins, four analogues are currently accepted namely ochratoxin A (OTA), ochratoxin B (OTB), ochratoxin C (OTC), and ochratoxin TA (OTTA). Of these, OTA is the most commonly found in food and feed products. Ochratoxin A is dihydroisocoumarin ring with amide linkage to phenylalanine. In maize, OTA is mainly produced by *A. niger* and *P. verrucosum* (Soares *et al.*, 2013). OTA polyketide biosynthetic cluster is still an active research niche, since its complete elucidation of the pathway is still scarce. However, certain putative genes have been confirmed as the key genes for OTA biosynthesis (Ferrara *et al.*, 2020).

Trichothecenes are *Fusarium*-derived sesquiterpenoid compounds with approximately 200 compounds already identified in nature. Trichothecenes are classified into types A, B, C, and D. In maize, types A (mainly HT-2 and T-2) and B (mainly deoxynivalenol [DON]) are the most common. Unlike other notable mycotoxins mentioned earlier which are under polyketide class, trichothecenes are terpenoid, and have terpenoid biosynthetic pathways with total genes varying between genera of the producers, and in some cases, they

also vary between species in the same genus (Proctor *et al.*, 2018). Deoxynivalenol, also known as 'vomitoxin', is a type B trichothecene which have carbonyl group at position C-8 (Afsah-Hejri *et al.*, 2013). In maize, this toxic compound is mainly produced by *F. graminearum*. Type A trichothecenes namely HT-2 and T-2, have ester function at C-8 (Munkvold *et al.*, 2019).

Zearalenone (ZEN) is an oestrogenic compound, also known as F-2 toxin. The structure of ZEN consists of resorcinol moiety fused to 14-member macrocyclic lactone. In maize, ZEN is commonly produced by *Fusarium* spp., including *F. graminearum* and *F. culmorum* (Huffman *et al.*, 2010). Table 2.2 depicts the classes, biosynthetic pathway, and the chemical structures of these mycotoxins which are commonly found in maize.

Table 2.2: Common mycotoxins, their metabolite classes, biosynthetic pathways, and chemical structures

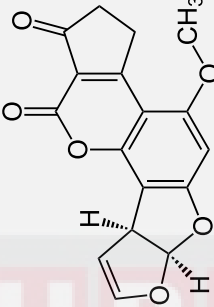
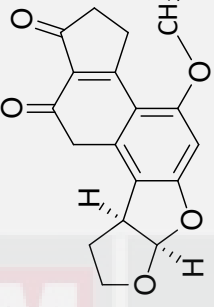
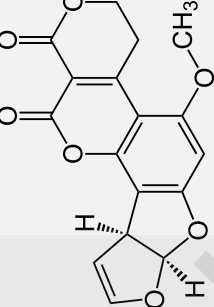
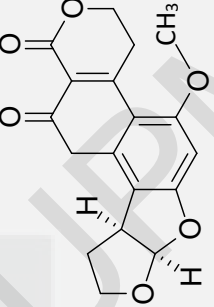
Mycotoxin	Class	Biosynthetic pathway	Chemical structure
Aflatoxin B ₁	Polyketide	Polyketide pathway Biosynthetic genes: 25 genes Regulatory genes/transcription factor: <i>aflR</i>	
Aflatoxin B ₂			
Aflatoxin G ₁			
Aflatoxin G ₂			

Table 2.2: Continued

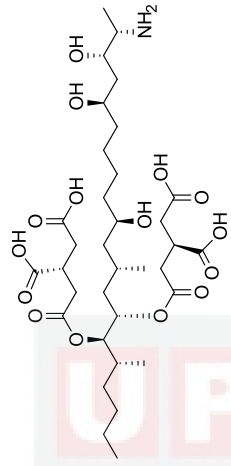
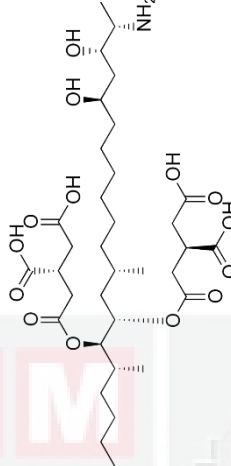
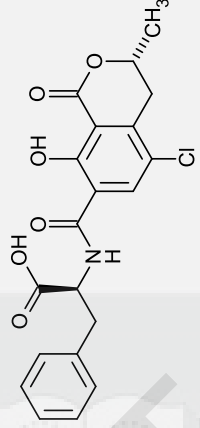
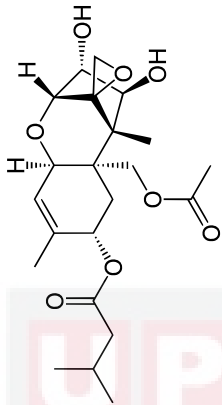
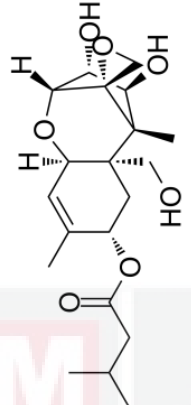
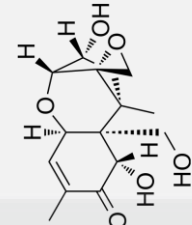
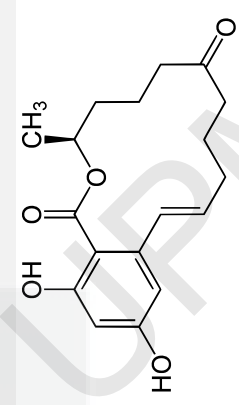
Fumonisin B ₁	Polyketide pathway Biosynthetic genes: 17 genes Regulatory genes/transcription factor: <i>FUM21</i> Key genes for synthesis: <i>FUM1</i>, <i>FUM6</i>, <i>FUM8</i> Polyketide	
Fumonisin B ₂	Polyketide pathway Biosynthetic genes: 17 genes Regulatory genes/transcription factor: <i>FUM21</i> Key genes for synthesis: <i>FUM1</i>, <i>FUM6</i>, <i>FUM8</i> Polyketide	
Ochratoxin A	Polyketide pathway Key genes for synthesis: <i>otaA</i>, <i>otaB</i>, <i>otaC</i>, <i>otaD</i>, <i>otaR1</i> Polyketide	

Table 2.2: Continued

HT-2	Terpenoid	Terpenoid pathway Biosynthetic genes: varies Regulatory genes/transcription factor: <i>TRI6</i> , <i>TRI10</i>	
T-2			
Deoxynivalenol	Terpenoid	Terpenoid pathway Biosynthetic genes: varies Regulatory genes/transcription factor: <i>TRI6</i> , <i>TRI10</i>	
Zearalenone	Polyketide	Polyketide pathway Regulatory genes/transcription factor: <i>ZEA1</i> , <i>ZEA2</i> , <i>ZEB1</i> , <i>ZEB2</i>	

Mycotoxin production by the mycotoxigenic fungi in crop commodities either during pre-harvest or post-harvest in storage conditions are often governed by the abiotic factors including environmental conditions (e.g., temperature, water activity, carbon dioxide) and substrate composition/availability. Other factors such as mechanical and insect damages are also important, since the damages can provide entry for fungal infestation which eventually initiate mycotoxin production and accumulation. For mycotoxins to be formed, the conidia of mycotoxigenic fungi will have to land on crop commodities through various modes of dispersal, commonly through wind dispersal, rain splash, and insect vectors as earlier mentioned. Once under favourable condition, these conidia will germinate and continue to proliferate, and subsequently produce the mycotoxins. Mycotoxins are commonly produced in the late exponential or early stationary phase of the fungal growth (Ismaiel & Papenbrock, 2015). Other trigger of mycotoxin production can also be upon competition with other fungi over a niche or a substrate (Magan & Aldred, 2007).

2.3.2 Toxicity to humans and animals, and regulations

Mycotoxin toxicity to humans and animals can be divided into two types: acute and chronic. Acute toxicity occurs upon a short-term exposure to the mycotoxin, has rapid onset, and obvious toxic response. Acute toxicity is often indicated by fever, vomiting, abdominal pain, hypertension, and worst, death. Chronic toxicity, on the other hand, occurs long after exposure in low doses to the mycotoxin, and often linked to cancers. Teratogenicity, carcinogenicity, immune-toxicity, liver cirrhosis, kidney failure, and stunted growth are some of

the conditions that indicate chronic mycotoxin exposure (Ostry *et al.*, 2017). In terms of their carcinogenicity toward humans and animals, the International Agency for Research on Cancer (IARC), which is under the auspices of the World Health Organisation (WHO) of the United Nations (UN), and serves as the authoritative source of information on causes of cancers, has classified mycotoxins into several groups (Ostry *et al.*, 2017), as shown in Table 2.3.

Table 2.3: International Agency for Research on Cancer (IARC) Monographs evaluations of carcinogenic hazard of selected mycotoxins

Group	Description	Mycotoxin
Group 1	Carcinogenic to humans. Sufficient evidence in humans. Causal relationship established.	Aflatoxin B ₁ , B ₂ , G ₁ , G ₂ , M ₁
Group 2A	Probably carcinogenic to humans. Limited evidence in humans. Sufficient evidence in experimental animals.	-
Group 2B	Possibly carcinogenic to humans. Limited evidence in humans. Less than sufficient evidence in experimental animals.	Fumonisin B ₁ , B ₂ Fusarin C Ochratoxin A Sterigmatocystin Citrinin Cyclochlorotine Deoxynivalenol Fusarenone X Kojic acid Luteoskyrin Nivalenol Patulin Penicillic acid Rugulosin Zearalenone T-2 toxin
Group 3	Not classifiable as to its carcinogenicity to humans. Insufficient evidence in humans and Insufficient evidence in experimental animals.	
Group 4	Probably not carcinogenic to humans. Evidence suggests lack of carcinogenicity in humans and experimental animals.	-

(Adapted from Ostry *et al.*, 2017).

Other than being exposed to humans directly by direct consumption of the contaminated food (primary exposure), mycotoxins especially aflatoxins can also be exposed to humans indirectly (secondary exposure) by consuming the milk of ruminants feeding on the contaminated feed (secondary exposure). For example, goats feeding on maize-based feed contaminated by *Aspergillus* section *Flavi*. Aflatoxin B₁ (AFB₁) will then be produced on the feed, consumed by the goats, metabolised and biotransformed into AFM₁ in the goats' system, and secreted through milk (hence the name M). Although not actually toxic towards adults, children are particularly sensitive towards AFM₁ which could lead to stunted growth (Azman *et al.*, 2021).

In animals, aflatoxins have been linked to hepatocellular carcinoma. Ochratoxin A has been shown to be a potent teratogen and nephrotoxin in poultry and swine. Fumonisin have been linked with neural tube defects and acute toxicity in swine and equine. Trichothecenes have been associated with mycotoxicosis in animals with symptoms such as haemorrhage, oedema, and reduced milk and egg production. The type-B trichothecene, DON, has been shown to induce feed refusal and vomiting in swine, while ZEN has been implicated with hypoestrogenism in swine (Munkvold *et al.*, 2019; Zain, 2011).

To reduce the risk of exposure to mycotoxins, many countries have set regulatory limits for allowable mycotoxins in foods and feeds (*i.e.*, maximum permitted proportion). For example, the European Union, as one of the organisations with strictest regulations on mycotoxins has set limits for total aflatoxins and AFB₁ in cereals at 4–15 and 2–12 µg/kg, respectively; AFM₁ in

milk for infants at 0.05 µg/kg. Others such as OTA is set at 5 and 3 µg/kg for unprocessed and processed cereals, respectively. DON and ZEN in cereals are set to a limit at 200–1,750 µg/kg and 20–400 µg/kg, respectively (Ksenija, 2018). For fumonisins, the maximum limits for maize intended for human consumption is at 1 mg/kg, maize-based cereals and snacks at 0.8 mg/kg, and maize by-products for animal feeds at 60 mg/kg (Chen *et al.*, 2021). In Malaysia, the Malaysian Food Regulation (1985) has set a standard on AFB₁ in cereal-based food for infants at 0.1 µg/kg, AFM₁ in milk at 0.5 µg/kg, and AFM₁ in infant formula at 0.025 µg/kg. For OTA, the limit in cereal-based food for children and infants is set at 0.5 µg/kg (Malaysian Food Regulation, 1985). However, to date, there is no maximum permitted limit has been set for animal feeds in Malaysia, which presents an opportunity for further research since ruminant, poultry, and swine industries as well as the aquaculture sector which mostly use plant-based feed contribute significantly to Malaysia's economy.

2.3.3 Occurrence in Malaysian commodities

Malaysia is one of the Southeast Asian countries with tropical weather of warm and humid all year round, thus predisposing the agricultural commodities to mycotoxigenic fungi and their subsequent mycotoxin contamination. The earliest recorded incidence of local mycotoxin contamination was in 1960 when outbreak of disease in swine farms caused severe liver damage to the animals, and aflatoxins were implicated as the culprit (Afsah-Hejri *et al.*, 2013). Later in 1988, aflatoxin contamination and aflatoxicosis was reported in humans for the first time. This aflatoxicosis case killed 13 children (Cheng,

1992) who consumed aflatoxin-contaminated *lau shu fun/loh shi fun* (rat tail noodles/silver needle noodles made from maize starch or tapioca starch or rice starch). Although no newer cases of similar magnitude have been reported, mycotoxigenic fungi and mycotoxins are continuously present in local and imported agricultural crops and post-harvest products (Khayoon *et al.*, 2012; Nithiyaa *et al.*, 2012).

Table 2.4 lists the occurrence data of mycotoxins on Malaysian foods and feedstuffs during 2007–2020. Cereals, spices/chillies and spice-based products, and peanut and peanut-based products were the three most tested foods, with all showing considerable amounts of mycotoxin contamination. It then became apparent that the concern was more towards aflatoxins, being in Group 1 carcinogen. A more comprehensive review on mycotoxin contamination of Malaysian food and agricultural produce has been published elsewhere (Afsah-Hejri *et al.*, 2013).

Table 2.4: Occurrence of mycotoxins on Malaysian foods and feeds (2007–2020)

Food/Feed (number of sample, <i>n</i>)	Mycotoxin	<i>n</i> positive, <i>n</i> above limit	Reference
Grain maize and maize-based poultry feed (<i>n</i> = 51)	Aflatoxins	<i>n</i> positive: 3/51; <i>n</i> above limit: none	Nasaruddin <i>et al.</i> , 2020
Spices and spice- based products (<i>n</i> = 90)	Aflatoxins, ochratoxin A	AFs <i>n</i> positive: 19/90; <i>n</i> above limit: 19/90 OTA <i>n</i> positive: 37/90; <i>n</i> above limit: 25/90	Ahmad-Zaidi <i>et al.</i> , 2019

Table 2.4: Continued

Commercial food and beverage ($n = 120$); apple, grape, orange, and pomegranate juices; wheat and barley flour; dried figs, raisins, chili powder, and spices; non-roasted peanut; roasted pistachio	Aflatoxins, ochratoxin A	AFs n positive: 19/120; n above limit: 14/120 OTA n positive: 3/120; n above limit: None	Alsharif <i>et al.</i> , 2019
Raw peanuts ($n = 87$), peanut-based products ($n = 91$)	Aflatoxins	Raw peanuts n positive: 36/87; n above limit: 20/87 Peanut-based products n positive: 50/91; n above limit: 9/91	Norlia <i>et al.</i> , 2018
Milk and dairy products ($n = 53$)	Aflatoxin M ₁	n positive: 19/53 n above limit: 4/53	Nadira <i>et al.</i> , 2017
Cow's, goat's, human's milk ($n = 33$)	Aflatoxin M ₁	n positive: 2/33 n above limit: none	Shuib <i>et al.</i> , 2017a
Cow's milk ($n = 102$)	Aflatoxin M ₁	n positive: 4/102 n above limit: 3/102	Shuib <i>et al.</i> , 2017b
Commercial vegetable oil ($n = 25$)	Aflatoxins, ochratoxin A, deoxynivalenol, zearalenone	AFs n positive: none n above limit: none OTA n positive: none n above limit: none DON n positive: none n above limit: none ZEN n positive: 15/25 n above limit: none	Sharmili <i>et al.</i> , 2016
Commercial spice ($n = 58$)	Aflatoxins, ochratoxin A	AFs n positive: 50/58 n above limit: 15/58 OTA n positive: 27/34 n above limit: 1/34	Ali <i>et al.</i> , 2015

Table 2.4: Continued

		AFs <i>n</i> positive: 46/50 <i>n</i> above limit: 35/50	
Red yeast rice (traditional Chinese medicine; <i>n</i> = 50)	Aflatoxins, ochratoxin A, citrinin	OTA <i>n</i> positive: 50/50 <i>n</i> above limit: none	Samsudin & Abdullah, 2013
		CIT <i>n</i> positive: 50/50 <i>n</i> above limit: 50/50	
Commercial dried chilli (<i>n</i> = 80)	Aflatoxins, ochratoxin A	AFs <i>n</i> positive: 52/80 <i>n</i> above limit: 9/80 OTA <i>n</i> positive: 65/80 <i>n</i> above limit: 13/80	Jalili & Jinap, 2012
Commercial rice (<i>n</i> = 5), peanut (<i>n</i> = 9) and chilli (<i>n</i> = 10)	Aflatoxins	Rice <i>n</i> positive: 4/5 <i>n</i> above limit: 3/5 Peanut <i>n</i> positive: 4/9 <i>n</i> above limit: 2/9	Khayoon <i>et al.</i> , 2012
Commercial coffee and cereal (<i>n</i> = 45)	Ochratoxin A	Chili <i>n</i> positive: 9/10 <i>n</i> above limit: 3/10 OTA <i>n</i> positive: 25/45 <i>n</i> above limit: 1/45 AFs <i>n</i> positive: 60/100 <i>n</i> above limit: 2/100	Lee <i>et al.</i> , 2012
Commercial cereal (<i>n</i> = 100)	Aflatoxins B ₁ , B ₂ , G ₁ , G ₂ , ochratoxin A, zearalenone, deoxynivalenol, fumonisins B ₁ , B ₂ , T-2, HT-2	OTA <i>n</i> positive: 40/100 <i>n</i> above limit: 2/100 DON <i>n</i> positive: 36/100 <i>n</i> above limit: none ZEN <i>n</i> positive: 25/100 <i>n</i> above limit: none FB ₁ <i>n</i> positive: 19/100 <i>n</i> above limit: none	Soleimany <i>et al.</i> , 2012a

Table 2.4: Continued

Commercial cereal (<i>n</i> = 80)	Aflatoxins B ₁ , B ₂ , G ₁ , G ₂ , ochratoxin A, zearalenone, deoxynivalenol, fumonisins B ₁ , B ₂ , T-2, HT-2	FB ₂ <i>n</i> positive: 13/100 <i>n</i> above limit: none	Soleimany <i>et al.</i> , 2012b
		T-2 <i>n</i> positive: 16/100 <i>n</i> above limit: none	
		HT-2 <i>n</i> positive: 16/100 <i>n</i> above limit: none	
		AFs <i>n</i> positive: 40/80 <i>n</i> above limit: 2/80	
		OTA <i>n</i> positive: 24/80 <i>n</i> above limit: 1/80	
		DON <i>n</i> positive: 24/80 <i>n</i> above limit: none	
		ZEN <i>n</i> positive: 15/80 <i>n</i> above limit: none	
		FB ₁ <i>n</i> positive: 13/80 <i>n</i> above limit: none	
		FB ₂ <i>n</i> positive: 11/80 <i>n</i> above limit: none	
		T-2 <i>n</i> positive: 11/80 <i>n</i> above limit: none	
Commercial food products (<i>n</i> = 95)	Aflatoxin B ₁	HT-2 <i>n</i> positive: 9/80 <i>n</i> above limit: none	Reddy <i>et al.</i> , 2011
		<i>n</i> positive: 69/95 <i>n</i> above limit: none	
Grain maize for animal feed (<i>n</i> = 80)	Aflatoxin B ₁ , fumonisins	AFB ₁ <i>n</i> positive: 65/80 <i>n</i> above limit: 18/80	Reddy & Salleh, 2011
		FUM <i>n</i> positive: 80/80 <i>n</i> above limit: none	
Commercial food and feed (<i>n</i> = 39)	Fumonisin	<i>n</i> positive: 5/39 <i>n</i> above limit: none	Khayoon <i>et al.</i> , 2010a

Table 2.4: Continued

Animal feed ($n = 42$)	Aflatoxins	AFs n positive: 8/42 n above limit: 3/42	Khayoon <i>et al.</i> , 2010b
Commercial wheat ($n = 15$) and barley ($n = 15$)	Aflatoxin B ₁	Wheat n positive: 3/15 n above limit: none Barley n positive: 1/15 n above limit: none	Reddy & Salleh, 2010
Commercial cereal ($n = 60$)	Aflatoxins, ochratoxin A, zearalenone	AFs n positive: 24/60 n above limit: 1/42 OTA n positive: 9/60 n above limit: 2/42 ZEN n positive: 11/60 n above limit: none	Rahmani <i>et al.</i> , 2010
Commercial noodle ($n = 135$)	Deoxynivalenol	n positive: 110/135 n above limit: none	Moazami & Jinap, 2009
Raw shelled peanut ($n = 145$)	Aflatoxins	AFs n positive: 73/145 n above limit: 33/145	Sulaiman <i>et al.</i> , 2007

Based on Table 2.4, AFs and FBs constitute a major proportion of mycotoxin contaminants in grain maize and maize-based products. Other mycotoxins, which were DON, HT-2, T-2, OTA, and ZEN were reported to a lesser extent. Besides mycotoxin contamination, Reddy & Salleh (2011) also found approximately 87% ($n = 69/80$) of the grain maize samples were contaminated by *A. flavus* and 47% ($n = 37/80$) by *F. verticillioides*. Another study by Zainudin & Perumal (2015) on maize flour found that all *F. verticillioides* isolates investigated ($n = 16/16$) were able to produce FB₁, and a high number of *A. flavus* isolates were able to produce AFB₁ ($n = 29/40$). Moreover, they also reported that *F. proliferatum*, *F. semitectum*, and *F. verticillioides* were able to

produce moniliformin (MON, $n = 3/3$, $n = 6/6$, and $n = 6/7$, respectively) and beauvericin (BEA, $n = 3/3$, $n = 4/6$, and $n = 5/7$, respectively) in the samples.

Similar trends of mycotoxin contamination in maize were observed in other Southeast Asian countries with tropical climates. In Indonesia, a study conducted during 1985–1986 found approximately 96% of 52 maize samples and 95% of 290 poultry feed samples from feed mills contained AFs. Moreover, ZEN and OTA were also found in 22% and 2% of the maize samples, respectively (Widiastuti *et al.*, 1988a). A subsequent study by the same author on the same maize samples found cyclopiazonic acid (CPA) in 81% ($n = 21/26$) of the samples (Widiastuti *et al.*, 1988b). Later in 1995, Ali *et al.* (1998) found 100% ($n = 16/16$) AFs, 69% ($n = 11/16$) FBs, and 12% ($n = 2/16$) each of DON, nivalenol (NIV), and ZEN from maize samples of central Java. All AF-contaminated samples were also co-contaminated with FBs. In addition, 75% ($n = 12/16$) and 56% ($n = 9/16$) of the samples were contaminated with *A. flavus* and fumonisin-producing species (*i.e.*, *F. moniliforme*, *F. proliferatum*, and *F. nygamai*), respectively. In Thailand, high FBs (FB₁: 89% and FB₂: 67%) and AFs (AFB₁: 83%, AFB₂: 55%) were found in 18 post-harvest maize samples intended for animal and human consumption. In most samples, the levels of FBs were higher than AFs, and 72.2% of samples were contaminated with both mycotoxins (Yoshizawa *et al.*, 1996). Later, Tansakul *et al.* (2013) detected FBs and BEA in 100% ($n = 58/58$) samples of DDGS used for animal feed, while approximately 83% ($n = 48/58$) and 48% ($n = 28/58$) samples were also contaminated with ZEN and DON, respectively. In Southern Vietnam, a study conducted in 2005 by Thieu *et al.* (2008) found 92% of AFB₁ and 33%

of ZEN from 12 samples of maize for feed. Later in 2009, a study conducted on dried maize kernel from wholesale, retail, and households of Northern Vietnam found *A. flavus*, *A. parasiticus* and *F. verticillioides* in 29% ($n = 30/102$), 4% ($n = 4/102$), and 26% ($n = 27/102$) of the samples. Moreover, they also found AFs (26%, $n = 27/102$) and FBs (23%, $n = 24/102$) in the samples, respectively (Huong *et al.*, 2016). Recently, a long and extensive three-year worldwide survey of mycotoxins in feedstuff had verified a high occurrence of FBs (83%) and AFs (71%), followed by DON (45%), ZEN (20%), and OTA (12%) in maize samples of Southeast Asia (*i.e.*, Malaysia, Indonesia, Philippines, Thailand, and Vietnam) (Rodrigues & Naehrer, 2012).

To prevent or manage contamination by mycotoxigenic fungi and their subsequent mycotoxin production, particularly during pre-harvest, various control measures has been implemented. These control measures are generally divided into: cultural, physical, chemical, and biological, and have been employed either individually or collectively before, during, or after crop cultivation as part of Good Agricultural Practices (GAP) and Integrated Pest Management (IPM) strategies.

Figure 2.3 summarises the control measures which encompass cultural, physical, chemical, and biological, falling under the umbrella of IPM (Frische *et al.*, 2018). The IPM pyramid ideally works from the bottom up, in which preventive measures (*e.g.*, the use of tolerant/resistant crop cultivars; crop care such as farm irrigation and crop rotation; promoting natural antagonists against crop pests [conservation biocontrol]), and the curative measures of

physical and biological controls always take priority and precedent before the use of chemicals. Only when the economic threshold is exceeded, as assessed by the risk analysis and monitoring, and when the other curative strategies are not sufficient or effective for control, the plant protection approach shift to chemical controls. An “economic threshold” is the pest’s population level infesting the farm or the extent of crop damage inflicted by the pests at which the value of the crop destroyed exceeds the cost of controlling the pest (Zalom, 2010).

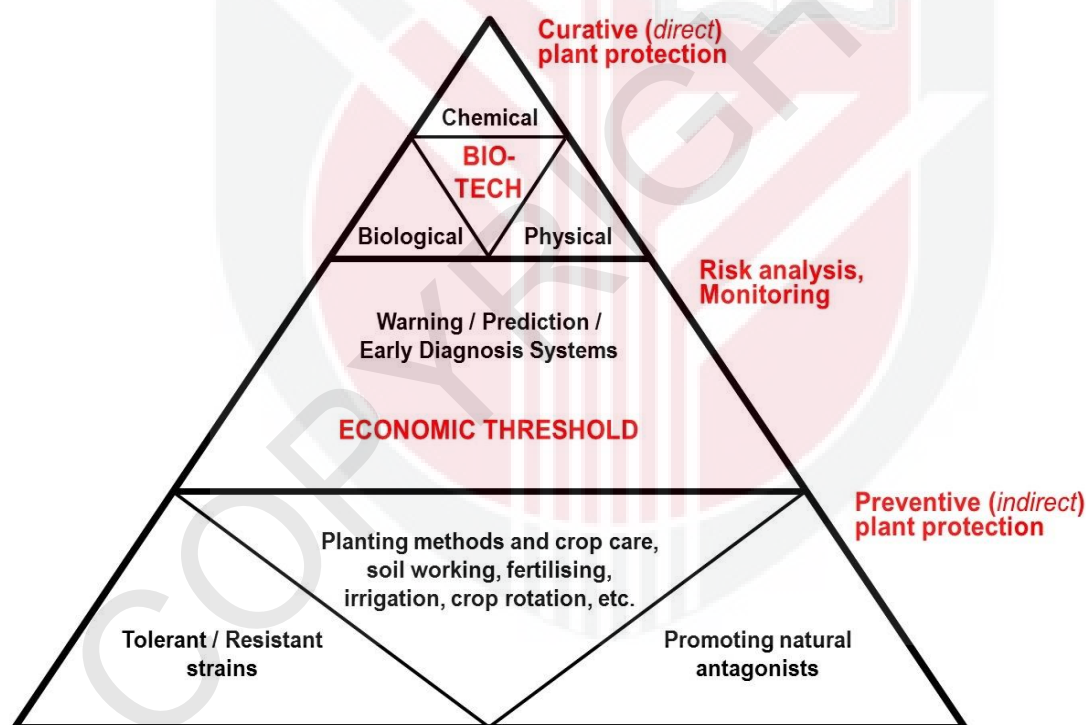


Figure 2.3: Principles and approaches of Integrated Plant Protection (IPP) otherwise known as Integrated Pest Management (IPM)
(Adapted from Frische *et al.*, 2018)

2.4 Cultural and physical control

Historically, cultural and physical control were the earliest and major means for controlling pests and diseases in agricultural commodities. The earliest documented cultural and physical control dated circa 1,500 B.C. where farmers manipulated planting times and dates to prevent pest attack. Today, these type of control forms an integral part of the IPM strategy (Sorensen *et al.*, 2016).

2.4.1 Definition of cultural and physical control

Cultural control refers to the modification of the targeted pests' habitat or environment to reduce the pest establishment and/or population. Cultural control involves good farm practices as well as farming machinery to alter the farming areas, thus reducing the pests' inoculum and/or their establishment. Physical control, on the other hand, refers to the use of equipment and devices as well as hands-on techniques that form a protective barrier between the crops and the pests (Sorensen *et al.*, 2016; Khan & Ram, 2014; Vincent *et al.*, 2003). This control measure can function in two ways, either by direct physical impact on the targeted pest, or by altering the pests' physical environment, thus rendering it unfavourable for growth. In other words, physical control mainly works by targeting the physiological and behavioural of pests (Sorensen *et al.*, 2016). Together, cultural control acts as a preventive strategy, while physical control acts as a curative strategy (Sorensen *et al.*,

2016). In IPM, cultural control always takes precedent before any other curative strategies, including the physical control measures (see Figure 2.3).

2.4.2 Applications of cultural and physical control

Cultural control includes controlled irrigation to minimise stresses on the crops, crop rotation, crop monitoring, growing pest-resistant crop varieties/cultivars through selective breeding, intercropping, seed selection, soil ploughing and tilling, timely harvesting, trap cropping, and companion planting (*i.e.*, companion crops planted nearby the main crops to divert pests away from infesting the main crops) Holden *et al.* (2012).

Physical control can further be divided into two: active and passive. Active method is when the control strategy requires a continuous input over the period of control, while passive method only requires a one-time application for control establishment (Vincent *et al.*, 2003). Active physical control measures include the uses of mechanical machinery and devices for pre-harvest cleaning of the cultivation area before crop plantation, hand collection and destruction of pest's egg masses, and pneumatic (*i.e.*, using blown or aspirated air to dislodge insect pest from the targeted crop). Passive physical control measures include the use of physical barriers such as nettings or trappings, trenches, fences, or even scarecrows (Sorensen *et al.*, 2016; Vincent *et al.*, 2003).

2.4.3 Limitations of cultural and physical control

Cultural and physical control have flaws and drawbacks, and must be appropriately modified. For cultural control, specifically mycotoxin control pre-harvest, tillage, irrigation, managing date of planting, and breeding programs either conventional or genetically modified have been indicated as insufficient, hard to implement, and may be impractical for controlling and minimising mycotoxins in targeted crops (Abbas *et al.*, 2011). Furthermore, physical control on a much larger scale requires enormous time and manpower, which will most likely contribute to higher operational costs. In fact, physical control measures have been indicated as equally insufficient as cultural control measures (Vincent *et al.*, 2003). Besides, although physical control is regarded as one of the curative strategies in controlling pests and diseases, often they could not adequately destroy the pests until much damages have been done (Vincent *et al.*, 2003). In the case when both control measures are not enough for controlling the pests, diseases, and mycotoxin contamination in the targeted area or on targeted crop, chemical control will usually be introduced.

2.5 Chemical control

The application of chemical control was first documented circa 1,000 B.C. (some 500 years after the first record of physical control) when the Greek Homer used sulphur to prevent insects and mites in plantation. Fast forward, the dawn of commercialised chemical control emerged in the 19th century. The first modern synthetic chemical pesticide for plant pathogen was recorded

during World War II. During this time, chemical pesticides reached their peak as two newly-introduced synthetic insecticide and herbicide, dichlorodiphenyltrichloroethane (DDT) and 2,4-dichlorophenoxyacetic acid (2,4-D), respectively, revolutionised pest management (Hajek & Eilenberg, 2018). Today, chemical control remains one of the most widely used control measures against pests and diseases. However, since chemical pesticides are always associated with some serious flaws and drawbacks which will be further discussed in sub-Section 2.5.3, often chemical control measures are only applied as the last resort within the IPM strategy.

2.5.1 Definition of chemical control

Chemical pest control refers to the use of chemically-synthesised pesticides, which can be employed either as solid (*e.g.*, by luring the pests to eat chemically-poisoned baits), liquid (*e.g.*, by spraying formulated pesticide suspensions onto the crops or soil), or gaseous (*e.g.*, by suffocating pests with chemically-infused fumigants). Apart from chemically-synthesised compounds, there are also plant-derived or plant-based control agents known as phytochemicals that pose lesser threat to the environments and human health as compared to their more recalcitrant chemically-synthesised counterparts. These phytochemicals, which include alkaloids, chromenes, cucurbitacins, cyclopropanoid acids, phenolics, polyacetylenes, quassinoids, saponins, terpenes, and their various derivatives, are usually applied as antifeedants and/or deterrents for insects (Koul, 2008). Additionally, there are also semiochemicals (*e.g.*, pheromones and allelochemicals) that are defined

as substances used in plant–insect or insect–insect interactions as complementary components to insecticide approaches in IPM. Semiochemicals are used to effectively modify insect pests' behaviour by affecting their survival and/or reproduction in the effort to control their infestations on crops (El-Ghany, 2019).

2.5.2 Applications of chemical control

Chemical pesticides, either produced synthetically or naturally, can further be divided into the following: herbicide (*i.e.*, to kill weeds), insecticide (*i.e.*, to kill insects), nematicide (*i.e.*, to kill nematodes), and fungicide (*i.e.*, to kill fungi). The usage of pesticides is indispensable in agricultural settings since they are very effective in controlling agricultural pests, thus increasing agricultural yield and production. Pesticides including fungicides are often formulated into liquid, powder, or granular, and have been applied before and during plantation period by various methods such as spraying, fumigation in soil, in-furrow application, seed pelleting or dressing, and baiting (Hajek & Eilenberg, 2018).

2.5.3 Limitations of chemical control

Despite providing efficacy as well as rapid control of pests, chemical control has long been associated with many issues and drawbacks. These include high production and application costs, human health hazards, restriction by domestic and international regulatory limits, trade bans, compounds banned and phased out, residual effects, environmental pollution, resistance

development in pests, and potential elimination of beneficial natural enemies of the targeted pests (Bommarco *et al.*, 2011). In addition, in controlling mycotoxins such as aflatoxins, some researchers regarded the usage of chemical control as elusive and inefficient (Sweany, 2022). In fact, some fungicides, in certain environmental conditions and at sub-optimal level, may otherwise stimulate mycotoxins instead of inhibiting them, albeit successfully reducing their producer fungal growth (Magan & Aldred, 2007). Therefore, biological control may serve as an alternative, or at least as a complementary, for chemical control.

2.6 Biological control

The earliest documented usage of biological control was circa 300 A.D. when the Chinese used predatory ants in their citrus orchards. Later, the first large-scale application of biocontrol in modern history was in the late 1880s when the parasitic fly *Cryptochaetum iceryae* and vedalia beetle, *Rodolia cardinalis* were introduced to control the population of *Icerya purchas* (cottony-cushion scale insect; Barrat *et al.*, 2018) in California citrus orchards. However, biocontrol application died down during the chemical pesticides' revolution. Nevertheless, its application gradually improved following public awareness of the excessive and unregulated usage of synthetic chemical pesticides (Barrat *et al.*, 2018). Since then, biocontrol has become one of the most important elements in the IPM strategy, which also forms the primary basis of the present study.

2.6.1 Definition of biological control

Biological control, or simply biocontrol, is the utilisation of beneficial living organism (or microorganism) to reduce or suppress the population density of another organism (*i.e.*, pest, pathogen) to a level below the crops' damaging level (van Lenteren *et al.*, 2018). To put simply, biocontrol is the management of pest and pathogen using their natural enemies. If properly applied, biocontrol could be a long-term and self-sustaining treatment method for managing pests (Sahayaraj, 2014) that will consequently contribute to lower agro-operational costs. Biocontrol has been widely applied to control insects, weeds, and plant pathogens (*e.g.*, bacteria, fungi; Barrat *et al.*, 2018). For biocontrol agents against plant pathogens, the term "antagonist" is more suited and frequently used by plant pathologists to describe the agents, since their mechanism of action against the plant pathogens are often indirect, for example, by antibiosis or resource competition, rather than directly by killing the pathogens (Hajek & Eilenberg, 2018). Therefore, the termed "antagonist" and "biocontrol agent" will be used interchangeably in the present study to indicate the same meaning, and where context allows.

At present, there are four widely accepted fundamental strategies in biocontrol (van Lenteren *et al.*, 2018): (a) natural, in which natural enemies of the pests are not frequently noticed and consciously manipulated by humans but their presence actually helps to regulate the population density of their host or prey; (b) importation (classical), in which foreign/exotic natural enemies of the pests are intentionally reared and released into new habitats or ecosystems to

regulate the population density of their host or prey; (c) augmentation, in which natural enemies of the pests, which are unable to survive and/or persist in the new habitats or ecosystems, are reared in large population, and mass-released (inundative augmentation) or periodically released in smaller numbers (inoculative augmentation) to regulate the population density of their host or prey; and (d) conservation, in which natural enemies of the pests are aided (for example, providing them with shelters, and reducing the use of chemicals that might kill them) to maintain and optimise their survival and/or effectiveness in regulating the population density of their host or prey.

2.6.2 Applications of biological control

Biocontrol agents against plant pathogen or phytopathogens encompasses four organisms: insects, bacteria, fungi, and yeasts. Against mycotoxigenic fungi, the latter three, also known as microbial biocontrol agents, are the most common. Of all these agents, the mycoparasitic fungi *Trichoderma* spp., such as *T. asperellum*, *T. atroviridae*, *T. gamsii*, *T. hamatum*, *T. harzianum*, *T. polysporum*, *T. stromaticum*, *T. virens*, and *T. viride*, are species that are most frequently applied against the mycotoxigenic and phytopathogenic fungi. Besides *Trichoderma*, soilborne bacteria such as *Bacillus* spp. and *Streptomyces* spp., and yeasts such as *Aureobasidium pullulans* and *Saccharomyces cerevisiae* are also frequently used (van Lenteren *et al.*, 2018). Against mycotoxins, the non-aflatoxigenic strains of *Aspergillus flavus* is the most effective and promising biocontrol agent to control aflatoxins, and these strains have been patented, registered, and applied in many parts of the

world, particularly in the United States of America and several countries in Africa with resounding success. However, other agents such as the yeast *Pichia anomala* was also reported to be effective against aflatoxins, particularly on treenuts (Mohale, 2013).

Among microbial biocontrol agents, the usage of fungi has gained attention as some beneficial fungal strains (e.g., *Trichoderma* spp.) have a broad-spectrum antagonistic activity against various phytopathogens. Besides, fungi have high reproductive rate, short generation time, are target-specific, and pose efficient control mechanisms against fungal pathogens, and may survive despite the absence of plant host by shifting their survival mode from parasitism into saprotrophism, therefore maintaining sustainability in the control environment (Thambugala *et al.*, 2020). Many fungal agents are also more stable during the harsh conditions of downstream processing (*i.e.*, during formulation) and field application due to their ability to develop conidia as survival strategy. Some mycotoxigenic species require lower moisture for growth and survival than the bacteria or yeasts. Therefore, using fungal biocontrol agents will be more suitable for effective control (Ayaz *et al.*, 2023; Mohale, 2013).

2.6.3 Limitations of biological control

Several drawbacks have been highlighted for biocontrol namely they do not function as rapidly as chemical pesticides, their application requires special skills/knowledge, or even worse, they prey on beneficial organism thus disrupting the natural food chain. The latter is what known as “non-target

effect” (Louda *et al.*, 2003). Simberloff in 2012 outlined four potential risks when introducing a biocontrol agent: (a) direct effect on non-targets; (b) indirect effect on non-targets; (c) dispersal of the introduced biocontrol agent to other area not originally intended; and (d) changed relationships between the introduced biocontrol agent and the native species (e.g., when the introduced biocontrol agent multiplies uncontrollably and becomes invasive) (Simberloff, 2012). To reduce the risk of non-target effect and invasive species, care and consideration should be taken into account when developing an efficient and successful biocontrol system. This includes selecting and developing new biocontrol agents from the native area where the control of targeted pest and diseases intended to be applied. By selecting indigenous/local antagonists, effective control could be achieved since the indigenous biocontrol agents may adapt, survive, and proliferate well in the niche’s microclimates (Moore, 2022). This was demonstrated in the present study where potential biocontrol agents were isolated from the same niche on which they were intended to be applied (e.g., grain maize plantation).

In 2011, Köhl and colleagues proposed nine sequential steps in selecting, developing, and introducing a new and effective biocontrol agent against plant pathogens. This could also be applied to select the new agents against mycotoxigenic species, with some additional steps required for controlling mycotoxins (Medina *et al.*, 2017). These sequential steps include: (a) assessment of targeted crop, disease, and market, (b) isolation of candidate antagonists, (c) rapid-throughput screening in terms of production, safety, and ecology, (d) database mining in terms of intellectual property protection, safety,

ecology, environmental risk, and marketing, (e) efficacy testing in bioassays for growth and mycotoxin reduction, (f) preliminary assessment of mass production, (g) pilot formulation, (h) upscaling mass production and full field testing, and (i) integration into cropping systems with constant monitoring of its efficacy and environmental risks. The present study managed to performed steps (a) to (e). Steps (f) to (i) were listed under recommendations for future work.

2.7 Current status of biological control in Malaysia

Biocontrol has a long history in Malaysia. The earliest available online resource dates back to more than 40 years ago in 1978 on the use of the beetle *Metrogaleruca obscura* and the wasp *Eurytoma attiva* to control the invasive weed *Cordia curassavica* (tropical black sage) through defoliation and seed destruction, respectively (Simmonds, 1980). Since then, Malaysian agricultural authorities, particularly the Department of Agriculture (DOA) and Malaysian Agricultural Research and Development Institute (MARDI), both under the jurisdiction of the Ministry of Agriculture and Agro-based Industry, have been conducting numerous research initiatives to combat pest and pathogen infestation on economically-important crops in Malaysia, such as oil palm and rice paddy. However, despite Malaysian climatic conditions (tropical; warm and humid all year round) being highly favourable for fungal proliferation, including that of mycotoxigenic fungi, on staple crop commodities pre- and post-harvest, to the best of our knowledge thus far, there has been very little, if any, research

related to the use of biocontrol agents to control mycotoxigenic fungi and their associated mycotoxin contamination in Malaysia.

As part of a good IPM, biocontrol of phytopathogenic organisms has been widely applied in Malaysia with all the four biocontrol strategies (*i.e.*, natural, importation, augmentation, conservation) have been documented. The role of natural enemies in controlling native pests and pathogens has been recognised in major crops of Malaysia, especially oil palm, cocoa, coconut and rice. The introduction of parasitoid wasps *Diadegma semiclausum* and *Diadromus collaris* in the 70's to control *Plutella xylostella* (diamondback moth whose larvae destructively infest cruciferous vegetables especially cabbages) had suppressed the drawbacks of the reduction in chemical pesticide usage. The introduction of beneficial plants such as *Cassia cobanensis* (cassia), *Turnera subulata* (white buttercup) and *Antigonon leptopus* (Mexican creeper) has provided shelter and supplementary food such as nectar, and encouraged the proliferation of predators and parasites. The conservation of natural or introduced enemies has also been important in insect pest management in Malaysia.

2.7.1 Biological control against phytopathogenic organisms

Table 2.5 simplifies more than 40 years of research on the biological control agents, their targeted pests/pathogens/diseases, and their potential mechanisms of action and/or applications against phytopathogenic organisms in Malaysia. Figure 2.4 further illustrates the distribution of the targeted crop

commodities on which the researches on potential biological control agents against phytopathogenic organisms have been focused. From Figure 2.4, it is apparent that two crop commodities, namely oil palm and rice paddy, evidently received appreciable attention and emphasis in terms of protection and prevention against diseases.

Table 2.5: Available publications on the use of biological control agents against phytopathogenic organisms in Malaysia (1980–2023)

Biocontrol control agent	Targeted pest / pathogen / disease	Application / potential mechanism of action	Reference
(a) Ascomycete			
<i>Beauveria bassiana</i>	<i>Atteva sciodoxa</i> (tiger moth) whose larvae infest long jack	Spraying of conidial suspension targeting larvae	Abood <i>et al.</i> , 2010
<i>Beauveria bassiana</i> and <i>Paecilomyces fumosoroseus</i>	<i>Plutella xylostella</i> (diamondback moth) whose larvae infest cabbages	Spraying of conidial suspension	Ibrahim & Low, 1993
<i>Diaporthe miricidae</i> , <i>Trichoderma asperellum</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Seedling treatment	Sim <i>et al.</i> , 2019
<i>Exserohilum longirostratum</i>	<i>Echinochloa crus-galli</i> (barnyard grass), weed infesting rice paddy fields	Spraying of conidial suspension	Ng <i>et al.</i> , 2012
<i>Exserohilum monoceras</i>	<i>Echinochloa crus-galli</i> (barnyard grass), weed infesting paddy fields	Spraying of conidial suspension	Kadir <i>et al.</i> , 2008
<i>Exserohilum prolatum</i>	<i>Rottboellia cochinchinensis</i> (itch grass), a noxious weed impacting crops	Soil treatment (spraying) with conidial suspension	Alloub <i>et al.</i> , 2009

Table 2.5: Continued

<i>Exserohilum rostratum</i>	<i>Cyperus iria</i> (flat sedge), weed infesting paddy fields	Spraying of conidial suspension	Kundat <i>et al.</i> , 2010
<i>Fusarium oxysporum</i> (non-pathogenic)	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i> (ascomycete) causing <i>Fusarium</i> wilt in bananas	As an endophyte	Ting <i>et al.</i> , 2009
<i>Isaria fumosorosea</i>	<i>Coptotermes curvignathus</i> and <i>C. gestroi</i> (termites) infesting trees	Conidia germinate and penetrate into the termite cuticle	Jessica <i>et al.</i> , 2019
<i>Isaria fumosorosea</i> and <i>Metarhizium anisopliae</i>	<i>Atteva sciodoxa</i> (tiger moth) whose larvae infest long jack	Spraying with conidial suspension	Sajap <i>et al.</i> , 2014
<i>Metarhizium anisopliae</i> var. <i>anisopliae</i>	<i>Coptotermes curvignathus</i> (termite) infesting oil palms	Conidia germinate and penetrate into the termite cuticle	Hoe <i>et al.</i> , 2009
<i>Metarhizium anisopliae</i> var. <i>major</i>	<i>Oryctes rhinoceros</i> (rhinoceros beetle) attacking oil palm fronds	Granules of mycelia and spores attacking beetle larvae	Moslim <i>et al.</i> , 2011b; Moslim <i>et al.</i> , 2009
<i>Paecilomyces carneus</i> and <i>P. farinosus</i>	<i>Pteroma pendula</i> (bagworm moth) whose larvae infest oil palms	Spraying of conidial suspension targeting larvae	Bakeri <i>et al.</i> , 2009
<i>Paecilomyces lilacinus</i>	<i>Meloidogyne incognita</i> (nematode) causing root-knot in black peppers	Spraying of conidial suspension	Pau <i>et al.</i> , 2012
<i>Trichoderma harzianum</i>	<i>Fusarium proliferatum</i> and <i>F. verticillioides</i> (ascomycete) causing <i>Fusarium</i> ear rot in maize	Production of volatile and non-volatile inhibitory compounds	Suhaida & Nur Ain Izzati, 2013
<i>Trichoderma harzianum</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Production of volatile and non-volatile inhibitory compounds	Siddiquee <i>et al.</i> , 2009; Nur Ain Izzati & Abdullah, 2008; Sundram <i>et al.</i> , 2008

Table 2.5: Continued

<i>Trichoderma harzianum</i>	<i>Macrophomina phaseolina</i> (ascomycete) causing charcoal rot disease in soybeans	Production of volatile and non-volatile inhibitory compounds	Khalili <i>et al.</i> , 2016
<i>Trichoderma virens</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Production of volatile and non-volatile inhibitory compounds	Angel <i>et al.</i> , 2016
<i>Trichoderma</i> sp.	<i>Botryodiplodia theobromae</i> (ascomycete) causing stem-end rot in mangos	Fruit treatment (spraying) with conidial suspension	Suhanna <i>et al.</i> , 2013
<i>Trichoderma</i> spp.	<i>Fusarium fujikuroi</i> (ascomycete) causing bakanae disease in rice	Production of volatile and non-volatile inhibitory compounds	Ng <i>et al.</i> , 2015
<i>Zoophthora radicans</i>	<i>Plutella xylostella</i> (diamondback moth) whose larvae infest cabbages	Spraying of conidial suspension	Furlong <i>et al.</i> , 1995
(b) Basidiomycete			
<i>Clitopilus prunulus</i> , <i>Grammothele fuligo</i> , <i>Lentinus tigrinus</i> , <i>Pycnoporus sanguineus</i> and <i>Trametes lactinea</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Root treatment of oil palm seedlings	Naidu <i>et al.</i> , 2018
<i>Fomes</i> sp., <i>Trametes elegans</i> , <i>Trametes lactinea</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Seedling treatment	Darlis <i>et al.</i> , 2023
<i>Ganoderma orbiforme</i> , <i>Neonothopanus nambi</i> and <i>Schizophyllum commune</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Competition for substrate and space, and production of non-volatile inhibitory compounds	Naidu <i>et al.</i> , 2016
<i>Schizophyllum commune</i>	Wood-degrading fungi on rubber wood	Production of inhibitory compounds	Teoh <i>et al.</i> , 2017 Peng & Don, 2013; Teoh <i>et al.</i> , 2012

Table 2.5: Continued

(c) Yeast			
110 species were screened, of which 29 species showed antagonism	<i>Colletotrichum gloeosporioides</i> (ascomycete) causing anthracnose in papayas	Production of hydrolytic enzymes and antimicrobial compounds	Hamizah <i>et al.</i> , 2013
(d) Bacterium			
Actinomycetes	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Production of inhibitory compounds	Shariffah-Muzaimah <i>et al.</i> , 2018; Nur Azura <i>et al.</i> , 2016; Shariffah-Muzaimah <i>et al.</i> , 2015; Tan <i>et al.</i> , 2002
<i>Bacillus amyloliquefaciens</i> and <i>Pseudomonas pachastrellae</i>	<i>Phytophthora capsici</i> (oomycete) and <i>Fusarium solani</i> (ascomycete) infesting black peppers	Production of extracellular hydrolytic enzymes	Kota <i>et al.</i> , 2015
<i>Bacillus thuringiensis</i> subsp. <i>Kurstaki</i>	<i>Metisa plana</i> (bagworm moth) whose larvae infest oil palms	Soil treatment (spraying) with bacterial suspension	Salim <i>et al.</i> , 2015
<i>Bacillus</i> spp. and <i>Enterobacter</i> spp.	<i>Fusarium solani</i> f. sp. <i>Piperis</i> and <i>F. oxysporum</i> f. sp. <i>piperis</i> (ascomycetes) causing foot rot and stem blight in black peppers	Production of volatile and diffusible bioactive antifungal compounds	Edward <i>et al.</i> , 2013
<i>Bacillus</i> spp., <i>Burkholderia</i> spp., and <i>Pseudomonas</i> spp.	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	As an endophyte	Ramli <i>et al.</i> , 2016 Seung <i>et al.</i> , 2015;
<i>Burkholderia cepacia</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Production of proteolytic enzymes	Azadeh <i>et al.</i> , 2010

Table 2.5: Continued

<i>Burkholderia cepacia</i> and <i>Pseudomonas aeruginosa</i>	<i>Colletotrichum gloeosporioides</i> (ascomycete) causing anthracnose in papayas	Production of volatile and non-volatile inhibitory compounds	Rahman <i>et al.</i> , 2007
<i>Burkholderia</i> sp.	<i>Phytophthora capsici</i> (oomycete) causing foot rot and stem blight in black peppers	Production of hydrolytic enzymes and antimicrobial compounds	Ahmad & Ahmadu, 2017
<i>Burkholderia</i> sp. and <i>Pseudomonas</i> sp.	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i> (ascomycete) causing Fusarium wilt in bananas	As endophytes which induces host defence enzymes	Mohd Fishal <i>et al.</i> , 2010
<i>Enterobacter asburiae</i> , <i>E. cancerogenus</i> , and <i>E. cloacae</i>	<i>Phytophthora capsici</i> (oomycete) causing foot rot and stem blight in black peppers	Production of volatile inhibitory compounds	Toh <i>et al.</i> , 2016
Lactic acid bacteria	<i>Colletotrichum capsici</i> (ascomycete) causing anthracnose in chilli	Seed treatment of chilli	El-Mabrok <i>et al.</i> , 2012
<i>Pseudomonas aeruginosa</i>	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	As an endophyte	Sathyapriya <i>et al.</i> , 2012; Bivi <i>et al.</i> , 2010
<i>Pseudomonas fluorescens</i>	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i> (ascomycete) causing Fusarium wilt in bananas	Production of inhibitory compounds	Mohammed <i>et al.</i> , 2011
<i>Pseudomonas</i> spp.	<i>Rhizoctonia solani</i> (basidiomycete) causing sheath blight of rice	Soil treatment (spraying) with bacterial suspension	Akter <i>et al.</i> , 2016; Akter <i>et al.</i> , 2014
<i>Serratia marcescens</i>	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i> (ascomycete) causing Fusarium wilt in bananas	Soil treatment with inoculum which induces host defence enzymes	Ting <i>et al.</i> , 2010

Table 2.5: Continued

<i>Stenotrophomonas maltophilia</i>	<i>Pyricularia oryzae</i> (rice blast fungus) infesting rice	Seed treatment and foliar spray, induction of host resistance, plant growth promotion	Fariman <i>et al.</i> , 2022
<i>Streptomyces glauciniger</i>	Phytopathogenic fungi	Production of chitinase	Awad <i>et al.</i> , 2014
<i>Streptomyces griseus</i>	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i> (ascomycete) causing Fusarium wilt in bananas	Soil treatment (spraying) with bacterial suspension	Zacky & Ting, 2015; Zacky & Ting, 2013
<i>Streptomyces violaceusniger</i>	<i>Fusarium oxysporum</i> f. sp. <i>cubense</i> (ascomycete) causing Fusarium wilt in bananas	Production of inhibitory compounds	Getha & Vikineswary, 2002
<i>Streptomyces</i> spp.	<i>Colletotrichum capsici</i> , <i>Colletotrichum acutatum</i> , and <i>Colletotrichum gloeosporioides</i> (ascomycete) causing anthracnose in chilli	Soil treatment (spraying) with bacterial suspension	Shahbazi <i>et al.</i> , 2014; Shahbazi <i>et al.</i> , 2013
<i>Streptomyces</i> spp.	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Seedling treatment, plant growth promotion	Shariffah-Muzaimah <i>et al.</i> , (2020)
<i>Streptomyces</i> spp.	<i>Pyricularia oryzae</i> (rice blast fungus) infesting rice	Production of inhibitory compounds	Awla <i>et al.</i> , 2017; Law <i>et al.</i> , 2017
(e) Insect			
<i>Acerophagus papayae</i> (wasp)	<i>Paracoccus marginatus</i> (papaya mealybug) infesting papayas	As an endoparasitoid	Mastoi <i>et al.</i> , 2018
<i>Apanteles metesae</i> (wasp)	<i>Metisa plana</i> (bagworm moth) whose larvae infest oil palms	As a parasitoid	Salmah <i>et al.</i> , 2012
<i>Calycomyza lantanae</i> and <i>Ophiomyia lantanae</i> (flies)	<i>Lantana camara</i> (big sage), invasive weed	Larvae of flies are leaf-eaters	Ooi, 1987

Table 2.5: Continued

<i>Cotesia vestalis</i> (wasp)	<i>Plutella xylostella</i> (diamondback moth) whose larvae infest cabbages	As a parasitoid	Kermani <i>et al.</i> , 2014
<i>Cosmolestes picticeps</i> and <i>Sycanus dichotomus</i> (bugs)	<i>Metisa plana</i> (bagworm moth) whose larvae infest oil palms	As predatory natural enemies	Jamian <i>et al.</i> , 2017
<i>Diachasmimorpha longicaudata</i> , <i>Fopius arisanus</i> , <i>Fopius vandenboschi</i> , <i>Psytalia incise</i> and <i>Psytalia fletcheri</i> (wasps)	<i>Bactrocera carambolae</i> , <i>Bactrocera cucurbitae</i> , and <i>Bactrocera papaya</i> (fruit flies) infesting fruits	As a parasitoid	Yaakop <i>et al.</i> , 2015; Shariff <i>et al.</i> , 2014; Ibrahim <i>et al.</i> , 2013; Chinajariyawong <i>et al.</i> , 2000
<i>Dolichoderus thoracicus</i> (ant)	<i>Conopomorpha cramerella</i> (cocoa pod borer moth) whose larvae infest cocoa	Active and dispersive predatory behaviour of ants	Adnan <i>et al.</i> , 2018
<i>Dolichoderus thoracicus</i> and <i>Oecophylla smaragdina</i> (ants)	<i>Helopeltis theobromae</i> (mosquito bug) causing leaf tattering and fruit blemishes in cocoa	Active and dispersive predatory behaviour of ants	Way & Khoo, 1991
<i>Menochilus sexmaculatus</i> (ladybird beetle)	<i>Rhopalosiphum maidis</i> (aphid) infesting maize	Predatory behaviour of beetles	Ibrahim & Kueh, 2013
<i>Micromus tasmaniae</i> (lacewings)	Aphids infesting potatoes	Plant treatment (spraying) with egg suspension	Hussein, 1984
<i>Oecophylla smaragdina</i> (weaver ant)	<i>Hypsipyla robusta</i> (moth) whose larvae feed on timber shoots, flowers and barks	Active and dispersive predatory behaviour of ants	Lim <i>et al.</i> , 2008
<i>Oecophylla smaragdina</i> (weaver ant)	<i>Pteroma pendula</i> (bagworm moth) whose larvae infest oil palms	Active and dispersive predatory behaviour of ants	Pierre & Idris, 2013
<i>Tamarixia radiata</i> (wasp)	<i>Diaphorina citri</i> (Asian citrus psyllid) infesting citrus fruits	As a parasitoid	Sule <i>et al.</i> , 2014

Table 2.5: Continued

(f) Microsporidia			
<i>Nosema bombycis</i>	<i>Plutella xylostella</i> (diamondback moth), <i>Spodoptera exigua</i> (beet armyworm moth) and <i>Spodoptera litura</i> (tobacco cutworm moth or cotton leafworm moth) whose larvae infest fruits and vegetables	Spraying of spore suspension targeting larvae	Ghani <i>et al.</i> , 2013; Kermani <i>et al.</i> , 2013; Ramli <i>et al.</i> , 2011
(g) Shrub			
<i>Tinospora tuberculata</i>	<i>Echinochloa crus-galli</i> (barnyard grass), weed infesting paddy fields	Production of volatile inhibitory compounds	Aslani <i>et al.</i> , 2015
(h) Snail			
<i>Pomacea canaliculata</i> and <i>Pomacea maculata</i>	<i>Limnocharis flava</i> (yellow velvet leaf), weed infesting paddy fields	As herbivore	Gilal <i>et al.</i> , 2016
(i) Virus			
Baculovirus	<i>Spodoptera litura</i> (tobacco cutworm moth or cotton leafworm moth) whose larvae infest star fruits and vegetables	Spraying of viral occlusion bodies	Zabedah <i>et al.</i> , 2010
Baculovirus	<i>Plutella xylostella</i> (diamondback moth) whose larvae infest cabbages	Spraying of viral occlusion bodies	Abdul Kadir <i>et al.</i> , 1999
Cucumber Mosaic Virus	<i>Bemisia tabaci</i> (silverleaf whitefly) infesting chilli	Induction of host resistance	Saad <i>et al.</i> , 2019
Nudivirus	<i>Oryctes rhinoceros</i> (rhinoceros beetle) attacking oil palm fronds	Spraying of viral occlusion bodies	Moslim <i>et al.</i> , 2011a; Ramle <i>et al.</i> , 2005

Table 2.5: Continued

(j) Combination			
A consortium of functional microbiota in microbial-enriched compost tea	<i>Golovinomyces cichoracearum</i> (ascomycete) causing powdery mildew on melons	Spraying of melon seedlings with the compost tea	Naidu <i>et al.</i> , 2012
Arbuscular mycorrhizal fungus <i>Glomus mosseae</i> (Glomeraceae) and <i>Enterobacter</i> sp. (bacterium)	<i>Rigidoporus microporus</i> (basidiomycete) causing white root rot in rubber	Induction of host resistance	Shabbir <i>et al.</i> , 2021
<i>Bacillus thuringiensis</i> (bacterium) and <i>Metarhizium anisopliae</i> (ascomycete)	<i>Tirathaba rufivena</i> (bunch moth) whose larvae infest oil palms	Spraying with fungal and bacterial suspension	Mohamad <i>et al.</i> , 2017
<i>Bacillus</i> spp. (bacteria) and <i>Trichoderma</i> spp. (ascomycetes)	<i>Ganoderma boninense</i> (basidiomycete) causing basal stem rot in oil palms	Production of volatile and non-volatile inhibitory compounds	Angel <i>et al.</i> , 2018; Alexander <i>et al.</i> , 2017
<i>Bacillus subtilis</i> (bacterium) and <i>Trichoderma</i> spp. (ascomycetes)	<i>Rhizoctonia solani</i> (basidiomycete) causing various commercially significant plant diseases	Soil treatment (spraying) with fungal and bacterial suspension	Ali & Nadarajah, 2013
<i>Cataenococcus hispidus</i> (mealybug) and <i>Dolichoderus thoracicus</i> (ant)	<i>Helopeltis theobromae</i> (mosquito bug) causing leaf tattering and fruit blemishes in cocoa	Active and dispersive predatory behaviour of ant and bug	Ho & Khoo, 1997
<i>Eurytoma attiva</i> (wasp) and <i>Metrogaleruca obscura</i> (beetle)	<i>Cordia curassavica</i> (tropical black sage or wild sage), invasive weed	Defoliation by beetle and seed-destruction by wasp	Simmonds, 1980
<i>Pseudomonas aeruginosa</i> (bacterium) <i>Trichoderma harzianum</i> and <i>Trichoderma virens</i> (ascomycetes)	<i>Colletotrichum truncatum</i> (ascomycete) causing damping-off in soybeans	Seed treatment with conidial suspension	Begum <i>et al.</i> , 2010

Publications were obtained based on a literature search on SCOPUS®. Search keywords: “biological control”, “Malaysia”. Biological control agents are sorted based on types of organisms.

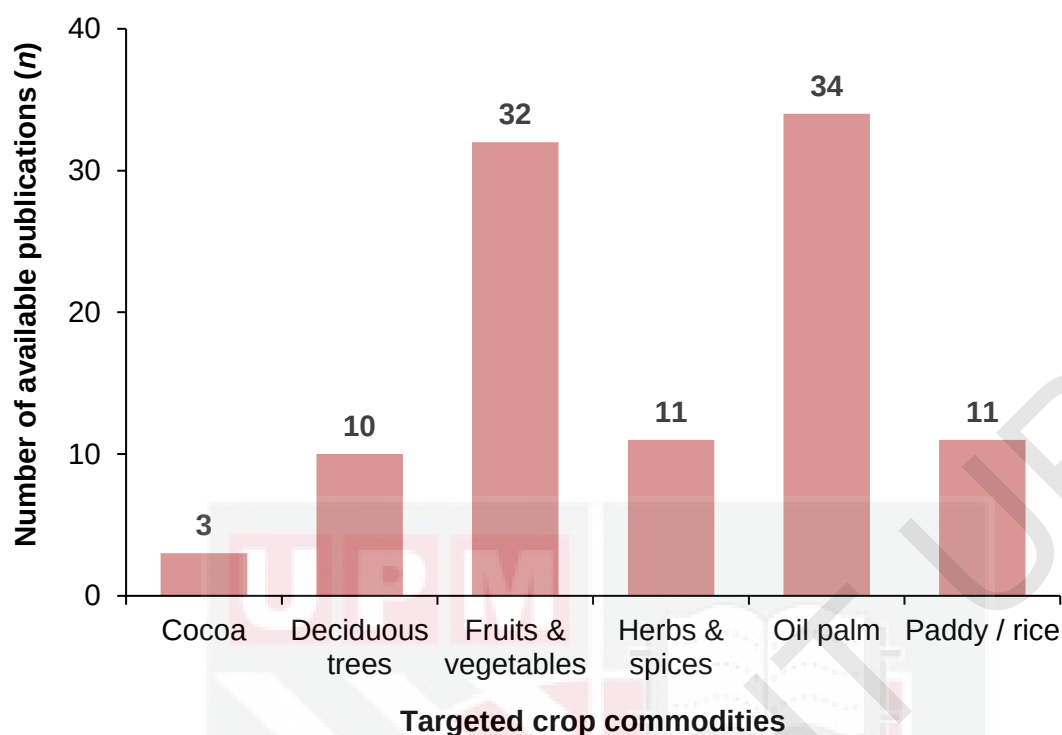


Figure 2.4: Distribution of targeted crop commodities on which scientific investigations on potential biological control agents against phytopathogenic organisms have been conducted in Malaysia (1980 – 2023). Publications were obtained based on a literature search on SCOPUS®, as similarly listed in Table 2.5. Search keywords: “biological control”, “Malaysia”

The major pests of oil palm in Malaysia include bagworm moths (*Metisa plana*, *Pteroma pendula*), bunch moths (*Tirathaba rufivena*), rats, rhinoceros beetles (*Oryctes rhinoceros*), and termites (*Coptotermes curvignathus*), while the most important diseases are *Ganoderma* basal stem rot (caused by *Ganoderma boninense*) and *Marasmius* bunch rot (caused by *Marasmius palmivorus*). To mitigate the *Ganoderma* basal stem rot, the Malaysian Palm Oil Board (MPOB), a government agency responsible for the research and development of palm oil industry in Malaysia, has introduced the Integrated *Ganoderma* Management (IGM) which incorporates several elements including sanitation, biocontrol, chemical control, and the use of fertiliser with beneficial trace

elements. The integration of biocontrol in the system is based on the understanding that low incidence of disease has been achieved in the presence of natural competitors/predators that keep the parasitic fungi under control (Bivi *et al.*, 2010). As an effort to further strengthen this approach, MPOB has also developed and commercialised their biocontrol formulations namely GanoEF biofertiliser (contains endophytic *Hendersonia* GanoEF1 fungus, mechanism of action: production of inhibitory bioactive compounds, Nurrashyeda *et al.*, 2015); and Embio™ actinoPLUS (contains *Streptomyces* sp., mechanism of action: production of inhibitory bioactive compounds, Shariffah-Muzaimah *et al.*, 2015).

For paddy, the rice blast disease caused by *Pyricularia oryzae* is considered a major endemic in rice-planting areas in Malaysia and across the world. Currently, the planting of resistant varieties and the application of fungicides are the most commonly used methods in controlling the disease in rice-growing areas in Malaysia (Abed-Ashtiani *et al.*, 2018). However, due to the fact that both practices fail to provide a long-term solution for the disease control, growing amount of research have been conducted to incorporate biocontrol approach as an alternative instead. Recently, an endophytic actinomycete, *Streptomyces* sp. strain UPMRS4 isolated from the rhizosphere of irrigated paddy fields in Malaysia has succeeded in suppressing the rice blast disease through the production of inhibitory bioactive compounds, thus inducing the systemic resistance of paddy against *P. oryzae* (Awla *et al.*, 2017).

Figure 2.5 depicts the types of potential biocontrol agents against phytopathogenic organisms in Malaysia, which are dominated by bacteria, ascomycetes and insects. The use of bacteria as a biocontrol agent especially *Bacillus* spp., *Burkholderia* spp. and *Pseudomonas* spp. has shown considerable success in Malaysia (Table 2.5). Ubiquitous in nature, these bacteria exist as both non-parasitic and parasitic pathogenic species; the latter as potent producers of various enzymes and antimicrobial substances that inhibit important phytopathogens while at the same time promote plant growth by the production of volatile substances (Beneduzi *et al.*, 2012).

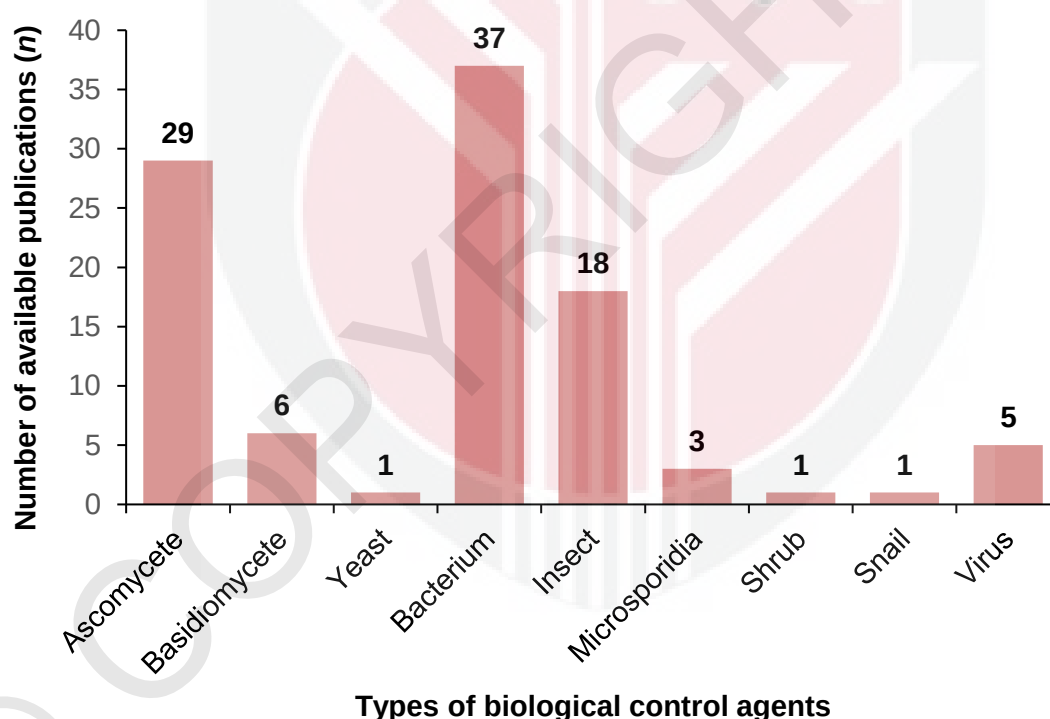


Figure 2.5: Distribution of potential biological control agents against phytopathogenic organisms in Malaysia (1980–2023). Publications were obtained based on a literature search on SCOPUS®, as similarly listed in Table 2.5. Search keywords: “biological control”, “Malaysia”

Furthermore, considering that majority of crop pests are insects, the use of entomopathogenic fungi, especially from the Division Ascomycota, has proven efficient on many crop species. Species like *Beauveria bassiana*, *Isaria fumosorosea*, *Metarhizium anisopliae*, *Paecilomyces* spp. and *Trichoderma* spp. have been efficacious against beetles, moths, and termites infesting a multitude of crop commodities in Malaysia (Table 2.5). In lethally parasitising the pest insects, or severely disabling them, entomopathogenic ascomycetes usually initiate the infection in the form of airborne conidia which naturally or artificially (*i.e.*, through human intervention) land onto the insects' exoskeleton. Under suitable ecophysiological conditions, these spores germinate and grow into hyphae, thereby colonising the insect's cuticle. These hyphae are capable of producing hydrolysing enzymes which bore a tunnel through which the hyphae could penetrate into the insects' body cavity, and there proliferate further, thus killing the host (Fernandes *et al.*, 2012). Entomopathogenic fungi can grow over a wide range of temperatures, and some strains have the appropriate ecophysiological growth parameters for use in climates such as Malaysia (Borisade & Magan, 2015). Being environmentally safe, the use of entomopathogenic ascomycetes draws worldwide interest for biological control of insect pests including in Malaysia. Apart from insects, these entomopathogenic fungi have also been shown to parasitise other phytopathogenic ascomycetes as well as nematodes (Table 2.5).

For insect species that are used to control insect pests, the biological control agents come either as insectivorous predators or insect parasitoids. Insectivorous predators are mainly free-living species that aggressively prey

on a large number of insects during their lifetime, thus controlling the pest density. Given that many major crop pests are insects, many of the predators used in biological control are insectivorous species (Table 2.5). The ladybird beetles (*Menochilus sexmaculatus*) are voracious predators of the aphids (*Rhopalosiphum maidis*) infesting maize. Various predatory ant species such as *Dolichoderus thoracicus* have been successful in controlling the cocoa pod borer moths (*Conopomorpha cramerella*) whose larvae infest cocoa, and mosquito bugs (*Helopeltis theobromae*) which cause leaf tattering and fruit blemishes in cocoa; and *Oecophylla smaragdina* which prey on bagworm moths (*Pteroma pendula*) whose larvae infest oil palms, and *Hypsipyla robusta* whose larvae feed on timber shoots, flowers and barks.

For insect parasitoids, their mechanism of action is generally by laying their eggs (oviposition) on (or in) the body of an insect host, on (or in) which their developing larvae will feed, consequently decapitating the host. Parasitoids are almost always endopterygote insects which have complete metamorphosis stages during their lifecycle; as parasitoid larvae and free-living adults. Parasitoids are generally classified into endoparasitoids which live within their host's body, or ectoparasitoids which feed on their host's body from outside. Majority insect parasitoid that has been used in Malaysia is from the wasp family such as *Acerophagus papayae*, *Apanteles metesae*, *Cotesia vestalis*, *Diachasmimorpha longicaudata*, *Fopius arisanus*, *F. vandenboschi*, *Psytalia incisi*, *P. fletcheri* and *Tamarixia radiata* which have shown considerable success in reducing the density of moth caterpillars, bugs, fruit flies, and psyllids infesting various economically important fruits and vegetables such as

cabbages, carambolas (star fruit), melons, oranges and papayas. Wasps have been shown to exhibit both endo– and ectoparasitoid behaviours.

Although the majority of insect parasitoids are wasps (Hymenoptera) and flies (Diptera) (Eggleton & Belshaw, 1992), to the best of our knowledge thus far, there has been no available record on the use of the latter as a biological control agent in Malaysia. This could either be due to the fact that this particular application is totally new in Malaysia, therefore suitable for future investigation; or that ongoing trials and researches are not yet conclusive and published.

2.7.2 Biological control against mycotoxigenic fungi

Although the biological control against phytopathogenic organisms has a long history in Malaysia, as earlier discussed, the same could not be entirely said against mycotoxigenic fungi. To the best of our knowledge, the application of biocontrol against mycotoxigenic fungi is almost non-existent in Malaysia with only a handful of publications available online on the subject matter (Samsudin & Magan, 2016; Samsudin *et al.*, 2016; Samsudin *et al.*, 2017; Khan *et al.*, 2021; Rahman *et al.*, 2022). These studies examined the use of *Clonostachys rosea* and *Streptomyces* sp. against growth of *Fusarium verticillioides* and the subsequent fumonisin contamination in maize, and also non-aflatoxigenic *A. flavus* against the aflatoxigenic counterparts in sweet maize and peanut. The only other work of almost similar nature was published slightly earlier on the use of the mycoparasite *Trichoderma harzianum* against *F. proliferatum* and *F. verticillioides* in controlling *Fusarium* ear rot in maize (Suhaida & Nur Ain

Izzati, 2013). They did not, however, specifically examine or quantify the impact on control of any mycotoxins. Because these fungal pathogens, under Malaysian climatic conditions, would certainly contaminate maize with fumonisins during silking, this would have been useful additional information. It is possible that this has not been recognised as an important research area in Malaysia so far. However, as Malaysia does have legislations for mycotoxin contamination of some commodities, this could be a major driver for practically related research for the reduction of mycotoxins using biocontrol strategies in the very near future. Given the fact that maize is one of the most contaminated crop commodities by members of *Aspergillus* section *Flavi* (Pitt & Hocking, 2009; Samson *et al.*, 2010), which almost invariably produce the Group 1 carcinogenic aflatoxins, and that this crop is also relevant in Malaysian agricultural sector, further investigation on the development of an efficient control against aflatoxigenic fungal colonisation on this crop is highly warranted.

As described in Table 2.5, several biocontrol agents have been investigated on their effects against the phytopathogenic *Fusarium* spp. More detailed examination shows that some of these *Fusarium* spp. are in fact also mycotoxigenic (e.g., *F. proliferatum*: beauvericin, fumonisin, moniliformin; *F. solani*: moniliformin, trichothecenes; *F. verticillioides*: fumonisin, fusaric acid, moniliformin), according to Desjardins (2006). Therefore, opportunities do exist for replicating such studies with biocontrol agents and investigate their effects in controlling mycotoxin contamination. In addition, as most studies have focused on the use of effective biocontrol agents against plant diseases, it may

be beneficial to also screen these for mycotoxin control (Medina *et al.*, 2017). Nevertheless, previous studies have suggested that several potential bacterial antagonists may, under certain abiotic conditions, stimulate mycotoxin production instead of inhibiting it (Al-Saad *et al.*, 2016). Thus, care is needed when screening for mycotoxin control. There are also some cases in which the fungal pathogens could “disarm” the applied antagonist. For example, it was shown that deoxynivalenol-producing *F. culmorum* and *F. graminearum* could repress the expression of genes that regulate the chitinase enzyme production in the biocontrol agent *Trichoderma atroviride* (Lutz *et al.*, 2003). This is not unusual because pathogens also possess diverse responses to counteract antagonists, which include detoxification and active efflux of antibiotics, and repression of biosynthetic genes involved in biological control (Duffy *et al.*, 2003).

In other parts of the world, however, the application of biocontrol agents to combat mycotoxigenic fungi is more widely practiced. Among the earliest and excellent reviews that discussed the potential use of non-mycotoxigenic fungi, yeasts and bacteria in controlling mycotoxigenic fungi was published by Cleveland *et al.* (2003). The review also discussed the indirect controls of mycotoxigenic fungi by controlling their vectoring insects or developing insect-resistance plant cultivars (*e.g.*, Bt maize) because insect damage provides entry points for mycotoxigenic fungi to colonise the crop, thus resulting in increased mycotoxin contamination. Another review on the dynamics and mechanisms of action of biocontrol agents against mycotoxigenic fungi was critically discussed by Medina *et al.* (2017). They suggested that the

mechanism of action and the inoculum dose required for effective control of mycotoxigenic fungi may be different from that for phytopathogenic fungi. Nevertheless, they concur that the primary mechanisms of action for the control of mycotoxigenic fungi or phytopathogenic organisms usually include niche/habitat exclusion, competition for nutrient, and production of inhibitory volatiles. In fact, Köhl *et al.*, (2019) suggested that biocontrol agents utilise a cascade of different interacting approaches for efficacy. Based on the available literatures, we could summarise the biological control agents into three major groups: (a) non-toxigenic strains of known mycotoxin producer (e.g., non-aflatoxigenic *A. flavus*), (b) mycoparasitic microfungi, and (c) phyllosphere or soilborne bacteria.

The use of non-aflatoxigenic *A. flavus* and *A. parasiticus* to control their toxigenic counterparts began in the early 90's in the United States of America (Brown *et al.*, 1991). Since then, this application has found success in other parts of the world where aflatoxin contamination is rampant and threatening the countries' economics, such as in Kenya (Marchera & Ndwiga, 2015), Italy (Mauro *et al.*, 2018), Argentina (Alaniz Zanon *et al.*, 2018), and Thailand (Tran-Dinh *et al.*, 2018). Its modes of action are either through competitive exclusion (*i.e.*, toxigenic strain displacement) (Damann 2015), or through thigmoregulation (*i.e.*, touch inhibition) (Huang *et al.*, 2011). However, in securing an efficient biocontrol agent, it should be noted that lack of mycotoxin biosynthesis because of the deletion of key biosynthetic gene clusters may not be the only criterion; rather the non-aflatoxigenic candidate must also be an effective coloniser of the crop commodity onto which it would be applied (to

competitively exclude its competitors), and that it must also be able to reduce the mycotoxin contamination (Horn & Dorner, 2011). Recent investigation proposed yet another mechanism for the antagonism of the non-aflatoxigenic strains which is active extrolite (*i.e.*, uncharacterised compounds) secretion (Moore *et al.*, 2019).

Another successful area for research and applications is the use of mycoparasitic fungi. As described in Table 2.5, *Trichoderma* spp. feature predominantly as a competent biocontrol agent against a wide variety of phytopathogenic organisms. Similar trait is also exhibited against mycotoxigenic fungi and the subsequent mycotoxin production (Veenstra *et al.*, 2019; Braun *et al.*, 2018; Saravanakumar *et al.*, 2018). Prevalently found in the rhizo- and phyllospheres, the free-living and fast-growing *Trichoderma* spp. exist in mutualistic endophytic relationships with plants. As a biocontrol agent, *Trichoderma* spp. demonstrate antagonism through antibiotic production, parasitism (*e.g.*, penetration and killing), inducing host-plant resistance (*Trichoderma* spp. are also known as Plant Growth Promoting Fungi), and habitat/nutrient competition (*Trichoderma* spp. are naturally effective decomposers and aggressive colonisers, thus leaving scarce macro- and micro-nutrients from soil for other competitors). *Trichoderma* spp. also produce a wide array of enzymes, chief among which is the fungal-cell-wall-degrading chitinase (Harman, 2006). The ability of *Trichoderma* spp. to produce chemically diverse secondary metabolites that could modify the environmental condition into one that is inhospitable for their competitors has also been extensively reviewed by Keswani *et al.* (2014) and Reino *et al.*

(2008). In the present study, non-aflatoxigenic *A. flavus* and *Trichoderma* spp. were successfully isolated, identified, and tested against mycotoxigenic strains also isolated from the same habitat (*i.e.*, local, indigenous).

Finally, the use of soilborne bacteria that produce various antibiotics and fungal-cell-wall-degrading enzymes. As in the control of phytopathogenic organisms, *Bacillus* spp. also exhibit potency and prowess in the control of mycotoxigenic fungi. This is mainly attributed to the production of cyclic lipopeptides which display amphiphilic properties that could disrupt the fungal cell wall and cell membrane (Siahmoshteh *et al.*, 2017). Other chemical metabolites produced by *Bacillus* spp. could also inhibit fungal nutrient acquisition, mycelial growth and conidial germination (Volpon *et al.*, 2000; Li *et al.*, 2009; Cao *et al.*, 2012). Recent investigation also elucidates the ability of *Bacillus* spp.'s enzymes in biodegrading the mycotoxins (Afsharmanesh *et al.*, 2018). Besides *Bacillus* spp., lactic acid bacteria have also been shown to possess anti-mycotoxigenic properties, including the production of organic acids which may acidify environmental pH thus inhibiting fungal growth and mycotoxin production; antifungal compounds (*e.g.*, propionate and cyclic dipeptide); and competitive exclusion of fungal pathogens (Guimarães *et al.*, 2018; Oliveira *et al.*, 2014). The efficiency of *Streptomyces* spp. in combatting mycotoxigenic fungi is also of note. The production of various bioactive molecules such as antibiotics (*e.g.*, candicidin, phospholactomycin, tubercidin, pyrrole-2-carboxylic acid), lytic enzymes and volatile organic compounds (*e.g.*, phenylethyl alcohol and (+)-*epi*-bicyclosesquiphellandrene) has greatly contributed to this (Mari *et al.*, 2016; Nguyen *et al.*, 2018; Hoster *et al.*, 2005).

The growth inhibition of mycotoxigenic fungi and the subsequent mycotoxin production by *Streptomyces* spp. in wheat (Palazzini *et al.*, 2017; Palazzini *et al.*, 2007; Nourozian *et al.*, 2006) and in peanut (Sultan and Magan, 2011) have been demonstrated. In the present study, however, bacterial biocontrol agents were not isolated and tested against the mycotoxigenic fungi. This could also be recommended for future works.

2.7.3 Emerging application in biological control

Recently, some studies have proposed the usage of other chemical compound derived from biocontrol agents that have already been utilised against the aflatoxin production by *Aspergillus* section *Flavi* (Josselin *et al.*, 2022; Moore *et al.*, 2019). Specifically, scientists found that extrolites produced by the non-aflatoxigenic *A. flavus*, primarily as one of the mechanisms deployed against the aflatoxin production, can also be utilised beyond the application of this beneficial strains in the agricultural system. Simply put, effective extrolites against aflatoxins can be synthesised and extracted from the non-aflatoxigenic *A. flavus*, and further used as natural-based chemical pesticides. These extrolites have currently include the volatile organic carbons and other yet unknown secondary metabolites (Josselin *et al.*, 2022). This discovery provides opportunity for research beyond the application of living organisms, to include usage of natural chemical products against mycotoxigenic fungi and mycotoxin in agricultural crops of Malaysia. However, this was not tested in the present study, and is worth exploring in the near future.

In summary, it is apparent that biocontrol studies in Malaysia was highly focused on the application against the phytopathogenic organisms, but not against mycotoxigenic fungi, which encapsulates the novelty of the present study. This could either be due to the fact that (a) this particular application is totally new in Malaysia, and is not yet recognised as an important research area, (b) ongoing trials are not yet conclusive and published, or (c) the safety consideration and risk analysis on whether or not to introduce biological control in Malaysian agro-ecosystem is taking longer than anticipated. This is rather perplexing because (a) Malaysian tropical climate is conducive for fungal proliferation including that of the mycotoxigenic fungi, (b) reports on mycotoxin contamination of Malaysian foods and crop commodities are abound, (c) Malaysia does have legislations for mycotoxin contamination, (d) in other parts of the world, the application of biological control agents to combat mycotoxigenic fungi is more widely practiced and accepted, and (e) the application of biological control agents against plant diseases has long and successful history in Malaysia.

CHAPTER 3

MYCODIVERSITY OF INDIGENOUS MYCOTOXIGENIC FUNGI AND POTENTIAL BIOLOGICAL CONTROL AGENTS FROM GRAIN MAIZE AGRO-ECOSYSTEM

3.1 Introduction

Fungi are ubiquitous in nature. In agricultural environment, fungi interact widely with other microorganisms and their plant hosts. This interaction outcome can be positive, negative, or neutral depending on their life strategy. Between fungi and other plant microbiome, their interaction may result into some being mutual, neutral, parasitic, competitive, or commensal (Boddy, 2016). Towards plant hosts, fungi may exist as commensal, symbiont, or pathogen. These complex interactions of fungi between selves, other microbiome, and the plant hosts, which are also governed by the abiotic environmental factors, lead to diverse and dynamic fungal community structure in an ecosystem (Bahram & Netherway, 2022; Zeilinger *et al.*, 2016).

Like other cereals, Malaysian grain maize is naturally susceptible to pathogenic fungal infestation pre-harvest. The tropical climate of Malaysia, which is hot and humid all year round, further provides favourable conditions for this fungal proliferation, following which the grain maize yields and quality would be severely affected. Fungi from the genera *Aspergillus*, *Fusarium*, and *Penicillium* have often been associated with maize pre- and post-harvest. Besides being a pathogen, these fungal species can also produce some of the most relevant mycotoxins in maize, such as aflatoxins, fumonisins,

ochratoxins, trichothecenes, and zearalenone (García-Díaz *et al.*, 2020; Munkvold *et al.*, 2019).

Most previous research on fungal species and the potential mycotoxin accumulation of the mycotoxigenic strains in Malaysian maize pre-harvest (sweet maize) rarely discussed the colonisation of fungal species other than *Fusarium* spp. (Darnetty & Salleh, 2013; Hsuan *et al.*, 2011; Nur Ain Izzati *et al.*, 2011; Zainudin *et al.*, 2011). Although that is understandable since members of *Fusarium* genus are notorious contaminants of maize, the tropical climate of Malaysia is also a suitable breeding ground for many other fungal species that may thrive in this weather condition. Further, this tropical environment may very well stimulate greater proliferation and mycotoxin production of other mycotoxigenic species such as *Aspergillus* section *Flavi* which are important mycotoxigenic fungi that produce deleterious aflatoxins (Cotty & Jaime-Garcia, 2007). Therefore, it is important to establish more thorough information on the fungal community structure that exists within the Malaysian maize agro-ecosystem, specifically in the pioneer grain maize farms since Malaysia is embarking towards this direction, as earlier mentioned. Such information may assist in better prediction of possible fungal contamination and the subsequent mycotoxin production within the said agro-ecosystem. This, in turn, may facilitate the development and establishment of proper management and control strategies, including biocontrol, in order to minimise mycotoxigenic fungal-related diseases and their mycotoxin formation in the farms (Nesci *et al.*, 2006). The isolation of certain indigenous fungal species that could become potential antagonists to control the indigenous

mycotoxigenic fungi and their mycotoxin will help to achieve this control objective (Nesci *et al.*, 2006).

Therefore, the present Chapter was aimed: (1) to investigate the fungal community structure present in two grain maize agro-ecosystem from the pioneer grain maize farms in Terengganu, Malaysia with special emphasis on the mycotoxigenic species; and (2) to evaluate the mycotoxigenic potential of the mycotoxigenic fungal isolates from both grain maize farms. To the best of our knowledge, this was the first work that could provide information on fungal community structure and the occurrence of mycotoxigenic species, as well as their mycotoxigenic potentials in the agro-ecosystems of pioneer grain maize plantations in Malaysia.

3.2 Materials and methods

3.2.1 Sampling

Sampling was conducted during the cropping season of March to August 2017 at two grain maize farms in Terengganu. Both grain maize farms, namely Kampong Dadong (latitude: 4° 16' 47.2512" N, longitude: 103° 13' 44.238" E) within the town of Chukai in Kemaman District, and Rhu Tapai (latitude: 5° 30' 48.15" N, longitude: 102° 58' 48.018" E) in Kuala Terengganu District, are lowlands located at the southern and north-east regions of Terengganu, respectively. Both farms were gazetted by the Malaysian government as pioneer farms intended for mass-cultivation and commercialisation of grain

maize. Both farms used grain maize hybrid GWG 888. The soil at Kampong Dadong is riverine alluvium soil (USDA taxonomy: ultisol, texture class: fine sandy clay), while that of Rhu Tapai is beach ridges interspersed with swales soil (BRIS; USDA taxonomy: spodosol, texture class: sandy). Both farms practiced a conventional farming system. Soil tillage was conducted twice before sowing. Fertilisers and chemical pesticides (herbicides, fungicides, and insecticides) were applied before sowing and during plantation. In Kampong Dadong, maize plantation was rotated with rice paddy. After harvesting of paddy and before maize sowing, a microbial decomposer was applied on the field to decompose the rice straw (Warris *et al.*, 2017).

The data on monthly temperature (°C), rainfall (mm), and relative humidity (%) of both grain maize farms during the March to August 2017 cropping season were provided by the Malaysian Meteorological Department, Ministry of Energy, Science, Technology, Environment, and Climate Change (Table 3.1). Since there was no on-site meteorological station on both grain maize farms, and to ensure data accuracy, the weather observations were collected from the closest meteorological station to both farms. For Kampong Dadong grain maize farm, the weather data were collected at the Hospital Kemaman meteorological station (located approximately 7 km radius from Chukai, Terengganu), while for the Rhu Tapai farm, the weather data were collected at the Kuala Terengganu meteorological station (located approximately 20 km radius from Rhu Tapai, Terengganu). The monthly temperatures of both grain maize farms during the cropping season were uniform with very little variation.

Table 3.1: Monthly mean temperature (°C), rainfall (mm), and relative humidity (%) during 2017 cropping season of grain maize from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia. Data were supplied by the Malaysian Meteorological Department, Ministry of Energy, Science, Technology, Environment, and Climate Change, and obtained from the closest meteorological station to the grain maize farms

Month	Kampong Dadong			Rhu Tapai		
	Temp. (°C)	Rainfall (mm)	RH (%)	Temp. (°C)	Rainfall (mm)	RH (%)
March	27.3	173.6	79.6	27.7	147.8	79.1
April	28.2	33.2	79.4	28.4	32.2	81.1
May	28.4	204.4	79.8	28.6	152.6	81.0
June	27.9	180.2	81.0	28.0	77.4	82.1
July	27.9	203.4	81.2	28.0	132.0	82.7
August	27.7	164.2	81.4	27.5	206.2	83.4
T _{mean} (°C)		27.9			28.0	
T _{range}		1.1			1.1	
CR (mm)		959.0			748.2	
RH _{mean} (%)		80.4			81.6	
RH _{range}		2			4.3	

T_{mean}: mean temperature, T_{range}: temperature range, CR: cumulative rainfall, RH: mean relative humidity, RH_{range}: range of relative humidity. The highest monthly temperature and relative humidity was subtracted with the lowest counterparts to obtain the range of temperature and relative humidity, respectively.

High relative humidity and rainfall were also recorded at both grain maize farms. This climatic trend corresponds to the Af climate (*i.e.*, tropical, fully humid) as categorised by the Köppen–Geiger climate classification (Kottek *et al.*, 2006).

Sampling on Kampong Dadong and Rhu Tapai grain maize farms was conducted one-time at the late maturation stage of the grain maize nearing the harvesting period during August 2017. The late maturation stage refers to the

last window within the R6 developmental stage of grain maize in which the grain reached physiological maturity (Zhao *et al.*, 2012). Four sample types were collected from each farm, namely cob, tassel, debris, and soil (Appendix A). On each farm, a centre plot that was far from the main road was selected, and three sub-samples for each sample type were aseptically collected from a diagonal section across the plot (*i.e.*, one point at the plot edge, one point at the centre, and one point at the other plot edge). Where the sub-sampling was considered, the sampling was conducted within the area of three adjacent grain maize plants. Overall, a total of 24 individual samples were collected (2 farms $\times$ 4 sample types $\times$ 3 sub-samples = 24 samples). Each cob, tassel, debris, and soil consist a total of 6 individual samples from both farms. All individual samples were analysed separately. The sub-sampling design was selected and performed in the present Chapter because of the spatial (and temporal) heterogeneity distribution (also known as patchy distribution) of the microbial communities including fungi in agricultural field, even within the same plot. Besides, analysing each individual samples separately will increase the chances of isolating more distinct and/or 'rare' or less abundant fungal species, that otherwise hard to be detected when analysing a composite sample composed of the pooled sub-samples (Castle *et al.*, 2019). Detection of more distinct fungal species permits isolation of novel fungi that might exhibit good antagonistic activity against the indigenous mycotoxigenic fungi and their subsequent mycotoxins.

All samples selected in the present Chapter represent the niche where both mycotoxigenic fungi and fungal antagonist often resided, both below- and

above-ground, and the plant tissues. The below-ground soil sample and the above-ground plant debris, hosts various saprotrophs that utilise nutrients in the soil and dead organic matter in the debris (Ismaiel *et al.*, 2024; Nji *et al.*, 2023; Vorob'eva & Toropova, 2020). Some fungi could further exploit and utilise plant resources including maize cobs and kernels. Fungi could also propagate by the production and release of airborne spores which may land onto the upper part of the maize plant including tassel. Therefore, sampling and analysis of the soil, debris, kernel, and tassel in the present Chapter have consider the possibility of the fungal occupation in this said samples. Besides, isolation of good fungal antagonists requires that the candidates be able to colonise either or both soil and the internal plant tissues for effective biocontrol application in the field (Ismaiel *et al.*, 2024). The soil samples were collected from the upper layer (≈ 13 cm depth) of the plot using trowel. Soil was collected upon which microbial activity primarily occurs (Carranza *et al.*, 2016). Maize cobs, tassels, and debris (i.e., dead maize plant materials such as leaves and stalks) were also aseptically collected. All samples were placed in sterile polyethylene sampling bags and kept in the ice box to maintain sample integrity before immediately transported to the laboratory, and refrigerated at 4°C. The grain maize kernels were threshed from the cobs, placed in another sterile polyethylene bag, and also refrigerated at 4°C. Samples were analysed within one to two days after sampling.

3.2.2 Isolation and enumeration of indigenous fungi

Quantitative enumeration of fungal load by dilution plating of each debris, kernel, soil, and tassel samples was performed on Dichloran Rose Bengal Chloramphenicol (DRBC; Oxoid, Basingstoke, UK) agar. Prior to homogenisation, grain maize kernels were left to soak with sterile 0.1% peptone water (w/v, 1 g of peptone in 1,000 mL of distilled water) for 60 minutes. Soaking was done to soften the kernels before stomaching, therefore allowing access to both outside and inside of the kernels and subsequently their fungal load (Pitt & Hocking, 2009). For homogenisation, 10 g of each sample were mixed with 90 mL of sterile 0.1% peptone (Oxoid, Basingstoke, UK) water, and homogenised for two minutes using a stomacher (Seward Ltd., Worthing, UK). Then, serial dilutions of 10^{-2} to 10^{-4} were performed, and 0.1 mL aliquot from each dilution were spread-plated on DRBC in triplicate (International Commission on Microbiological Specifications for Foods, 2017, 2008). Inoculated plates were incubated in the dark at 30°C for seven days. Following incubation, plates with 10–100 colony-forming units (CFU) were used to calculate the fungal populations using the Equation 3.1, and results were expressed as CFU per gram of sample (CFU/g). Next, fungal colonies of different morphologies were sub-cultured onto fresh Potato Dextrose Agar (PDA; Oxoid, Basingstoke, UK), and incubated at 30°C for seven days to obtain axenic culture (*i.e.*, pure) for further identification.

$$CFU/g = \frac{\text{average number of colonies} \times \text{dilution factor}}{\text{volume of aliquot}}$$

Equation 3.1: Formula for colony forming unit per gram (CFU/g)

Direct plating was employed to enumerate and isolate fungi from the particulate plant sample in the present work, namely the grain maize kernels and tassels, since these particulate samples contain fungi on both the surface (*i.e.*, epiphytes) and also within the intracellular and intercellular tissues (*i.e.*, endophytes) (Pitt & Hocking, 2009). In particular, to assess fungi resided within the plants' tissue, surface sterilisation was performed on both samples. Surface sterilisation would discard the epiphytes, and allowing easier isolation of the endophytes (Alam *et al.*, 2021). Direct plating of both samples was performed on Dichloran-Glycerol 18 (DG-18; Oxoid, Basingstoke, UK) agar and PDA supplemented with 1 mg/L (w/v) chloramphenicol (Merck, Darmstadt, Germany). DG-18 was used to isolate moderately xerophilic fungi. Prior to inoculation, the tassel was cut to 0.5–1.0 cm length. Next, both samples were surface sterilised by immersing in 5% (v/v) sodium hypochlorite (RandM Chemicals, London, UK) separately for five minutes, followed by rinsing twice with sterile distilled water. Thereafter, five of each kernel and tassel were aseptically inoculated at equal distance on both growth media. All plates were incubated in the dark at 30°C for seven days. Following incubation, the isolation frequency (%) of a species recovered from the kernel and tassel samples was calculated based on Equation 3.2 (Pitt & Hocking, 2009).

Isolation frequency (%)

$$= \frac{\text{number of infected kernels or tassels}}{\text{total number of kernels or tassels per plate}} \times 100$$

Equation 3.2: Formula for isolation frequency (%)

Next, fungal colonies of different morphologies were sub-cultured onto fresh PDA, and incubated at 30°C for seven days to obtain axenic culture for further identification.

3.2.3 Morphological and molecular identification of fungal isolates

Fungal isolates were distinguished macro-morphologically by their colony colour (reverse and obverse), texture and size, and pigmentation. The micro-morphological identification was performed using an optical microscope (Olympus, Tokyo, Japan) to distinguish fungal colonies based on their mycelial characteristic and/or spore formation and morphology. *Aspergillus* spp. were morphologically identified based on keys provided by Pitt & Hocking (2009). *Penicillium* spp. were morphologically identified based on keys provided by Pitt & Hocking (2009), Samson *et al.* (2010), and Visagie *et al.* (2014). *Fusarium* spp. were morphologically identified based on keys provided by Leslie & Summerell (2008). The remaining fungal isolates were morphologically identified based on keys provided by Ediriweera *et al.* (2021), Jung *et al.* (2014), Hanafusa *et al.* (2016), Pitt & Hocking (2009), Samuels *et al.*, (2010), and Watanabe (2010). Appendix B – H depict the morphological keys for all

mycotoxigenic isolates and the potential biocontrol agents utilised and determined in this thesis.

Thereafter, all fungal isolates were grouped based on similar morphology. A representative isolate (*i.e.*, one isolate) of each distinct morphology were molecularly identified/validated using polymerase chain reaction (PCR). Briefly, fungal mycelia/spores from axenic solid cultures were suspended in 10 mL of Potato Dextrose Broth (PDB; Oxoid, Basingstoke, UK), and incubated with shaking at 100 rpm and 30°C for one to five days, depending on the fungal growth characteristic of either fast- or slow-grower. For example, *Aspergillus* spp. are fast-growing fungi, thus were incubated for only one day, while *Fusarium* spp. are slow-growing fungi, thus were incubated for longer duration. Then, fungal cultures were harvested by filtration using sterile filter paper No. 1 (Whatman, Kent, UK), followed by washing with sterile distilled water before left to dry under a laminar flow. Subsequently, the dried mycelia were grounded to a fine powder in liquid nitrogen with a mortar and pestle before transferred into a 2-mL Eppendorf tube. The genomic DNA extraction was performed using the DNeasy plant mini kit (Qiagen, Maryland, USA) following the manufacturer's instructions, and the purified DNA was stored at -20°C until further analyses.

PCR reaction for internal transcribed spacer (ITS) region was performed using a primer pair ITS1: 5' TCC GTA GGT GAA CCT GCG G 3' and ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3' (product size: 600 bp; White *et al.*, 1990). The PCR reaction mixture was prepared in a total volume of 25 µL, which contained

12.5 μ L of 1 $\times$ HotStarTaq Plus Master Mix (Qiagen, Maryland, USA), 2.5 μ L of 1 $\times$ CoralLoad concentrate, which contained orange and red gel-tracking dye (Qiagen, Maryland, USA), 1.25 μ L (0.5 μ M) of each primer, 2.0 μ L of DNA template, and 6.75 μ L of RNase-free water (Qiagen, Maryland, USA). The HotStarTaq Plus Master Mix consisted of HotStarTaq Plus DNA polymerase, PCR buffer with 3 mM magnesium chloride ($MgCl_2$), and 400 μ M of each dNTP. The amplification program was carried out using SuperCycler SC-200 (Kyrattec, Mansfield, Australia) under the following condition: pre-denaturation at 95°C for two minutes; 30 cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for one minute 20 seconds; followed by a final extension at 72°C for 10 minutes (Norlia *et al.*, 2019). Next, the amplicons were separated by gel electrophoresis using 1.5% agarose gel (Promega, Wisconsin, USA) stained with 3 μ L of ethidium bromide (Promega, Wisconsin, USA), and ran in 1 $\times$ Tris-acetate-ethylene diamine tetra acetic acid (TAE). The electrophoresis was conducted for 80 minutes at 90 V and 400 mA. The amplified genes were visualised under ultraviolet light using gel documentation (Syngene, Bangalore, India), and their size was estimated using Gel Pilot® 100 bp Plus DNA ladder (Qiagen, Maryland, USA). The amplicons were then submitted to a local service provider (MyTACG Bioscience Enterprise, Kuala Lumpur, Malaysia) for DNA purification and sequencing. The sequences obtained were edited and aligned using BioEdit version 7.2 before compared with other sequences previously deposited in the National Centre for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) tool (<https://blast.ncbi.nlm.nih.gov>, accessed on 29 January 2021). BLAST search results showing $\geq 99\%$

similarity with the isolates' ITS sequences were considered for assigning fungal isolates to the species name. The fungal species names were then validated with databases from Index Fungorum (<https://www.indexfungorum.org>, accessed on 29 January 2021) and Mycobank (<https://www.mycobank.org>, accessed on 29 January 2021), and fungal isolates were then assigned to the names used throughout the present study. All isolates were deposited at Laboratory of Food Mycology, Department of Food Science, Faculty of Food Science and Technology, UPM, and maintained on PDA for further analyses.

To deposit the sequences onto the GenBank database, the sequences were first transformed into FASTA format. The FASTA format line (also known as FASTA definition line) includes the ">" symbol, followed by sequence ID (SeqID), sequence source information (known as Source Modifier), and feature annotation (biological features, *e.g.*, genes, structural RNA). In the present study, the FASTA definition line was ">SeqID [organism = XXX] [strain = XXX] [isolation_source = XXX] [country = XXX] 18 S small subunit ribosomal RNA, partial sequence; internal transcribed spacer 1, 5.8 S ribosomal RNA, internal transcribed spacer 2, complete sequence; 28 S large subunit ribosomal RNA, partial sequence". Sequence was added after "hard return" (ENTER button). All sequences were kept in one file, and directly submitted to GenBank via their web-based submission tool, BankIt (<https://www.ncbi.nlm.nih.gov/WebSub/>, accessed on 29 January 2021). Following successful sequence deposition, the GenBank would release accession numbers that give the scientific community a unique label for each

species/strain with which they may retrieve the species/strain data from the GenBank online servers.

3.2.4 Preparation of grain maize agar as semi-synthetic growth medium

Grain maize agar (GMA) with grain maize as natural substrate was used as a semi-synthetic growth medium to mimic the mycotoxin-producing ability of fungal isolates on actual grain maize in nature, and to avoid the overproduction of mycotoxins that is usually the case for commercial fungal growth media. This is because commercial growth media, such as PDA and Malt Extract Agar (MEA) contain high amount of monosaccharide dextrose (simple sugar) which provides an easily accessible carbon source for the utilisation of mycotoxigenic species, leading to the overproduction of mycotoxins as compared to natural substrates such as maize (Yazid *et al.*, 2018).

The GMA was prepared using commercially-obtained GWG 888 grain maize kernels and technical agar (Oxoid, Basingstoke, UK). The technical agar, also known as bacteriological agar, is a bacteriological-grade agar use as a solidifying agent to prepare microbiological media. Briefly, grain maize kernels were ground to fine powder using a Waring blender (Waring, Torrington, USA). Next, approximately 30 g of fine grain maize powder and 15 g of technical agar were added into 1 L distilled water (Yazid *et al.*, 2018). The medium was autoclaved at 121°C and 15 psi for 15 minutes, before poured into 90 mm Ø Petri plates, and left to solidify. Solidified GMA plates were refrigerated at 4°C prior to fungal inoculation.

3.2.5 Mycotoxigenic potentials of fungal isolates

The mycotoxigenic potentials of *A. flavus* (*i.e.*, AFB₁, AFB₂, AFG₁, AFG₂), *A. niger* (*i.e.*, OTA), and *Fusarium* spp. (*i.e.*, DON, FB₁, FB₂, HT-2, T-2, ZEN) were determined by reverse-phase high-performance liquid chromatography (HPLC; Waters 600, Haverhill, USA) using a Gemini® C₁₈ column (5 µm, 250 × 4.6 mm; Phenomenex, Washington, USA). For mycotoxin extraction, 10 µL spore suspension of each isolate was inoculated centrally on GMA, and incubated in the dark at 30°C for 10 days. Next, five mycelial plugs were transferred into pre-weighed Eppendorf (Hamburg, Germany) tubes before the final weight was recorded. Prior to mycotoxin quantification, the mobile phases were filtered using a nylon membrane filter (0.45 µm; Merck, Darmstadt, Germany), and sonicated using an ultrasonic bath (Power-Sonic 420, Seoul, Korea) for 30 minutes to degas. Ultrapure water for the mobile phase was obtained from Elga Purelab Classic UV MK2 (Lane End, UK). Following quantification, data were processed using Empower 2 chromatography data software (Waters, Haverhill, USA).

3.2.5.1 Extraction and quantification of aflatoxins

The extraction and quantification of aflatoxins were performed following the method described by Bragulat *et al.* (2001) with slight modification. Briefly, 1,000 µL of absolute methanol (Merck, Darmstadt, Germany) was added to the Eppendorf tube containing mycelial plugs, and the mixture was vortexed using a vortex mixer (LMS Co. Ltd., Tokyo, Japan). Then, the tube was

incubated for 30 minutes at room temperature before centrifuged (Sartorius, Göttingen, Germany) at 5,590 *g* for five minutes. The resulting extract was filtered through a nylon syringe filter (0.22 μm ; Macherey-Nagel, Düren, Germany) into HPLC vials (Thermo Scientific, Massachusetts, USA). Aflatoxin separation and detection were performed with a mobile phase of methanol:acetonitrile:water (35:10:55, v/v/v) at 40°C, and a flow rate of 0.6 mL/min. The injection volume was 20 μL . A post-column (*i.e.*, photochemical reactor for enhanced detection, PHRED; Aura Industries, New York, USA) was employed for aflatoxin derivatisation. A fluorescence detector (Waters 2475, Massachusetts, USA) with the excitation and emission wavelengths of 365 nm and 435 nm, respectively was employed (Afsah-Hejri *et al*, 2011).

3.2.5.2 Extraction and quantification of ochratoxin A

The extraction and quantification of OTA were performed following the method described by Mohale *et al.* (2013). Briefly, 1,000 μL of absolute methanol was added to the Eppendorf tube containing mycelial plugs, and the mixture was shaken using orbital shaker (Scilogex, Connecticut, USA) for 60 min. The resulting supernatant was filtered using a 0.22 μm nylon syringe filter into HPLC vials. OTA separation was performed with a mobile phase of acetonitrile:water:glacial acetic acid (57:41:2, v/v/v), and at a flow rate of 1.0 mL/min. The injection volume was 20 μL . A fluorescence detector was employed for OTA detection with the excitation and emission wavelengths of 360 nm and 440 nm, respectively. Acetonitrile and glacial acetic acid used in

the present study were purchased from Merck (Darmstadt, Germany) and RandM Chemicals (London, UK), respectively.

3.2.5.3 Extraction, derivatisation, and quantification of fumonisins

The extraction, derivatisation, and quantification of fumonisins were performed following the method described by Visconti *et al.* (2001). Briefly, 1,000 μL of methanol:acetonitrile:water (25:25:50, v/v) was added to the Eppendorf tube containing mycelial plugs, vigorously vortexed before shaken using orbital shaker for 60 minutes, and filtered with nylon syringe filter (0.22 μm) into HPLC vials. Then, the filtrate was evaporated to dryness in a stream of nitrogen before re-dissolved in the mobile phase for HPLC analysis. Prior to analysis, the fumonisin standards and samples (50 μL) were derivatised with 100 μL ortho-phthalaldehyde (OPA; Sigma-Aldrich, Missouri, USA) for one minute. The derivatisation reagent was prepared fresh by dissolving 40 mg OPA in 1 mL absolute methanol before adding 5 mL of 0.1 M disodium tetraborate (Chemiz, Selangor, Malaysia) and 50 μL of 2-mercaptoethanol (Sigma-Aldrich, Missouri, USA) in a capped amber vial. Fumonisin separation was performed at a flow rate of 1.0 mL/min in a mobile phase of methanol: 0.1 M sodium dihydrogen phosphate (77:23, v/v) adjusted to pH 3.35 using phosphoric acid. Sodium dihydrogen phosphate was obtained from Chemiz (Selangor, Malaysia). The injection volume was 30 μL . Fumonisins were detected using a fluorescence detector with the excitation and emission wavelengths of 335 nm and 440 nm, respectively.

3.2.5.4 Extraction and quantification of trichothecenes

The extraction and quantification of DON were performed following the method described by Moazami & Jinap (2009) with slight modification. Briefly, 1,000 μL of acetonitrile: water (17:83, v/v) was added to the Eppendorf tube containing mycelial plugs, and vortexed for 30 seconds. Next, the tube was agitated using orbital shaker at 180 rpm for 60 minutes before centrifuged at 4,200 g for 15 minutes. The resulting supernatant was filtered through a 0.22 μm nylon syringe filter into HPLC vials. Then, the filtrate was evaporated to dryness in a stream of nitrogen before re-dissolved in 500 μL of absolute methanol. Trichothecene separation was performed with a mobile phase of acetonitrile:water (17:83, v/v), and at a flow rate of 1.0 mL/min. The injection volume was 20 μL . A photodiode array detector (Waters 2996, Massachusetts, USA) with a wavelength set at 218 nm was used to detect DON.

The extraction and quantification of HT-2 and T-2 toxins were performed following the method described by Medina & Magan (2011). Briefly, 1,000 μL of methanol:water (80:20 v/v) was added to the Eppendorf tube containing mycelial plugs. Next, the tube was agitated using orbital shaker in the dark for 90 minutes at 150 rpm before centrifuged at 8,000 g for 15 minutes. Following filtration using a nylon syringe filter (0.22 μm), the extract was transferred into HPLC vials, and evaporated to dryness in a stream of nitrogen. The dried extracts were re-dissolved in 300 μL of acetonitrile:water (50:50 v/v). Trichothecene separation was performed in gradient mode with the mobile phase of A (water) and B (acetonitrile). The gradient concentrations were

initiated by holding for the first three minutes with 30% B, after which the condition was changed linearly to 55% B for 18 minutes. The condition was then changed to 99% B for one minute, and maintained for five minutes as a cleaning step to improve the results. After cleaning, the condition was returned to the initial 30% B. The flow rate was 1 mL/min. The injection volume was 50 μ L. A photodiode array detector with a wavelength of 200 nm was employed to detect HT-2 and T-2 toxins.

3.2.5.5 Extraction and quantification of zearalenone

The extraction and quantification of ZEN were performed following the method described by Iqbal *et al.* (2014) with slight modification. Briefly, 1,000 μ L of acetonitrile:water (90:10, v/v) was added to the Eppendorf tube containing mycelial plugs, and vortexed for 30 seconds. Next, the tube was agitated using orbital shaker at 180 rpm for 60 minutes before centrifuged at 4,200 *g* for 15 minutes. Next, the supernatant was filtered through a 0.22 μ m nylon syringe filter into HPLC vials. The filtrate was evaporated to dryness in a stream of nitrogen before re-dissolved in 250 μ L of HPLC mobile phase. Zearalenone separation was performed with a mobile phase of acetonitrile:water:methanol (48:50:2, v/v/v), and at a flow rate of 1.0 mL/min. The injection volume was 20 μ L. ZEN was detected using a fluorescence detector with the excitation and emission wavelengths of 274 nm and 450 nm, respectively.

3.2.5.6 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Following mycotoxin detection, linear calibration curves were constructed by plotting the peak area (response) obtained from HPLC analysis against the concentrations of each standard calibrant solution, after which the actual mycotoxin production of fungal isolates was calculated. The standard calibrant solutions were prepared and diluted in different solvents as follows (*i.e.*, AFB₁, AFB₂, AFG₁, AFG₂ and OTA in methanol; HT-2 and T-2 in acetonitrile; FB₁, FB₂, DON and ZEN in acetonitrile:water). The mycotoxin standards of AFB₁, AFB₂, AFG₁, AFG₂, DON, FB₁, FB₂, OTA, and ZEN used in the present study were obtained from Sigma-Aldrich (Missouri, USA), while those of HT-2 and T-2 were obtained from Trilogy (Washington, USA) (Appendix J). Subsequently, linear regression was fitted to establish the correlation coefficient (R^2) (Appendix K – Q). The limit of detection (LOD) and limit of quantification (LOQ) of each chromatographic analysis were estimated following the method prescribed by the International Conference on Harmonization (ICH) by using the formula; $LOD = 3\sigma/s$ and $LOQ = 10\sigma/s$, in which σ was the standard deviation of blank responses, and s was the slope of the calibration curve (Shrivastava & Gupta, 2011). The linearity, LOD, and LOQ of all analyses are shown in Table 3.2. Overall, the calibration curves yielded a strong linear response ($R^2 > 0.99$) for all the mycotoxins analysed.

Table 3.2: Linearity, limit of detection (LOD), and limit of quantification (LOQ) for the mycotoxin analyses

Analyte	Calibrant solution	Linearity		^a LOD	^a LOQ
		Linear equation	<i>R</i> ²		
AFB ₁	0.5 – 100	$y = 936537x$	0.9986	0.007	0.02
AFB ₂	0.15 – 30	$y = 2007506.65x$	0.9987	0.003	0.01
AFG ₁	0.5 – 100	$y = 326195x$	0.9996	0.05	0.2
AFG ₂	0.15 – 30	$y = 657140x$	0.9994	0.02	0.07
OTA	0.5 – 100	$y = 19434.67x$	0.9997	0.2	0.8
FB ₁	0.05 – 25	$y = 15206645.27x$	0.9989	0.0003	0.0009
FB ₂	0.05 – 25	$y = 11905744.21x$	0.9968	0.0004	0.001
DON	0.1 – 5	$y = 25207.29x$	0.9955	0.009	0.03
HT-2	0.5 – 50	$y = 58650.53x$	0.9978	0.002	0.008
T-2	0.5 – 50	$y = 49356.43x$	0.9978	0.002	0.005
ZEN	0.1 – 10	$y = 628263x$	0.9976	0.002	0.007

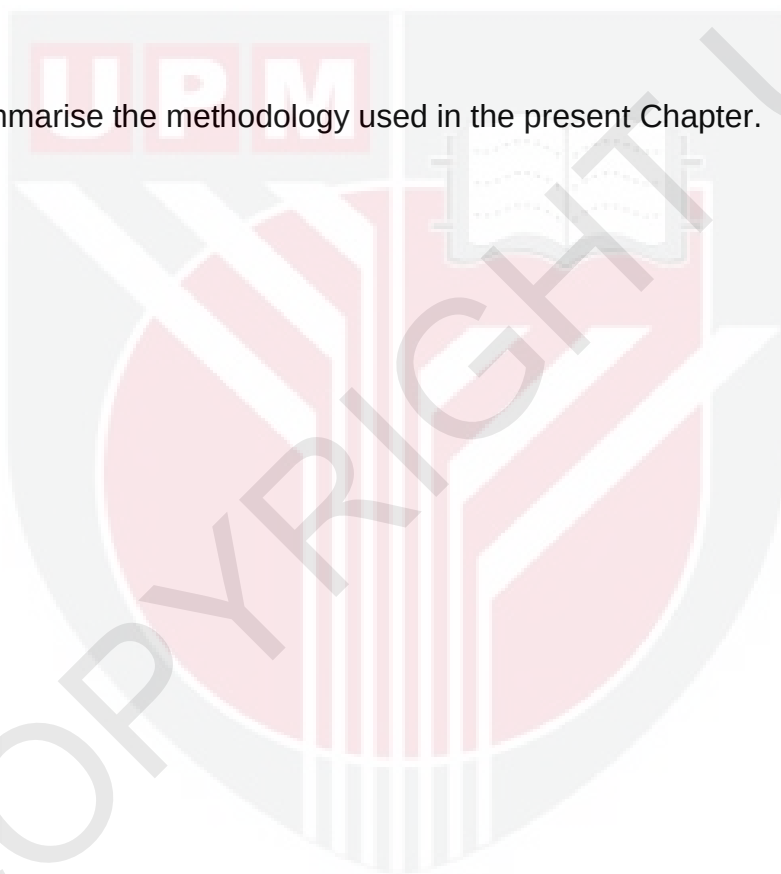
AFB₁: aflatoxin B₁; AFB₂: aflatoxin B₂; AFG₁: aflatoxin G₁; AFG₂: aflatoxin G₂; OTA: ochratoxin A; FB₁: fumonisin B₁; FB₂: fumonisin B₂; DON: deoxynivalenol; HT-2: HT-2 toxin; T-2: T-2 toxin; ZEN: zearalenone. ^aLOD and LOQ of AFB₁, AFB₂, AFG₁, AFG₂, and OTA were in ng/mL, while the LOD and LOQ of FB₁, FB₂, DON, HT-2, T-2, and ZEN were in µg/mL.

3.2.6 Experimental design and statistical analysis

Sampling of the two pioneer grain maize farms was non-replicated since it was conducted only once in August 2017. All 24 individual samples were analysed separately but data for total fungal load (log CFU/g) and the isolation frequency (%) of the three individual sample types from each farm were pooled. All experiments on fungal enumeration and mycotoxigenic potential were conducted in three replicates ($n = 3$). Measurements were then averaged and presented as mean $\pm$ SE (standard error). The CFU of fungal isolates were tested for normality using Kolmogorov–Smirnov test before log-transformed.

Next, statistical analysis for the total fungal load was carried out using one-way analysis of variance (ANOVA) with a 95% confidence interval, and $p < 0.05$ was accepted as a significant difference. Sample types served as independent variables. A *post hoc* Tukey's Honest Significant Difference (Tukey's HSD) were carried out with p -value of < 0.05 to compare the significant difference between means of treatments. The statistical software SPSS version 29.0 (SPSS Inc., Illinois, USA) was used to perform the analysis.

Figure 3.1 summarise the methodology used in the present Chapter.



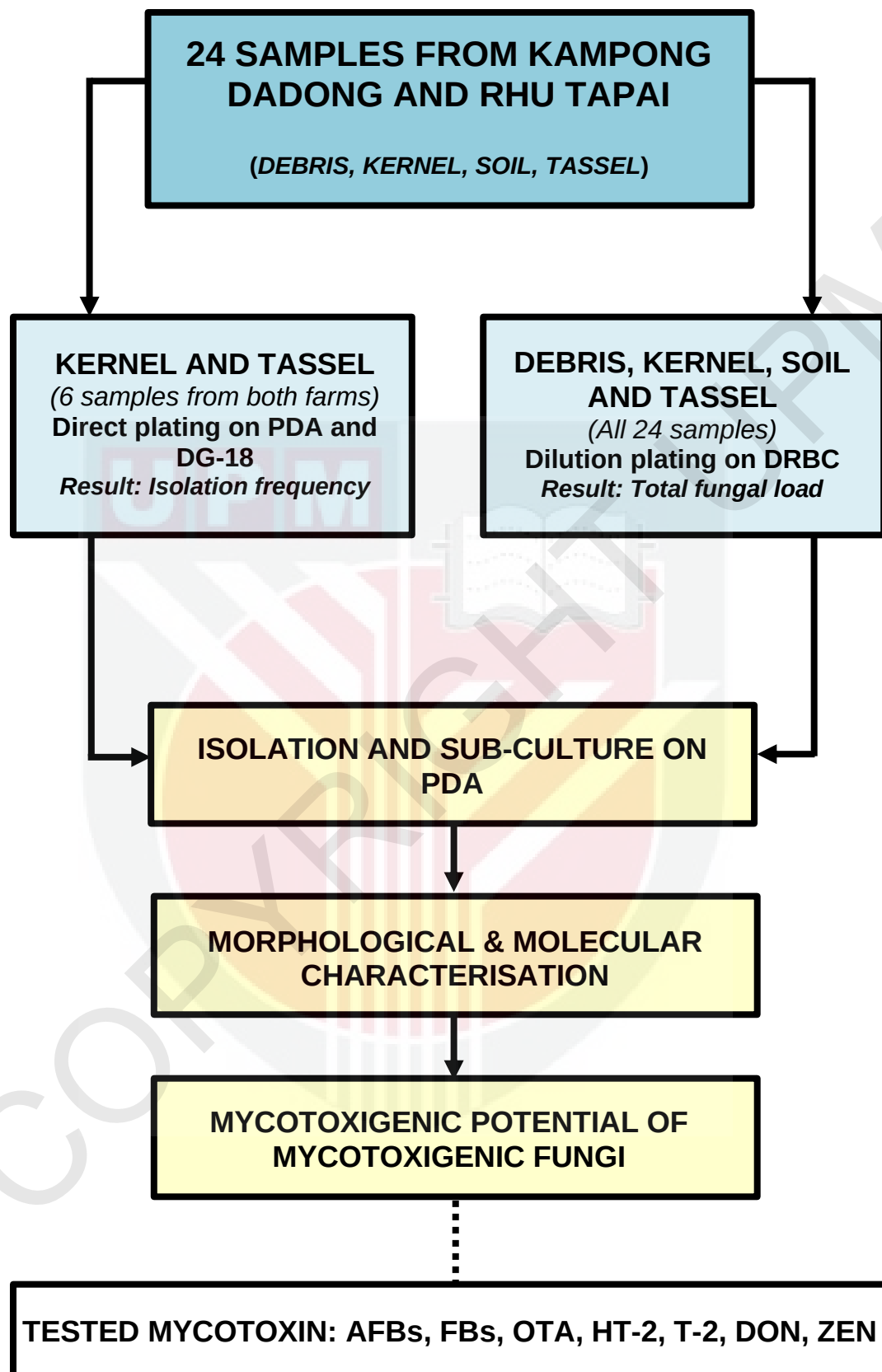


Figure 3.1: Flow diagram of the methodology used in Chapter 3

3.3 Results

3.3.1 Density and diversity of mycobiota isolated from Kampong Dadong and Rhu Tapai grain maize farms

Figure 3.2 depicts the total fungal loads (log CFU/g) on debris, kernel, soil, and tassel samples obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu following dilution plating on DRBC and incubation at 30°C for seven days. However, there was a significant difference between the total fungal loads of each sample analysed. For both farms, the tassel samples significantly yielded the highest total fungal loads ($\log 6.71 \pm 0$ CFU/g for Kampong Dadong and $\log 6.72 \pm 0$ CFU/g for Rhu Tapai), followed by debris, kernel, and soil samples.

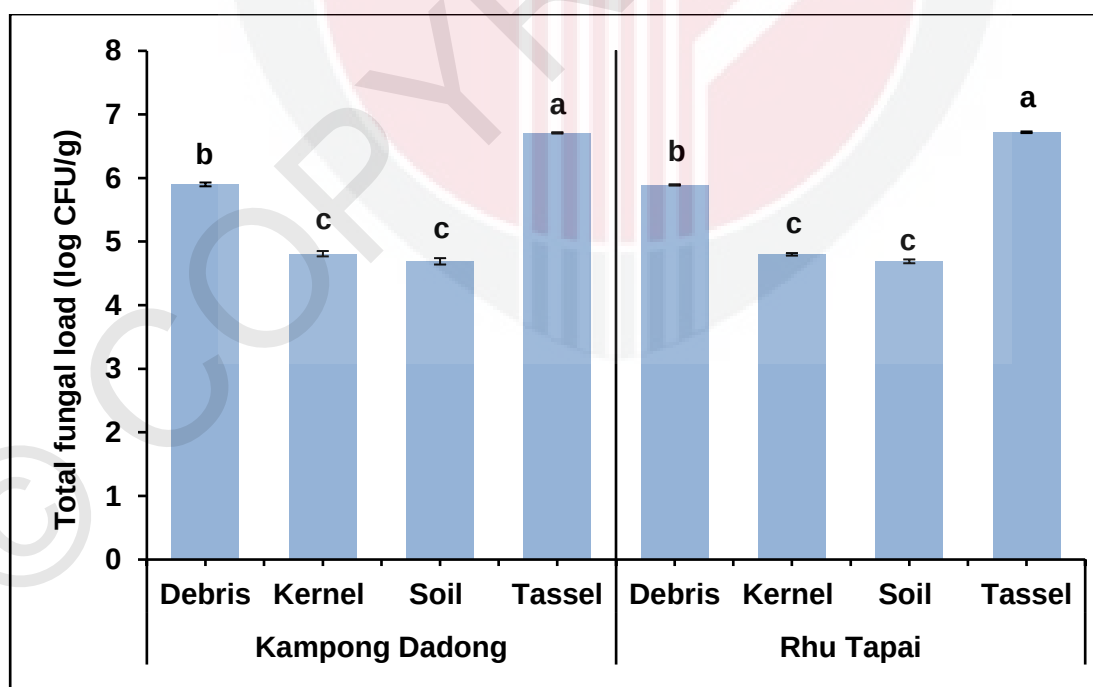


Figure 3.2: Total fungal loads (log CFU/g) of debris, kernel, soil, and tassel samples obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia following dilution plating on

Dichloran Rose Bengal Chloramphenicol agar (DRBC) and incubation at 30°C for seven days. Data are means of triplicate ($n = 3$) with bars indicating standard error ($\pm SE$). Different letters indicate significant difference ($p < 0.05$) by Tukey's Honest Significant Difference (Tukey's HSD)

A total of 131 fungal isolates were recovered from all samples of both farms. Based on sequence identification, approximately 13 fungal genera: *Aspergillus*, *Bjerkandera*, *Curvularia*, *Fusarium*, *Lasiodiplodia*, *Neosartorya*, *Penicillium*, *Phomopsis*, *Pyrrhoderma*, *Schizophyllum*, *Talaromyces*, *Trametes*, *Trichoderma*, and 30 fungal species were identified (Table 3.3). Table 3.4 summarises the qualitative distribution of fungal isolates on debris, kernel, soil, and tassel samples observed from Kampong Dadong and Rhu Tapai grain maize farms. Further, Table 3.5 lists the number of fungal isolates obtained from Kampong Dadong and Rhu Tapai grain maize farms. On both farms, Ascomycota was the dominant phylum, followed by Basidiomycota. Generally, the fungal species isolated from Kampong Dadong grain maize farm was slightly higher, that was 22 species with 64 total fungal isolates, as compared to those isolated from Rhu Tapai grain maize farm, that was 20 species with 67 total fungal isolates. The predominant species found from Kampong Dadong samples were *F. verticillioides* ($n = 10/12$ of samples; 83%) followed by the pathogenic fungi *L. theobromae* ($n = 9/12$ of samples; 75%), and *A. niger* ($n = 6/12$ of samples; 50%). Similarly, *F. verticillioides* ($n = 10/12$ of samples; 83%) was the predominant species found from Rhu Tapai samples, followed by *L. theobromae* ($n = 9/12$ of samples; 75%), and *A. flavus* ($n = 6/12$ of samples; 50%). Regarding mycotoxigenic fungi, five species were recovered from Kampong Dadong samples with their occurrence in the

Table 3.3: Fungal species, sequence similarity (%), GenBank accession number, and isolate code of fungal isolates obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia

No.	Fungi	Sequence similarity (%)	GenBank accession no.	Isolate code
1.	<i>Aspergillus aculeatus</i>	99.82	MW542998	Aa3TD*
2.	<i>Aspergillus flavus</i>	100	MW542999	Af2SR , Af1KD, Af3SD, Af5TD, Af6KR, Af7KR, Af4TR
3.	<i>Aspergillus niger</i>	100	MW543000	An1KD , An3KD, An4TD, An2KR, An5TR
4.	<i>Bjerkandera adusta</i>	100	MW543001	Ba42DD
5.	<i>Curvularia</i> sp.	99	MW543002	C13SR
6.	<i>Fusarium incarnatum</i>	100	MW543003	Fi110KD , Fi53TD, Fi56TR
7.	<i>Fusarium longipes</i>	99.44	MW543004	FI57TR
8.	<i>Fusarium proliferatum</i>	100	MW543005	Fp9DD
9.	<i>Fusarium verticillioides</i>	100	MW543006	Fv9TD , Fv79DD, Fv9DD, Fv70DD, Fv21KD, Fv25KD, Fv32KD, Fv90SD, Fv93SD, Fv106SD, Fv16TD, Fv3DR, Fv4DR, Fv21DR, Fv69DR, Fv25KR, Fv26KR, Fv58SR, Fv89SR, Fv90SR, Fv92SR, Fv99SR, Fv111SR, Fv22TR
10.	<i>Lasiodiplodia theobromae</i>	100	MW543007	Lt107DR , Lt73DD, Lt12KD, Lt13SD, Lt15SD, Lt21SD, Lt14TD, Lt16TD, Lt12KR, Lt55KR, Lt100SR, Lt101SR, Lt102SR, Lt5TR, Lt15TR, Lt55TR
11.	<i>Neosartorya fischeri</i>	100	MW543008	Nf94SR , Nf43DD, Nf72DD, Nf9SD, Nf13SD, Nf14SD, Nf16SD, Nf17SD, Nf20SD, Nf49SD, Nf105SD, Nf47DR, Nf82SR, Nf84SR, Nf88SR, Nf95SR, Nf98SR
12.	<i>Penicillium</i> sp.	97.01	MW543014	P83SR , P102DR, P59SR, P93SR

Table 3.3: Continued

13.	<i>Penicillium</i> sp.	100	MW543009	Pd8TR , Pd8KR
14.	<i>Penicillium charlesii</i>	100	MW543010	Pc30KD
15.	<i>Penicillium citreonigrum</i>	100	MW543011	Pc74SD , Pc104SD, Pc108SD
16.	<i>Penicillium daleae</i>	100	MW543012	Pd66SR , Pd76DD, Pd60SR
17.	<i>Penicillium janthinellum</i>	99.64	MW543013	Pj81SR , Pj27SD, Pj46SD, Pj86SD, Pj104DR, Pj37SR, Pj68SR, Pj75SR, Pj87SR, Pj90SR, Pj91SR
18.	<i>Penicillium polonicum</i>	99.6	MW543015	Pp51SD , Pp52SD, Pp93SD, Pp112SD, Pp36KR
19.	<i>Penicillium rubens</i>	100	MW543016	Pr85SR , Pr80DD, Pr32TD, Pr34TD, Pr50TD, Pr108DR, Pr67SR, Pr91SR
20.	<i>Phomopsis</i> sp.	99.46	MW543017	P47KD
21.	<i>Pyrrhoderma noxium</i>	99.24	MW543018	Pn45SR
22.	<i>Schizophyllum commune</i>	99.20	MW543019	Sc40DD , Sc29DD
23.	<i>Talaromyces islandicus</i>	99.13	MW543020	Ti38KD
24.	<i>Talaromyces radicus</i>	99.49	MW543021	Tr35KR
25.	<i>Talaromyces purpureogenus</i>	100	MW543022	Tp71DD , Tp94TD, Tp65SR
26.	<i>Trametes cubensis</i>	100	MW543023	Tc28DD
27.	<i>Trichoderma asperelloides</i>	100	MW543024	Ta39KR
28.	<i>Trichoderma asperellum</i>	100	MW543025	Ta31KR
29.	<i>Trichoderma harzianum</i>	99.67	MW543026	Th33SR , Th20SR, Th24KD, Th36SR
30.	<i>Trichoderma yunnanense</i>	99.83	MW543027	Ty34KD

Total number of isolates = 131. Isolates in bold were selected for molecular identification and BLAST sequence analysis. In the isolate code, the first two letters correspond to their genus and species initials, the number is a running sequence, the next letter corresponds to the source of isolation (D = debris; K = kernel; S= soil; T = tassel), and the final letter corresponds to the grain maize farms from which the sample was taken (D = Kampong Dadong; R = Rhu Tapai).

Table 3.4: Qualitative distribution of fungal isolates on debris, kernel, soil, and tassel samples obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia. Debris, kernel, soil, and tassel each consist of three individual samples per farm

Species	Kampong Dadong				Rhu Tapai			
	Debris	Kernel	Soil	Tassel	Debris	Kernel	Soil	Tassel
Total (n = 12)								
Total (n = 12)								
Ascomycota								
<i>Aspergillus aculeatus</i>	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3
<i>A. flavus</i>	0/3	1/3	1/3	3/3	0/3	3/3	1/3	2/3
<i>A. niger</i>	0/3	3/3	0/3	3/3	0/3	3/3	0/3	2/3
<i>Curvularia</i> sp.	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3
<i>Fusarium incarnatum</i>	0/3	2/3	0/3	1/3	0/3	0/3	0/3	3/3
<i>F. longipes</i>	0/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3
<i>F. proliferatum</i>	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
<i>F. verticillioides</i>	2/3	3/3	2/3	3/3	2/3	3/3	3/3	2/3
<i>Lasiodiplodia theobromae</i>	1/3	3/3	2/3	3/3	1/3	3/3	2/3	3/3
<i>Neosartorya fischeri</i>	2/3	0/3	3/3	0/3	1/3	0/3	3/3	0/3
<i>Penicillium</i> sp.	0/3	0/3	0/3	0/3	1/3	0/3	3/3	0/3
<i>Penicillium</i> sp.	0/3	0/3	0/3	0/3	0/3	3/3	0/3	1/3
<i>P. charlesii</i>	0/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3
<i>P. citreonigrum</i>	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3
Total (n = 12)								

Table 3.4: Continued

<i>P. daleae</i>	1/3	0/3	0/3	0/3	0/3	1/12	0/3	0/3	1/3	0/3	1/12
<i>P. janthinellum</i>	0/3	0/3	2/3	0/3	0/3	2/12	1/3	0/3	3/3	0/3	4/12
<i>P. polonicum</i>	0/3	0/3	3/3	0/3	0/3	3/12	0/3	3/3	0/3	0/3	3/12
<i>P. rubens</i>	1/3	0/3	0/3	3/3	0/3	4/12	1/3	0/3	3/3	0/3	4/12
<i>Phomopsis</i> sp.	0/3	2/3	0/3	0/3	0/3	2/12	0/3	0/3	0/3	0/3	0/12
<i>Talaromyces islandicus</i>	0/3	1/3	0/3	0/3	0/3	1/12	0/3	0/3	0/3	0/3	0/12
<i>T. radicus</i>	0/3	0/3	0/3	0/3	0/3	0/12	0/3	1/3	0/3	0/3	1/12
<i>T. purpureogenus</i>	1/3	0/3	0/3	3/3	0/3	2/12	0/3	0/3	1/3	0/3	1/12
<i>Trichoderma asperelloides</i>	0/3	0/3	0/3	0/3	0/3	0/12	0/3	1/3	0/3	0/3	1/12
<i>T. asperellum</i>	0/3	0/3	0/3	0/3	0/3	0/12	0/3	1/3	0/3	0/3	1/12
<i>T. harzianum</i>	0/3	1/3	0/3	0/3	0/3	1/12	0/3	0/3	2/3	0/3	2/12
<i>T. yunnanense</i>	0/3	1/3	0/3	0/3	0/3	1/12	0/3	0/3	0/3	0/3	0/12
Basidiomycota											
<i>Bjerkandera adusta</i>	1/3	0/3	0/3	0/3	0/3	1/12	0/3	0/3	0/3	0/3	0/12
<i>Pyrrhoderma noxium</i>	0/3	0/3	0/3	0/3	0/3	0/12	0/3	0/3	1/3	0/3	1/12
<i>Schizophyllum commune</i>	1/3	0/3	0/3	0/3	0/3	1/12	0/3	0/3	0/3	0/3	0/12
<i>Trametes cubensis</i>	1/3	0/3	0/3	0/3	0/3	1/12	0/3	0/3	0/3	0/3	0/12

n: number of samples to which each fungus was present, regardless the media or method of isolation.

Table 3.5: Total fungi isolated from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia

Species	Kampong Dadong		Rhu Tapai	
	Sample	<i>n</i>	Sample	<i>n</i>
Ascomycota				
<i>Aspergillus aculeatus</i>	Tassel	1		
<i>A. flavus</i>	Kernel	1	Kernel	2
	Soil	1	Soil	1
	Tassel	1	Tassel	1
<i>A. niger</i>	Kernel	2	Kernel	1
	Tassel	1	Tassel	1
<i>Curvularia</i> sp.			Soil	1
<i>Fusarium incarnatum</i>	Kernel	1	Tassel	1
	Tassel	1		
<i>F. longipes</i>			Tassel	1
<i>F. proliferatum</i>	Debris	1		
<i>F. verticillioides</i>	Debris	3	Debris	4
	Kernel	3	Kernel	2
	Soil	3	Soil	6
	Tassel	2	Tassel	1
<i>Lasiodiplodia theobromae</i>	Debris	1	Debris	1
	Kernel	1	Kernel	2
	Soil	3	Soil	3
	Tassel	2	Tassel	3
<i>Neosartorya fischeri</i>	Debris	2	Debris	1
	Soil	8	Soil	6
<i>Penicillium</i> sp.			Debris	1
			Soil	3
<i>Penicillium</i> sp.			Kernel	1
			Tassel	1
<i>P. charlesii</i>	Kernel	1		
<i>P. citreonigrum</i>	Soil	3		
<i>P. daleae</i>	Debris	1	Soil	2
<i>P. janthinellum</i>	Soil	3	Debris	1
			Soil	7
<i>P. polonicum</i>	Soil	4	Kernel	1
<i>P. rubens</i>	Debris	1	Debris	1
	Tassel	3	Soil	3
<i>Phomopsis</i> sp.	Kernel	1		
<i>Talaromyces islandicus</i>	Kernel	1		

Table 3.5: Continued

<i>T. radicus</i>			Kernel	1
<i>T. purpureogenus</i>	Debris	1	Soil	1
	Tassel	1		
<i>Trichoderma asperelloides</i>			Kernel	1
<i>T. asperellum</i>			Kernel	1
<i>T. harzianum</i>	Kernel	1	Soil	3
<i>T. yunnanense</i>	Kernel	1		
Basidiomycota				
<i>Bjerkandera adusta</i>	Debris	1		
<i>Pyrrhoderma noxium</i>			Soil	1
<i>Schizophyllum commune</i>	Debris	2		
<i>Trametes cubensis</i>	Debris	1		
TOTAL		64 isolates	67 isolates	
		22 species	20 species	

n: number of isolates.

following order: *F. verticillioides* > *A. niger* > *A. flavus* > *F. incarnatum* > *F. proliferatum*, while only four mycotoxigenic species were recovered from Rhu Tapai samples in the following order: *F. verticillioides* > *A. flavus* > *A. niger* > *F. incarnatum*. Other fungal species, *Trichoderma* spp., that have potential as biocontrol agents as evidenced by previous publications covered in the Literature Review (sub-Section 2.7), were also recovered from both farms, albeit in lower frequency. Overall, *F. verticillioides*, *L. theobromae*, and *A. flavus* occurred in almost all samples. *A. flavus*, however, was not isolated from any of the tested soils on both farms. Three species, namely *A. niger*, *F. incarnatum*, and *Penicillium* sp. were consistently isolated from samples of the upper part of the grain maize plant (kernel and tassel), while four species, namely *N. fischeri*, *Penicillium* sp., *P. daleae*, and *P. janthinellum* were consistently isolated from the above- and lower-ground samples (soil and

debris). The other species were either not consistently present between both the plants parts and soil/debris (e.g., *P. rubens* found in debris and tassel samples only), or only present in one sample types (e.g., *F. proliferatum* only found in debris).

The isolation frequency (%) of fungal isolates on kernel and tassel samples obtained from Kampong Dadong and Rhu Tapai grain maize farms following direct plating on DG-18 and PDA and incubation at 30°C for seven days are depicted in Figures 3.3 and 3.4, respectively. Originally, DG-18 (0.95 a_w) was often employed to isolate moderately xerophilic fungi, particularly some fungi from the genera *Aspergillus* and *Penicillium* (Beuchat, 1992), while PDA was often employed as the general medium for fungal isolation (as it supports luxuriant fungal growth). The present study, however, employed both growth media for complimentary reason, and found that most species were able to grow on both of them, which could have possibly been due to the ability of most fungi obtained in the present study to grow in a wide range of water activity (Beuchat, 1992). Therefore, the % isolation frequency of fungal species was mainly reported on PDA first even if they grew on both growth media. However, for fungal species that did not grow on PDA, their % isolation frequency was reported on DG-18 instead. For moderate xerophiles, their % isolation frequency was also reported on DG-18 as they thrived on that medium. Altogether, *A. flavus*, *A. niger*, *F. verticillioides*, and *L. theobromae* were the fungal isolates frequently isolated from all kernel and tassel samples. *F. verticillioides* was isolated more frequently as compared to the other species in kernel and tassel samples of Kampong Dadong, and in kernel samples of

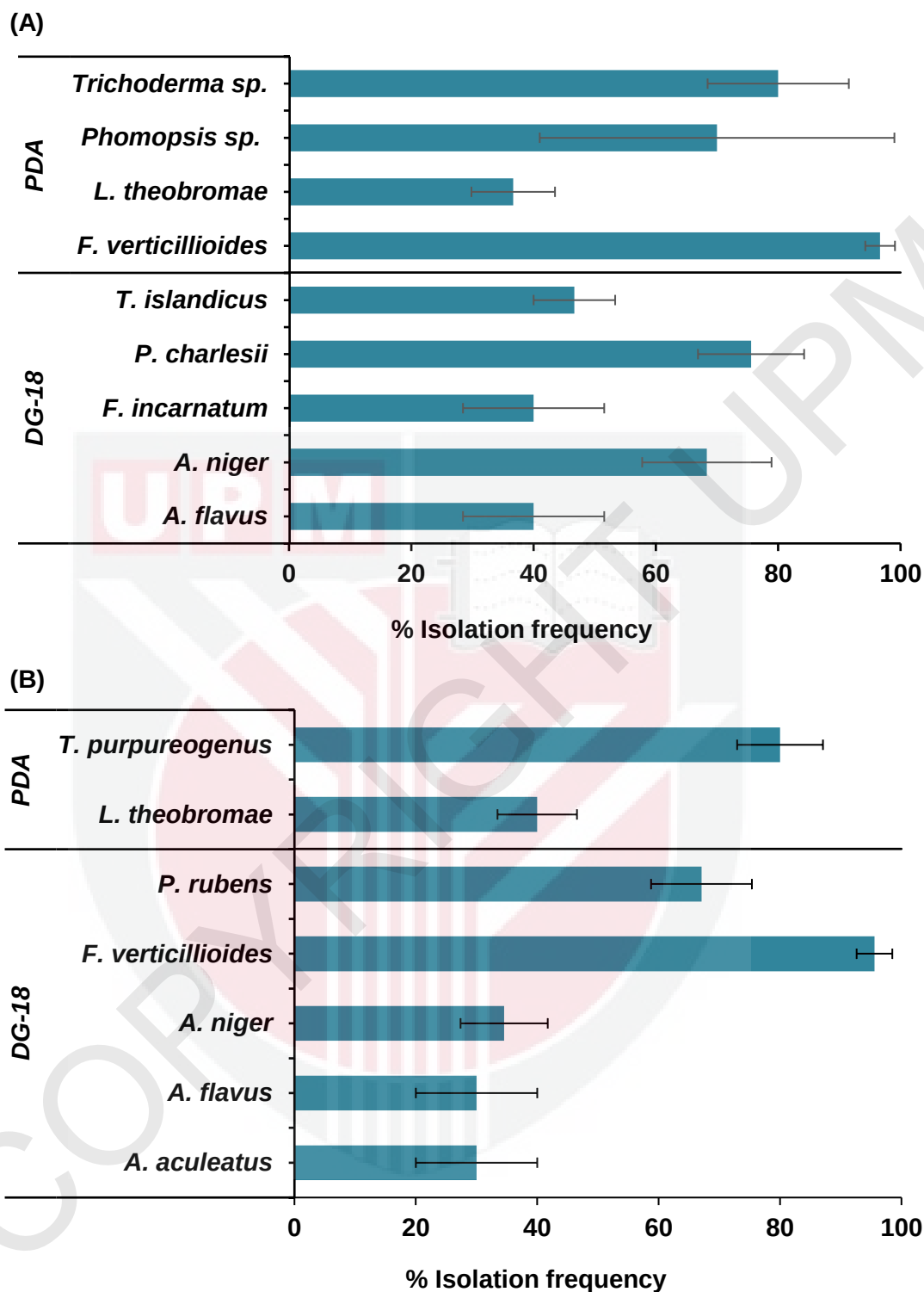


Figure 3.3: Isolation frequency (%) of fungal isolates on (A) kernel and (B) tassel samples obtained from Kampong Dadong grain maize farm in Terengganu, Malaysia following direct plating on Dichloran Glycerol 18% agar (DG-18) and Potato Dextrose Agar (PDA), and incubation at 30°C for seven days. Data are means of triplicate ($n = 3$) with bars indicating standard error ($\pm SE$)

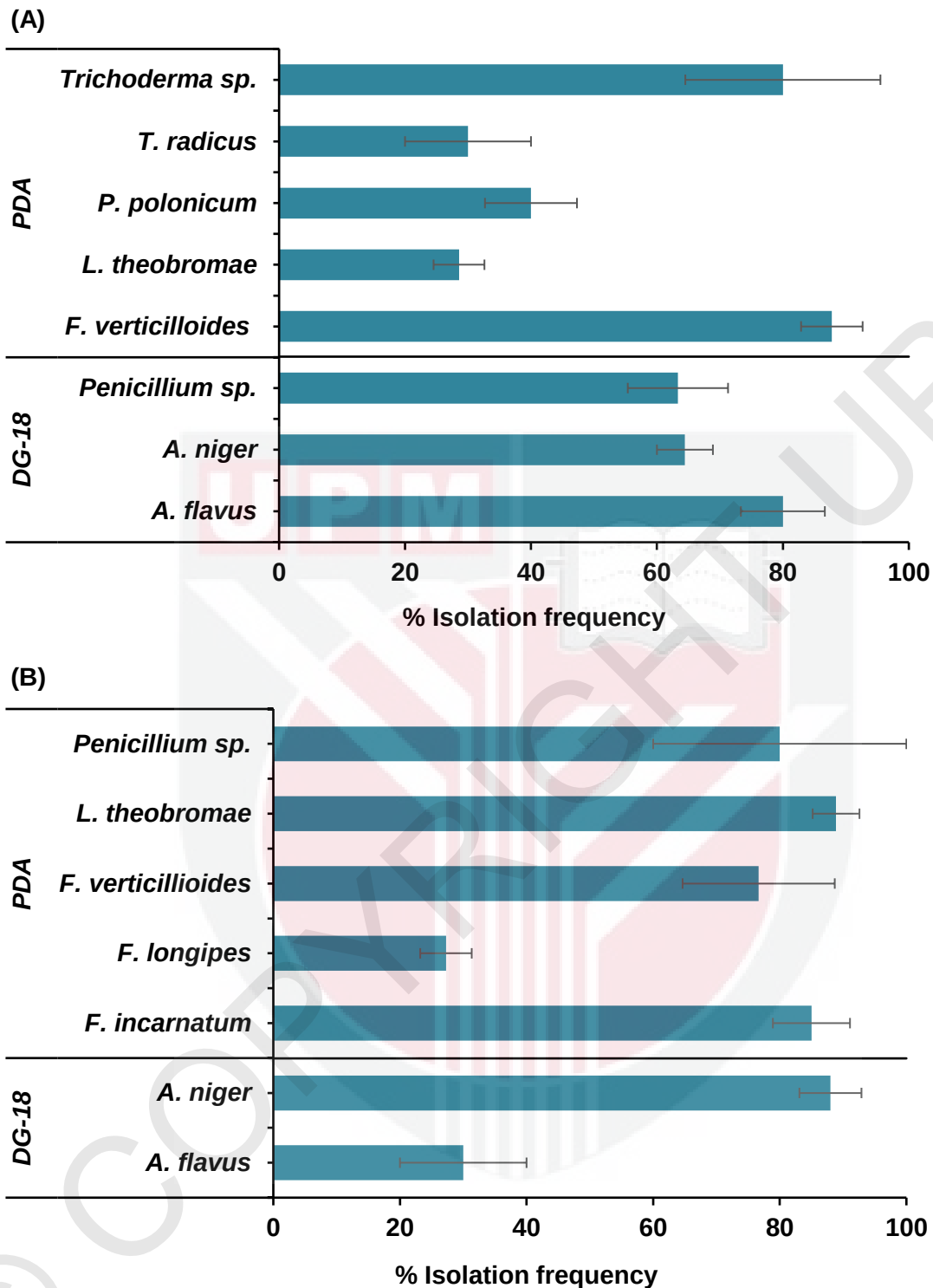


Figure 3.4: Isolation frequency (%) of fungal isolates on (A) kernel and (B) tassel samples obtained from Rhu Tapai grain maize farm in Terengganu, Malaysia following direct plating on Dichloran Glycerol 18% agar (DG-18) and Potato Dextrose Agar (PDA), and incubation at 30°C for seven days. Data are means of triplicate ($n = 3$) with bars indicating standard error ($\pm$ SE)

Rhu Tapai. In contrast, in tassel samples of Rhu Tapai, *L. theobromae* was the most frequently isolated species.

3.3.2 Mycotoxigenic potentials of mycotoxigenic species isolated from Kampong Dadong and Rhu Tapai grain maize farms

The mycotoxigenic fungal isolates, which comprised of seven *A. flavus* isolates (100% of isolated strains), five *A. niger* isolates (100% of isolated strains), three *F. incarnatum* isolates (100% of isolated strains), and one *F. proliferatum* isolate (100% of isolated strains) recovered on all samples obtained from both grain maize farms were tested for their mycotoxigenic potentials *in vitro*. For *F. verticillioides*, 14 of 24 (58.3% of isolated strains) were randomly selected from all samples from both farms for mycotoxin analyses (because of constraint on resources for analysis). Mycotoxigenic potentials of mycotoxigenic species are tabulated in Table 3.6 (*Aspergillus* spp.) and Table 3.7 (*Fusarium* spp.). For aflatoxins, three of seven *A. flavus* isolates produced AFB₁ ranging from 84 to 240.8 ng/g, of which the only one isolate also produced AFB₂, with the production pattern of AFB₁ > AFB₂. Both AFG₁ and AFG₂ were not detected. For OTA, all five *A. niger* isolates tested produced OTA ranging from 4,080.9 to 32,598.2 ng/g. For *Fusarium* mycotoxins, all three *F. incarnatum* isolates produced ZEN (29,717.3 – 68,550.3 µg/g), with one isolate produced HT-2 and T-2 toxins. For fumonisins, 13 of 14 *F. verticillioides* isolates produced both FB₁ and FB₂ (1,118.2 – 10,087.8 µg/g and 298.9 – 2,657.2 µg/g, respectively). One *F. proliferatum* isolate also produced FB₁ and FB₂. The fumonisin production pattern was FB₁ > FB₂. In addition, one of 15

Table 3.6: Aflatoxins and ochratoxin A (ng/g) production by *Aspergillus flavus* and *A. niger* isolates, respectively, obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia

Species	Farm	Sample	Isolate code	Mycotoxin (ng/g)				
				AFB ₁	AFB ₂	AFG ₁	AFG ₂	OTA
<i>A. flavus</i>	KD	Kernel	Af1KD	n.d.	n.d.	n.d.	n.d.	-
<i>A. flavus</i>	KD	Soil	Af3SD	84 ± 1.5	n.d.	n.d.	n.d.	-
<i>A. flavus</i>	KD	Tassel	Af5TD	n.d.	n.d.	n.d.	n.d.	-
<i>A. flavus</i>	RT	Kernel	Af6KR	126.1 ± 6.9	n.d.	n.d.	n.d.	-
<i>A. flavus</i>	RT	Kernel	Af7KR	240.7 ± 43.8	42.5 ± 1	n.d.	n.d.	-
<i>A. flavus</i>	RT	Soil	Af2SR	n.d.	n.d.	n.d.	n.d.	-
<i>A. flavus</i>	RT	Tassel	Af4TR	n.d.	n.d.	n.d.	n.d.	-
<i>A. niger</i>	KD	Kernel	An1KD	-	-	-	-	32,598.2 ± 2,399
<i>A. niger</i>	KD	Kernel	An3KD	-	-	-	-	23,915.7 ± 306.4
<i>A. niger</i>	KD	Tassel	An4TD	-	-	-	-	32,476 ± 5,807.1
<i>A. niger</i>	RT	Kernel	An2KR	-	-	-	-	21,870 ± 6,132.1
<i>A. niger</i>	RT	Tassel	An5TR	-	-	-	-	4,080.9 ± 433.7

AFB₁: aflatoxin B₁; AFB₂: aflatoxin B₂; AFG₁: aflatoxin G₁; AFG₂: aflatoxin G₂; OTA: ochratoxin A. KD: Kampong Dadong; RT: Rhu Tapai. n.d.: not detected; -: not analysed. Data are means of triplicate ($n = 3$), and expressed as mean ± standard error (± SE).

Table 3.7: Fumonisin, trichothecenes A, and zearalenone ($\mu\text{g/g}$) production by *Fusarium* spp. isolates obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia

Species	Farm	Sample	Isolate code	Mycotoxin ($\mu\text{g/g}$)				
				FB ₁	FB ₂	HT-2	T-2	ZEN
<i>F. incarnatum</i>	KD	Tassel	Fi53TD	n.d.	n.d.	n.d.	n.d.	68,550.3 $\pm$ 16,523.8
<i>F. incarnatum</i>	KD	Kernel	Fi110KD	n.d.	n.d.	631.8 $\pm$ 74.8	434.1 $\pm$ 171.4	29,717.3 $\pm$ 3,265.8
<i>F. incarnatum</i>	RT	Tassel	Fi56TR	n.d.	n.d.	n.d.	n.d.	36,116.1 $\pm$ 4,745.5
<i>F. proliferatum</i>	KD	Debris	Fp9DD	6,727.7 $\pm$ 1,089.8	2,021.7 $\pm$ 326.1	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Debris	Fv9DD	7,536.1 $\pm$ 1,293.7	1,009.1 $\pm$ 186.2	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Debris	Fv79DD	3,894.7 $\pm$ 3,444.6	451.6 $\pm$ 379	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Kernel	Fv21KD	9,508 $\pm$ 1,992.1	2,657.2 $\pm$ 485	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Kernel	Fv32KD	8,863.6 $\pm$ 270	2,030.1 $\pm$ 42.7	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Kernel	Fv25KD	1,384.8 $\pm$ 332	496.5 $\pm$ 65.8	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Soil	Fv106SD	n.d.	n.d.	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Tassel	Fv9TD	7,775.2 $\pm$ 1,496.9	1,848.8 $\pm$ 327	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	KD	Tassel	Fv16TD	5,705.1 $\pm$ 823.3	756.2 $\pm$ 102.6	n.d.	n.d.	n.d.
<i>F. verticillioides</i>	RT	Debris	Fv69DR	1,118.2 $\pm$ 87.1	646.6 $\pm$ 134.5	n.d.	n.d.	n.d.

Table 3.7: Continued

<i>F. verticillioidea</i>	RT	Kernel	FV25KR	4,471.1 ± 787.7	1,908.6 ± 292.9	n.d.	n.d.	n.d.
<i>F. verticillioidea</i>	RT	Soil	FV58SR	4,109.8 ± 1,666.8	914.4 ± 168.2	n.d.	n.d.	n.d.
<i>F. verticillioidea</i>	RT	Soil	FV92SR	10,087.8 ± 1,190.4	1,624.1 ± 194.2	n.d.	n.d.	n.d.
<i>F. verticillioidea</i>	RT	Soil	FV111SR	3,125.1 ± 391.6	608 ± 47.6	n.d.	n.d.	2,601.9 ± 466
<i>F. verticillioidea</i>	RT	Tassel	FV22TR	2,512.3 ± 753.2	298.9 ± 52.4	n.d.	n.d.	n.d.

FB₁: fumonisin B₁; FB₂: fumonisin B₂; HT-2: HT-2 toxin; T-2: T-2 toxin; ZEN: zearalenone. KD: Kampong Dadong; RT: Rhu Tapai. n.d.: not detected. Data are means of triplicate ($n = 3$), and expressed as mean ± standard error ($\pm SE$).

F. verticillioides isolates also produced ZEN (2,601.9 µg/g). None of the *Fusarium* spp. isolates were able to produce DON.

3.4 Discussion

In agro-ecosystem, some fungi are distributed across soils and plants (*i.e.*, generalist), while some others are only narrowly distributed to a specific substrate niche (*i.e.*, specialist). Besides their lifestyle, fungal distribution is confined within their specified biogeographical boundaries and ecological niches (*i.e.*, biotic and abiotic) (Peay *et al.*, 2010). Particularly, spatial and temporal distribution patterns of a fungal species in any given environment are dependent on their ecological niches (*e.g.*, abiotic: temperature and humidity; biotic: rival organisms, plant hosts), with some fungi becoming more dominant than the others (Bebber & Chaloner, 2022). In other words, fungal generalist will often be dominating their community structure across the agro-ecosystem, as they are more adaptable and having broader host range (Wang *et al.*, 2021). Generalist fungi are mostly from the phylum Ascomycota, a saprotroph, endophyte, or plant pathogen. All things considered, it is only natural that the dominant fungal species frequently found in maize pre-harvest are generalist, including *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Talaromyces* spp., and *Trichoderma* spp. (Katati *et al.*, 2023; Wang *et al.*, 2021). For maize cultivated in tropical climate, the mycotoxigenic *Fusarium* spp. (specifically *F. verticillioides*) has been reported as the most dominant genera (Katati *et al.*, 2023).

The present study showed that the mycotoxigenic species *F. verticillioides* dominated both pioneer grain maize farms in Terengganu, Malaysia. A high occurrence of the pathogenic *L. theobromae* was also observed on both farms. Besides *F. verticillioides*, the mycotoxigenic *A. flavus* was also occur in almost all samples, except in soil, despite it being a saprophyte. The occurrence of other mycotoxigenic species (*i.e.*, *A. niger*, *F. proliferatum*, *F. incarnatum*) was also observed albeit in low amount. The other species in phylum Ascomycota, namely *Penicillium* spp., *Talaromyces* spp., *Neosartorya* sp., *Phomopsis* sp., *Trichoderma* spp., *Curvularia* sp., and the other four species in phylum Basidiomycota, were only occurred in low amounts across all samples. Albeit both farms having located at different zones which may have different microclimates, (Kampong Dadong located near the central region of the Peninsular, while Rhu Tapai located near the coast), and cultivated on different texture of soil (Kampong Dadong: fine sandy clay; Rhu Tapai: sandy), there were no large difference between the fungal genus found on both farms. It is deduced that the low occurrence of fungi other than *F. verticillioides* and *L. theobromae*, and the absence of *A. flavus* in soils may be due to the culture-dependent method employed in the present study, which may favour the isolation of the most abundant species as compared to the less abundant and slow grower. Besides, isolation of fungi using culture-dependent method are limited to the media utilised for the process, and to only fungi that could produce conidia (Katati *et al.*, 2023).

The plant pathogen *L. theobromae* was found in all samples tested due to it being exist as both endophyte and pathogen. This species is ubiquitous in

tropical and sub-tropical regions, and prevalent in a wide range of plant hosts (Alves *et al.*, 2008). In maize, *L. theobromae* can cause ear rot disease (Ma *et al.*, 2016). Among many secondary metabolites produced by *L. theobromae*, a phytotoxin, known chemically as (3S,4R)-3-carboxy-2-methylene-heptan-4-olide, is associated with the black spot disease in tropical fruits (Salvatore *et al.*, 2020). However, its toxicity towards animals and humans is not yet known, and was not assessed in the present study.

Besides pathogens, fungal species that have potential as biocontrol agents, especially the mycoparasitic *Trichoderma* spp., were also isolated from both grain maize farms, and may be used in the development of an effective control measure to combat fungal infestation indigenously and biologically in the grain maize farms without the use of chemically synthesised fungicides. In sub-Section 2.7.1 of the Literature Review, the potentials of *Trichoderma* spp. to biologically control the fungal diseases on cocoa, oil palm, soybean, mango, and rice paddy within the Malaysian agro-ecosystems have been discussed. However, no work has been done thus far to control the proliferation of mycotoxigenic fungi in Malaysia. This thus opens a future research opportunity as an effort to sustainably manage and control mycotoxin contamination of economically relevant Malaysian agricultural commodities. The following Chapters of the present study also assessed *Trichoderma* spp.'s potentials against mycotoxigenic fungi and mycotoxin production.

The high occurrence of *F. verticillioides* in both grain maize farms demonstrated that this species, indeed, is endemic in maize (Munkvold *et al.*,

2019). A similar high occurrence had also been reported in many countries, including that in tropical regions (García-Díaz *et al.*, 2020; Degraeve *et al.*, 2016; Covarelli *et al.*, 2011; Ghiasian *et al.*, 2005). This result agreed with previous findings (Nur Ain Izzati *et al.*, 2011; Zainudin *et al.*, 2011), which reported that *F. verticillioides* was among the *Fusarium* spp. frequently found in Malaysian maize agro-ecosystems. Nevertheless, in contrast to the present findings, Nur Ain Izzati *et al.* (2011) reported that *F. proliferatum* was predominant in almost all maize farms investigated. Since both species have a similar life cycle, thus requiring similar ecophysiological conditions to survive (Reyes Gaige *et al.*, 2020), it is not surprising that both species could actively compete within the maize agro-ecosystems. The slight discrepancy between the findings of both studies may be attributed to the shift in weather conditions (*i.e.*, climate change) over the ten years period, which was becoming more favourable for *F. verticillioides* colonisation. According to Marín *et al.* (2010), *F. verticillioides* may be better adapted to higher temperature and water stress than *F. proliferatum*. Although this was not ascertained in the present study, it was apparent that the recent higher daily temperatures (Tang, 2019) may have affected the water activity of the microenvironments (*e.g.*, maize kernels, soil) in which those fungi inhabited, thus resulting in higher *F. verticillioides* occurrence in all samples from both grain maize farms assessed in the present study. Regardless, both species are significant fumonisin producers, thus indicating a definite risk of fumonisin contamination in the maize kernels, and by extension, in the animal feeds.

Apart from its occurrence in the maize kernel samples, *F. verticillioides*, being an endophyte and saprophyte (Bacon *et al.*, 2008), was also present in the debris, soil, and tassel samples, thus contributing to the high occurrence in both grain maize farms. The high occurrence of *F. verticillioides* in the debris samples may serve as a source of inoculum for its vertical contamination in maize through systemic infection (Blacutt *et al.*, 2018). Further, Cotton & Munkvold (1998) had described the long-term survival of this species (up to 630 days) in farm residues, either with a monocropping or rotation system, which may intensify the risk of *F. verticillioides* infection in maize farm should there be no proper crop residue management implemented.

As compared to *F. verticillioides*, *A. flavus* was found relatively lower in the present study. This was possibly due to the ability of the former species to dominate maize niches in a favourable growth environment. In fact, *F. verticillioides* may invariably outcompete many species co-existing within the maize niches, provided that they co-exist in a favourable environment for the growth of *F. verticillioides*, for example, under high humidity conditions. This has been documented by studies *in vitro* (Marín *et al.*, 1998) and *in planta* (Giorni *et al.*, 2019). In the case of *F. verticillioides* versus *A. flavus*, the former may effectively and rapidly utilise the carbon sources once it germinates and colonises the maize ecosystem, thus inducing the plant host resistance, such as the production of phytoalexins, as a defence mechanism against *A. flavus* (Marín *et al.*, 1998). This process thereby inhibits further *A. flavus* colonisation in the agro-ecosystem. *A. flavus* could only become predominant in drier environments, as reported in northern Italy during the 2003–2004 drought

episode (Giorni *et al.*, 2007). In the present study, high monthly rainfalls during the 2017 cropping season might have resulted in high soil and grain moisture, which was favourable for the proliferation of *F. verticillioides*. Furthermore, the Rhu Tapai grain maize farm, which recorded hotter and dryer climatic conditions (Table 3.1) throughout the sampling period in 2017, recorded higher *A. flavus* occurrence (Figure 3.4) as compared to Kampong Dadong grain maize farm.

Prior to grain maize cultivation, Malaysian maize production was dominated by sweet maize. Therefore, previous studies on the occurrence of fungal species and mycotoxigenic fungi pre-harvest were focused mainly on this type of maize. Among the earliest documented data, all had particularly emphasised the proliferation of *Fusarium* spp. (Nur Ain Izzati *et al.*, 2011; Zainudin *et al.*, 2011). Two similar studies that were conducted in seven and 12 states of Malaysia, respectively, found various *Fusarium* spp. contaminating maize ears, bracts, and kernels (Nur Ain Izzati *et al.*, 2011; Zainudin *et al.*, 2011). The species found were *F. equiseti*, *F. longipes*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. pseudograminearum*, *F. semitectum*, *F. solani*, *F. subglutinans*, and *F. verticillioides*. In both studies, *F. proliferatum* was predominant in almost all regions. Interestingly, also in both studies, *F. pseudograminearum*, the closely related species to *F. graminearum* (Clear *et al.*, 2006), was only isolated in the colder region of Malaysia (Cameron Highlands, with average temperature of 18°C–23°C). Recently, a study was conducted to determine the aflatoxigenic and non-aflatoxigenic *A. flavus* from sweet maize pre-harvest in Cameron Highlands. Among 40 *A. flavus* isolates,

45% ($n = 18/40$) were aflatoxigenic, and the remaining 55% ($n = 22/40$) were non-aflatoxigenic (Khan *et al.*, 2020).

Although grain maize and sweet maize may become infected by the same fungal pathogens (e.g., *F. verticillioides*), the severity of infection may vary based on to maize type. This variation is attributed to the maize unique characteristics. As compared to grain maize, sweet maize contains higher reducing sugar and sucrose, a low amount of the complex carbohydrate amylopectin, and higher kernel water content (Ranum *et al.*, 2014). Consequently, sweet maize is more susceptible, and will be more severely affected by fungal infection than grain maize. However, depending on the maize breed, some sweet maize may also be resistant to the infection and the subsequent mycotoxin accumulation (Santiago *et al.*, 2013). Nevertheless, both maize types are always prone to mycotoxigenic fungi that could result in devastating loss. Therefore, knowledge on what type of mycotoxigenic species infecting this crop may assist in selecting the best control strategy.

Regarding the fungal abundance in the analysed samples, tassels from both grain maize farms recorded the highest total fungal loads, which reflected the abundance of airborne spores during the cropping season. The spores are released from their primary inocula (*i.e.*, maize plant or debris) (Burge, 2002) by several mechanisms, including rain and wind (McCartney, 1994). Crandall and Gilbert (2017) opined that high relative humidity and warm air temperature, which are the dominant weather conditions in Malaysia, may result in a high abundance of airborne spores. Furthermore, airborne spores are usually

derived from plant pathogens and mycotoxigenic fungi (Bock *et al.*, 2004); hence their abundance in the atmosphere at the grain maize farms may heighten the risk of diseases and contamination of the maize plants. In fact, many cultivable fungal species recovered from the tassel samples of both grain maize farms analysed in the present study were either pathogenic (*i.e.*, *L. theobromae*) or mycotoxigenic (*i.e.*, *A. flavus*, *A. niger*, *F. incarnatum*, *F. verticillioides*). Additionally, during maize flowering season, maize ears or kernels may be susceptible to fungal infection *via* pollens from tassels that carry fungal spores through the silk channel (Thompson & Raizada, 2018), thus predisposing the maize to fungal diseases and mycotoxin accumulation during favourable weather conditions.

Regarding fumonisin production, almost all (93%) *F. verticillioides* isolates analysed in the present study, including one *F. proliferatum*, produced FB₁ and FB₂, with FB₁ being the major analogue. Fumonisin FB₁ usually constitute of 75% of the total fumonisins (Proctor *et al.*, 2006). These isolates are considered as high FB₁ producers (> 500 µg/g) (Nelson *et al.*, 1991), which corroborated the findings of Darnetty & Salleh (2013) who investigated the mycotoxigenic potentials of *Fusarium* spp. isolates from Indonesia and Malaysia. Nevertheless, this contradicted Ismail *et al.* (2017), who reported the prevalence of low fumonisin-producing (< 50 µg/g) *F. verticillioides* isolates recovered from maize kernels in Sabah and Sarawak, Malaysia. This discrepancy might have been attributed to the differences in fumonisin production profiles by different fungal strains of the same species. Similar observation on the variability in fumonisin production profiles has been

recorded in fumonisin-producing species from China (Duan *et al.*, 2016), Italy (Covarelli *et al.*, 2012), and Iran (Ghiasian *et al.*, 2005). Interestingly, those studies reported high variability in the yields of fumonisins from the isolates obtained between and within provinces or fields. These emphasise that strain specificity, rather than geographical origins, is more relevant to distinguish their fumonisin production profiles. Different strains may be genetically varied, thus possessing distinct capability in terms of fumonisin production. However, more research needs to be conducted to confirm the variation of fumonisin production profile by *Fusarium* spp. between farms in different microclimates.

Among all aflatoxin producers in the *Aspergillus* section *Flavi*, only *A. flavus* was recovered in the present study. This might have been due to the ubiquitous nature of this fungus in hot and dry environmental conditions. Previous studies on maize (Agbetiamah *et al.*, 2018; Razzaghi-Abyaneh *et al.*, 2006) peanut (Agbetiamah *et al.*, 2018), and cotton (Cotty, 1997) farms distinctly reported high *A. flavus* occurrence. In fact, those studies had indicated a very low to negative occurrence of *A. parasiticus*, the other prominent aflatoxin producer from the *Aspergillus* section *Flavi*. Among the *A. flavus* isolates recovered, 57% ($n = 4/7$) were non-aflatoxigenic, while the remaining were aflatoxigenic. These non-aflatoxigenic isolates may be utilised as potential biocontrol agents against their toxigenic counterparts. Nevertheless, this “non-aflatoxigenic” status could very well be a “false-negative”, and that these isolates were, in fact, very low aflatoxin producers, below the detection limits of the chromatography system employed in the present study. Therefore, a validation study is highly warranted to verify the

aflatoxigenic property of these isolates by determining the absence/presence of genes responsible for aflatoxin biosynthesis (Gallo *et al.*, 2012), which was carried out in Chapter 4.

Among *A. flavus* aflatoxigenic isolates, a low concentration of aflatoxins (< 0.5 µg/g) was detected. This might have been attributed to the complex interaction between the pathogens and climatic conditions, agricultural practices, as well as the plant host that further drive the natural selection of a certain group of strains (*i.e.*, vegetative compatibility group, VCG) over the other (Ortega-Beltran & Cotty, 2018). Since the *A. flavus* population is dynamic within the stated interaction, and no VCG may be dominant across years (Ortega-Beltran & Cotty, 2018), it could be suggested that a temporal study spanning different years of sampling period should be conducted in the Malaysian grain maize agro-ecosystems to accurately assess the risk of aflatoxin contamination. If indeed Malaysian grain maize agro-ecosystems are infested with low-aflatoxin producers instead of the higher ones, suitable prevention and mitigation strategies could, therefore, be suggested, particularly using the indigenous non-aflatoxigenic strains. It is worth noting that among the primary challenges in selecting excellent non-aflatoxigenic candidates against the toxigenic ones is the ability to effectively inhibit aflatoxin production, especially against the high-producing isolates. The selection would, therefore, be slightly easier if the aflatoxigenic strains are, in fact, the low-producing ones as recovered in the present study. No G-series aflatoxins were detected from *Aspergillus* spp. recovered from both grain maize farms assessed in the present study. This corresponded well with the absence of *A. parasiticus* from the recovered

mycobiota. As mentioned earlier, *A. flavus* produces B-type aflatoxins, while *A. parasiticus* produces both B- and G-types aflatoxins.

The co-occurrence and interaction between *A. flavus* and *F. verticillioides* in grain maize may trigger stress conditions (*i.e.*, due to niche competition), which consequently influence their mycotoxin production. This interaction, which is affected by climatic conditions, may stimulate/inhibit mycotoxin production of both species (Camardo Leggieri *et al.*, 2019; Giorni *et al.*, 2019). For example, *in planta* study on the co-occurrence of *F. verticillioides* and *A. flavus* revealed enhanced AFB₁ production in maize co-inoculated with both species during the hotter and rainier growing season of 2016 as compared to the one inoculated with *A. flavus* alone. In contrast, the co-inoculated species *F. verticillioides* produced low fumonisins during the growing season of the same year. In the subsequent year of the slightly drier and cooler season, AFB₁ production from the co-inoculation was significantly low, particularly near- and during the harvesting period. Simultaneously, fumonisin production from the co-inoculation was slightly enhanced at the harvesting period (Giorni *et al.*, 2019). This shows that the outcome of the interaction between both species in maize may vary, and is highly dependent on climatic conditions. That being said, Malaysia's tropical climate that seems favourable for *F. verticillioides* proliferation and fumonisin formation in grain maize, as reported in the present study, could also easily induce greater aflatoxin production by *A. flavus* in grain maize, thus contributing to higher aflatoxin levels in maize and maize products. However, more research needs to be done to confirm this hypothesis.

Other mycotoxigenic species have also shown potential as producers of mycotoxin in the grain maize agro-ecosystem of Malaysia. The occurrence of ochratoxigenic *A. niger* in maize had been previously reported (Palencia *et al.*, 2010). *F. incarnatum* (synonyms: *F. pallidoroseum*, *F. semitectum*) had also been reported to produce trichothecenes A and ZEN in maize (Munkvold, 2017). These species were both recovered in the present study. The other common *Fusarium* pathogen in maize is *F. graminearum*, which was not recovered in the present study. This also explains the absence of DON from the analysed samples. This could have been due to *F. graminearum* being more prevalent in colder geographical regions as compared to the tropical regions (Pfordt *et al.*, 2020; Broders *et al.*, 2007) as in Malaysia. Although various *Fusarium* spp. have been reported to produce ZEN, its production by *F. verticillioides* is very rare (Gupta *et al.*, 2018). In the present study, however, one *F. verticillioides* isolate was found to produce ZEN (Table 3.7). The occurrence of multi-mycotoxigenic isolates in samples obtained from both grain maize farms indicated the potential risk of the resulting animal feed being contaminated by multi-mycotoxins.

The sampling from both pioneer farms was done only one-time, therefore the sampling was not replicated. This is because the cultivation on Kampong Dadong for the next season (year 2018) was halted due to technical and financial constraints. Nevertheless, the present study provides an observational data (Lennon, 2011) that could be utilised as a background information for more thorough study in the future. It is therefore recommended that a wider spatial and temporal sampling, which covers diverse agro-

ecological zones and year-to-year climatic variations, could be undertaken to fully comprehend what we are up against in terms of phytopathogenic and mycotoxigenic fungi. Besides culture-dependent method, future studies can also utilise molecular analyses such as Next Generation Sequencing that providing more thorough information on the fungal community structure on the pioneer grain maize farms. Molecular differentiation method, such as multi-locus analysis (*i.e.*, in addition to ITS analysis), can also be employed in future works to better identify genetically close species, such as those within the *Aspergillus* and *Fusarium* genera.

It is important to note that the occurrence of the fungal producers during pre-harvest may serve as the primary inoculum for the subsequent mycotoxigenic fungi and mycotoxin contamination in agricultural commodities post-harvest. Although the occurrence of mycotoxigenic species in the field may not always indicate the presence of their toxigenic metabolites in maize and maize products post-harvest, there is still a risk that could occur under certain environmental conditions. Therefore, prevention strategy is important, and therefore, recommended to manage the fungal proliferation and subsequent mycotoxin accumulation earlier in the grain maize farms (Conte *et al.*, 2020).

3.5 Conclusions

The present study has successfully established the fungal community structure, including mycotoxigenic species of two pioneer grain maize farms in Terengganu, Malaysia. The toxigenic potentials of the mycotoxigenic fungal

isolates from both grain maize farms have also been elucidated. Overall, *F. verticillioides* was predominant in both grain maize farms. The fungal pathogen of maize, which is *L. theobromae*, was also isolated in high-frequency. Moreover, other mycotoxigenic species that may produce important mycotoxins in maize, namely *A. flavus*, *A. niger*, *F. incarnatum* and *F. proliferatum*, also occurred on both farms. Among the mycotoxigenic isolates tested for mycotoxin production, 13 of 14 *F. verticillioides* isolates, and one *F. proliferatum* isolate produced fumonisins, three of seven *A. flavus* isolates produced aflatoxins (aflatoxigenic), all *A. niger* and *F. incarnatum* isolates produced OTA and ZEN, respectively, one of three *F. incarnatum* isolates produced HT-2 and T-2, and one isolate of *F. verticillioides* produced ZEN. Several known fungal isolates that could further be analysed for their antagonism against the indigenous mycotoxigenic fungi and their mycotoxins were also isolated and determined. These were *Trichoderma* spp. and four strains of non-aflatoxigenic *A. flavus*. However, the non-aflatoxigenic isolates must be validated for their non-aflatoxigenicity by molecular identification of aflatoxin biosynthetic genes before utilisation as potential biocontrol agents. Herein, all seven isolated *A. flavus* from the present Chapter must be examined and validated for their aflatoxigenicity, since the non-aflatoxigenic status of the four strains could be uncertain by the limitation of the chromatographic analysis (*i.e.*, HPLC) and the utilisation of tested media, and there could in fact be more or lesser non-aflatoxigenic strains from all the seven isolates. This was duly performed in Chapter 4. Further, the potential antagonism of other indigenous fungal isolates was also tested against growth and mycotoxin production of the indigenous mycotoxigenic fungi in Chapter 5.

CHAPTER 4

MOLECULAR IDENTIFICATION AND AFLATOXIGENIC POTENTIAL OF INDIGENOUS *Aspergillus flavus* ISOLATES FROM GRAIN MAIZE AGRO-ECOSYSTEM

4.1 Introduction

Like many species in *Aspergillus* section *Flavi*, *A. flavus* displays intra-species diversity with regard to morphological characteristics, genetic materials, and secondary metabolite profiles (Uka *et al.*, 2019). With regard to their genotype, *A. flavus* varies between aflatoxigenic and non-aflatoxigenic strains. Unlike their aflatoxigenic counterpart, non-aflatoxigenic *A. flavus* is unable to produce aflatoxins due to deletion, either small or large, or single nucleotide polymorphism (SNP) across the gene cluster of the aflatoxin biosynthesis pathway (Adhikari *et al.*, 2016). This allows the latter to be used as excellent biocontrol agent against aflatoxin contamination. In the 1980s, the first biocontrol agent utilising non-aflatoxigenic *A. flavus* was discovered to control aflatoxin production in cotton fields (Moore, 2022; Cotty 1994). Henceforth, non-aflatoxigenic biocontrol have been studied and utilised as biocontrol agents against aflatoxins in crops in many African countries and Italy with considerable success (Moral *et al.*, 2020).

Non-aflatoxigenic *A. flavus* remains, to date, as the most effective and promising biocontrol agent introduced to control aflatoxin contamination in agricultural crops (Moore, 2022) since both genotypes share similar ecophysiological requirements like growth conditions, nutrients, and

habitats/niches. Furthermore, since the usage of chemical pesticides against aflatoxigenic *A. flavus* and aflatoxins, and the usage of resistant maize cultivars are often insufficient, non-aflatoxigenic *A. flavus* seems to be an excellent alternative (Sweany, 2022).

Members of *Aspergillus* section *Flavi* also comprise of species complexes (*i.e.*, closely-related species) that could be difficult to differentiate without proper approaches, while correct *A. flavus* identification is important for biocontrol application. In 2006, Samson and colleagues used polyphasic approach in *Aspergillus* species identification in order to minimise error that typically occurs when using only morphological (*i.e.*, phenotypic) or chemical (*i.e.*, metabolite) approach. This approach extends from the traditional fungal identification which exclusively utilises morphological and chemical profiles, and includes molecular (genotypic) characterisation, such as the sequence and phylogenetic analysis of multiple genomic regions. This extended approach complements the traditional ones, thus helping resolve fungal species into its valid taxa (Samson *et al.*, 2006).

In Chapter 3, seven *A. flavus* was isolated from the pioneer grain maize farms in Terengganu, Malaysia, and identified morphologically and molecularly using ITS. Among these seven strains, four were identified as non-aflatoxigenic and three were aflatoxigenic on 3% GMA. However, further validation to confirm the non-aflatoxigenicity of these isolated strains is needed beyond the use of HPLC since the absence of aflatoxins does not necessarily confirm that the strain is non-aflatoxigenic, rather it could be producing very low aflatoxins

below the HPLC detection and quantification limits. Therefore, the present Chapter aimed: (1) to determine aflatoxigenic and non-aflatoxigenic *A. flavus* isolates based on their molecular and chemical characteristics, and examine the phylogenetic relationship to confirm the taxonomic status of the putative *A. flavus*, and (2) to examine the antagonistic potential of indigenous non-aflatoxigenic isolates to reduce aflatoxin production using dual-culture assay *in vitro* on GMA.

4.2 Materials and methods

4.2.1 *A. flavus* isolates

Seven *A. flavus* isolates assessed in the present Chapter were previously isolated from the pioneer grain maize farms in Terengganu, Malaysia during the 2017 cropping season (as described in Chapter 3). These *A. flavus* isolates, namely Af1KD, Af2SR, Af3SD, Af4TR, Af5TD, Af6KR, and Af7KR, were obtained from kernel, soil, and tassel samples of Kampong Dadong and Rhu Tapai farms. All isolates appeared as yellow-green obverse, and pale brown reverse on PDA, with spherical/sub-spheroidal conidia (Samson *et al.*, 2010). A representative isolate was also molecularly identified (Af2SR) using PCR of ITS region and tested for aflatoxigenicity on 3% GMA. Three isolates could produce aflatoxins on 3% GMA, namely Af3SD, Af6KR, and Af7KR, while the remaining four, namely Af1KD, Af2SR, Af4TR, Af5TD, could not. The aflatoxigenicity of these *A. flavus* isolates was validated by the subsequent methodology.

4.2.2 Molecular analyses

4.2.2.1 DNA extraction

DNA extraction of the one-day old *A. flavus* isolates in PDB was performed as described in sub-Section 3.2.3.

4.2.2.2 Aflatoxin biosynthesis gene identification

Seven aflatoxin biosynthesis genes namely *pkSA*, *nor-1*, *ver-1*, *verA*, *aflR*, *aflJ*, and *omtA* were tested to distinguish *A. flavus* isolates into aflatoxigenic or non-aflatoxigenic genotypes (Table 4.1). These genes are located at early, middle, and late regions of the aflatoxin biosynthesis cluster. The PCR reaction for all genes was 20 μ L. The reaction mixture contained 10 μ L of EconoTaq Plus Green 2 $\times$ Master Mix (Lucigen Corporation, Wisconsin, USA), 0.4 μ L of each primer (0.2 μ M), 3 μ L of DNA template, and 6.6 μ L of RNase-free water. The EconoTaq Plus Green contained 0.1 units/ μ L EconoTaq DNA Polymerase, PCR buffer of pH 9.0, 3 mM magnesium chloride (MgCl_2), 400 μ M of each dNTPs, PCR enhancer/stabiliser, and blue and yellow tracking dyes. The amplification programme was carried out using SuperCycler SC-200. Following amplification, the amplicons were separated by gel electrophoresis, and observed under UV light to estimate their size using gel documentation and Gel Pilot® 100 bp Plus DNA ladder, as described in sub-Section 3.2.3.

Table 4.1: Targeted genes, primers, and PCR conditions for amplification of targeted aflatoxin biosynthesis genes of indigenous *Aspergillus flavus* isolates

Set	Target gene	Primer pair	Primer sequence	^a Size (bp)	Annealing temperature (°C)	Reference
^b M1	aflR	<i>aflr1</i>	5' TAT CTC CCC CCG GGC ATC TCC CGG 3'	1,032		
	<i>aflR</i>	<i>aflr2</i>	5' CCG TCA GAC AGC CAC TGG ACA CGG 3'			
	nor-1	<i>nor1</i>	5' ACC GCT ACG CCG GCA CTC TCG GCA C 3'		67	Criseo et al. (2001); Norlia et al. (2019)
	<i>aflD</i>	<i>nor2</i>	5' GTT GGC CGC CAG CTT CGA CAC TCC G 3'	400		
	ver-1	<i>ver1</i>	5' GCC GCA GGC CGC GGA GAA AGT GGT 3'	537		
	<i>aflM</i>	<i>ver2</i>	5' GGG GAT ATA CTC CCG CGA CAC AGC C 3'			
M2	pkSA	<i>pkSA1</i>	5' GCT GGG ATT CTG CAT GGG TT 3'	536		
	<i>aflC</i>	<i>pkSA2</i>	5' CAG TTG CTC CCA AGG AGT GGT 3'			
	omtA	<i>omt1</i>	5' CAG GAT ATC ATT GTG GAC GG 3'		61	Norlia et al. (2019); Yin et al. (2009)
	<i>aflP</i>	<i>omt2</i>	5' CTC CTC TAC CAG TGG CTT CG 3'	594		
	glcA	<i>glcA1</i>	5' GTA CGA TGC AAA TGG CGT CC 3'	851		
		<i>glcA2</i>	5' GAA GCT CTG TGT CGT TGG GA 3'			
^c S1	aflJ	<i>aflj1</i>	5' CTT CAA CAA CGA CCC AAG GTT 3'	435	55	Chang et al. (2005)
	<i>aflS</i>	<i>aflj2</i>	5' AGA TGA GAT ACA CTG CCG CA 3'			
S2	verA	<i>vera1</i>	5' CCG CAA CAC CAC AAG TAG CA 3'	423	55	Chang et al. (2005)
	<i>aflN</i>	<i>vera2</i>	5' AAA CGC TCT CCA GGC ACC TT 3'			

^aAmplicon size. ^bM: multiplex PCR. ^cS: singleplex PCR. In set, the number after symbol indicates the number of sets. In target gene, original gene names are in bold, and new gene names are in italic (Yu et al., 2004).

4.2.2.3 Genomic DNA amplification and sequencing

Polymerase chain reaction (PCR) and sequencing of *A. flavus* isolates' DNA were performed on two genomic regions: ITS (product size: 600 bp) with primer pair ITS1: 5' TCC GTA GGT GAA CCT GCG G 3' and ITS4: 5' TCC TCC GCT TAT TGA TAT GC 3' (White *et al.*, 1990); and partial β -tubulin (*benA*, product size: 495 bp) with primer pair Bt2a: 5' GGT AAC CAA ATC GGT GCT GCT TTC 3' and Bt2b: 5' ACC CTC AGT GTA GTG ACC CTT GGC 3' (Glass & Donaldson, 1995). The PCR reaction for both regions was 25 μ L. The reaction mixture contained 12.5 μ L of EconoTaq Plus Green 2 $\times$ Master Mix and 3 μ L of DNA template. For amplification of ITS and *benA* region, 1.25 μ L of each ITS primers (0.5 μ M) and 2.5 μ L of each *benA* primers (1 μ M) was added to the mixture. The remaining volume for both reaction mixtures was top-upped with RNase-free water.

The amplification programme was with the following conditions. For ITS region: pre-denaturation at 95°C for two minutes; 30 cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for one minute and 20 seconds; followed by final extension at 72°C for 10 minutes. For *benA* region: pre-denaturation at 95°C for one minute; 35 cycles of denaturation at 95°C for one minute, annealing at 61°C for one minute, and extension at 72°C for one minute; followed by final extension at 72°C for five minutes (Norlia *et al.*, 2019). Thereafter, the amplicons were separated by gel electrophoresis, and observed under UV light to estimate their size using gel documentation and Gel Pilot® 100 bp Plus DNA ladder, as described in sub-

Section 3.2.3. The amplicons were then submitted to local service provider (MyTACG Bioscience Enterprise, Malaysia) for DNA purification and sequencing. A commercial aflatoxigenic type strain, *A. flavus* NRRL 3357, was obtained from New South Wales (Australia), maintained axenically on PDA at 30°C until sporulation, and served as positive control.

4.2.2.4 Sequence alignment, model selection, and phylogenetic analysis

The sequences obtained were edited and trimmed using BioEdit v. 7.2.5 (Hall, 1999). Phylogenetic tree was built based on the sequences obtained in the present study, and the published sequences including reference strains of *Aspergillus* section *Flavi* species obtained from the NCBI database (Table 4.2). The species provided in the list are closely related to *A. flavus* and could also produce aflatoxins (Norlia *et al.*, 2019). The subsequent sequence alignment, model selection, concatenation of ITS and β -tubulin genomic regions, and building of the phylogenetic tree were done using Mega v. 11 software (Tamura *et al.*, 2021). All sequences were aligned using MUSCLE algorithm. Maximum likelihood (ML) tree was developed for individual and combined (concatenated) datasets. *A. niger* strain CBS 113.46 was used as the outgroup taxon. The best-fit nucleotide substitution model was determined by the lowest Bayesian Information Criterion (BIC) score, and indicated as following: ITS dataset = T92 + G (Tamura-3-parameter + discrete gamma distribution), length alignment = 418; β -tubulin dataset = K2 + G (Kimura-2-parameter + discrete gamma

Table 4.2: *Aspergillus* section *Flavi* with published sequences in NCBI database, and their accession number used for phylogenetic tree analysis of individual and combined (ITS, *benA*) genomic regions

Species	Strain	GenBank accession number	
		ITS	<i>benA</i>
<i>A. arachidicola</i>	CBS 117610	MF668184	EF203158
<i>A. arachidicola</i>	CBS 117614	KY937923	KY924665
<i>A. flavus</i>	NRRL 21882	HQ856223	MG825981
<i>A. flavus</i>	NRRL 3357	MF966967	M38265
<i>A. minisclerotigenes</i>	CBS 117635	KY937925	KY924667
<i>A. minisclerotigenes</i>	NRRL 29000	KY937929	KY924668
<i>A. niger</i>	CBS 113.46	FJ629351	FJ629302
<i>A. nomius</i>	NRRL 13137	AF027860	AF255067
<i>A. nomius</i>	NRRL 25393	AF027864	AF255068
<i>A. novoparasiticus</i>	AFc31	KC964099	KY924669
<i>A. novoparasiticus</i>	AFc32	KC964100	KY924670
<i>A. oryzae</i>	CBS 100925	MF668185	EF203138
<i>A. parasiticus</i>	CBS 100308	KJ175436	KJ175496
<i>A. parasiticus</i>	NRRL 492	KY937934	KY924674
<i>A. parvisclerotigenus</i>	AFc36	KC964102	KC954604
<i>A. parvisclerotigenus</i>	CBS 121.62	EF409240	EF203130
<i>A. pseudotamarii</i>	NRRL 25518	KY937937	KY924675
<i>A. pseudotamarii</i>	NRRL 443	AF004931	EF661476
<i>A. tamarii</i>	CBS 118098	KJ175442	KJ175500
<i>A. tamarii</i>	CBS 121599	KJ175443	KJ175501

distribution) length alignment = 350 bp; combined dataset = T92 + G, length alignment = 822 bp. The robustness of phylogeny was assessed by bootstrap method with 1,000 replicates, and bootstrap value of lower than 70% were not shown.

4.2.3 Aflatoxigenicity test

4.2.3.1 Qualitative assessment by coconut cream agar

The *A. flavus* isolates were examined qualitatively for aflatoxin production using 50% CCA. The CCA was prepared by adding coconut cream in distilled water in 1:1 ratio (v/v). Next, 15 g of technical agar was added to the mixture before autoclaved at 121°C for 15 minutes, poured into Petri plates, and left to solidify overnight. Then, approximately 10 µL of spore suspensions (10^6 spores/mL) were prepared in sterile distilled water with 0.01% v/v Tween 80, counted using a Neubauer haemocytometer, inoculated centrally on the 50% CCA, and incubated in the dark at 30°C for seven days. Following two, five, and seven days of incubation, the reverse side of the colony were observed for the presence of blue-fluorescence ring under ultra-violet light (UV light; λ 365 nm) which indicated aflatoxin biosynthesis (Dyer & McCammon, 1994).

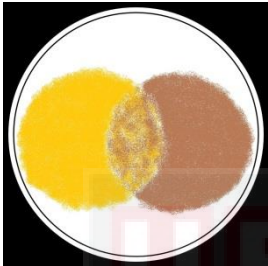
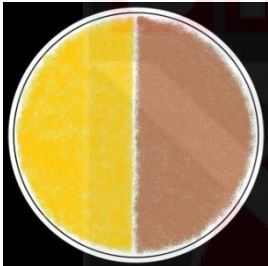
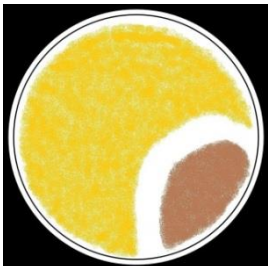
4.2.3.2 Quantitative assessment by high-performance liquid chromatography with fluorescence detector

For HPLC quantification, approximately 10 µL of the spore suspension (10^6 spores/mL) of each isolate were inoculated centrally on PDA and MEA. Thereafter, all plates were incubated in the dark for seven days at 30°C. Aflatoxin extraction and quantification, preparation of mobile phase, and data processing were performed as described in sub-Section 3.2.5.

4.2.4 Dual-culture assay on grain maize agar and determination of aflatoxin reduction

A 3% semi-synthetic GMA was prepared as described in sub-Section 3.2.4. Next, 5 μL of the aflatoxigenic and non-aflatoxigenic *A. flavus* spore suspensions (10^6 spores/mL) were aseptically inoculated 60 mm apart from each other, and 15 mm from the plate's periphery. The inoculated plates were then incubated for seven days at 30°C. At the end of incubation period, the types of interaction (Table 4.3) were scored using numerical scores namely mutual intermingling (1/1), mutual antagonism upon contact (2/2), mutual antagonism at a distance (3/3), dominance of one species upon contact (4/0), and dominance of one species at a distance (5/0) (Magan & Lacey, 1984; Samsudin & Magan, 2016). The interaction scores for each non-aflatoxigenic *A. flavus* isolates were then added to obtain Index of Dominance (I_D).

Table 4.3: Index of Dominance (I_D) as explained by interaction score of dual-culture assay between pathogen and antagonist. Descriptions and scores are adapted from Magan & Lacey (1984). Diagrammatic representations of colony growth are produced in-house. Standard diameter ($\varnothing$) of a Petri dish is 90 mm

Colony growth (qualitative)	Interaction score (quantitative)	Description of interaction
	1/1 <i>mutual intermingling</i>	The growth of one species (the pathogen) and that of another species (the antagonist) intermingle mutually, and without inhibition.
	2/2 <i>antagonism (mutual inhibition) upon contact</i>	The growth of one species is mutually inhibited by another species upon contact. Space exists between colonies at < 2 mm.
	3/3 <i>antagonism (mutual inhibition) at a distance</i>	The growth of one species is mutually inhibited by another species at a distant. Space exists between colonies at > 2 mm.
	4/0 <i>dominance (strong inhibition) of one species upon contact</i>	Inhibitor species continues to grow unchanged/unchallenged, or at a reduced rate, through/over the inhibited species.
	5/0 <i>dominance (strong inhibition) of one species at a distance</i>	Inhibitor species continues to grow unchanged/unchallenged, or at a reduced rate, through a clear zone away from the inhibited species.

The Percentage Inhibition of Radial Growth (PIRG, %) was also recorded by measuring the colony radii of mycotoxigenic fungi from both treatment and control plates, and computing them into the Equation 4.1 (Rahman *et al.*, 2009).

$$\%PIRG = \frac{R_1 - R_2}{R_1} \times 100$$

Equation 4.1: Formula for Percentage Inhibition of Radial Growth

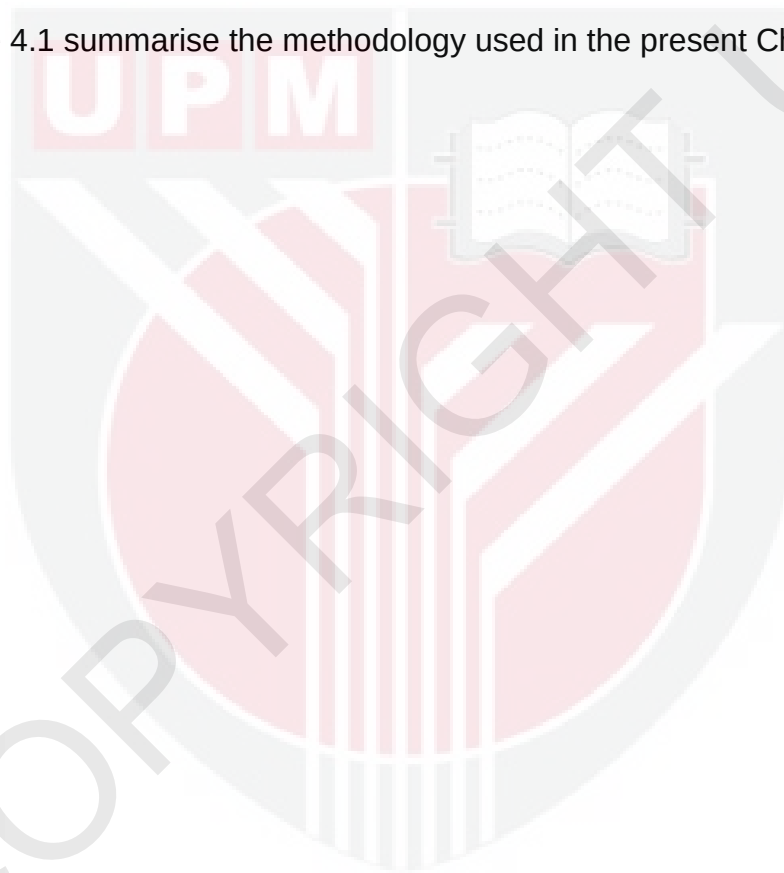
Where, R_1 was radius of mycotoxigenic fungus in control plate, and R_2 was radius of mycotoxigenic fungus in treatment plate. The radius of mycotoxigenic fungi in control and treatment plates were measured from the inoculation point towards the direction of antagonist colony (for treatment) or the supposed inoculation and growth area (for control). Thereafter, five agar plugs of the aflatoxigenic isolates were collected from the plate's assay for aflatoxin analysis as previously described in sub-Section 3.2.5.

4.2.5 Statistical analysis

All experiments were conducted in four replicates ($n = 4$). Measurements were averaged and reported as mean $\pm$ SE. Data of aflatoxin production on PDA and MEA, and AFB₁ reductions following dual-culture assay were examined for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. The statistical analysis for the aflatoxin production on PDA and MEA by each *A. flavus* isolates was done using independent samples t-test. For AFB₁ reductions, the

statistical analysis was done using one-way analysis of variance (ANOVA) with 95% confidence interval. p -value of < 0.05 was accepted as significant difference. Next, a *post hoc* Tukey's Honest Significant Difference (Tukey's HSD) were carried out with p -value of < 0.05 to compare the significant difference between groups. The statistical software SPSS v. 29.0 was used to perform all statistical analyses.

Figure 4.1 summarise the methodology used in the present Chapter.



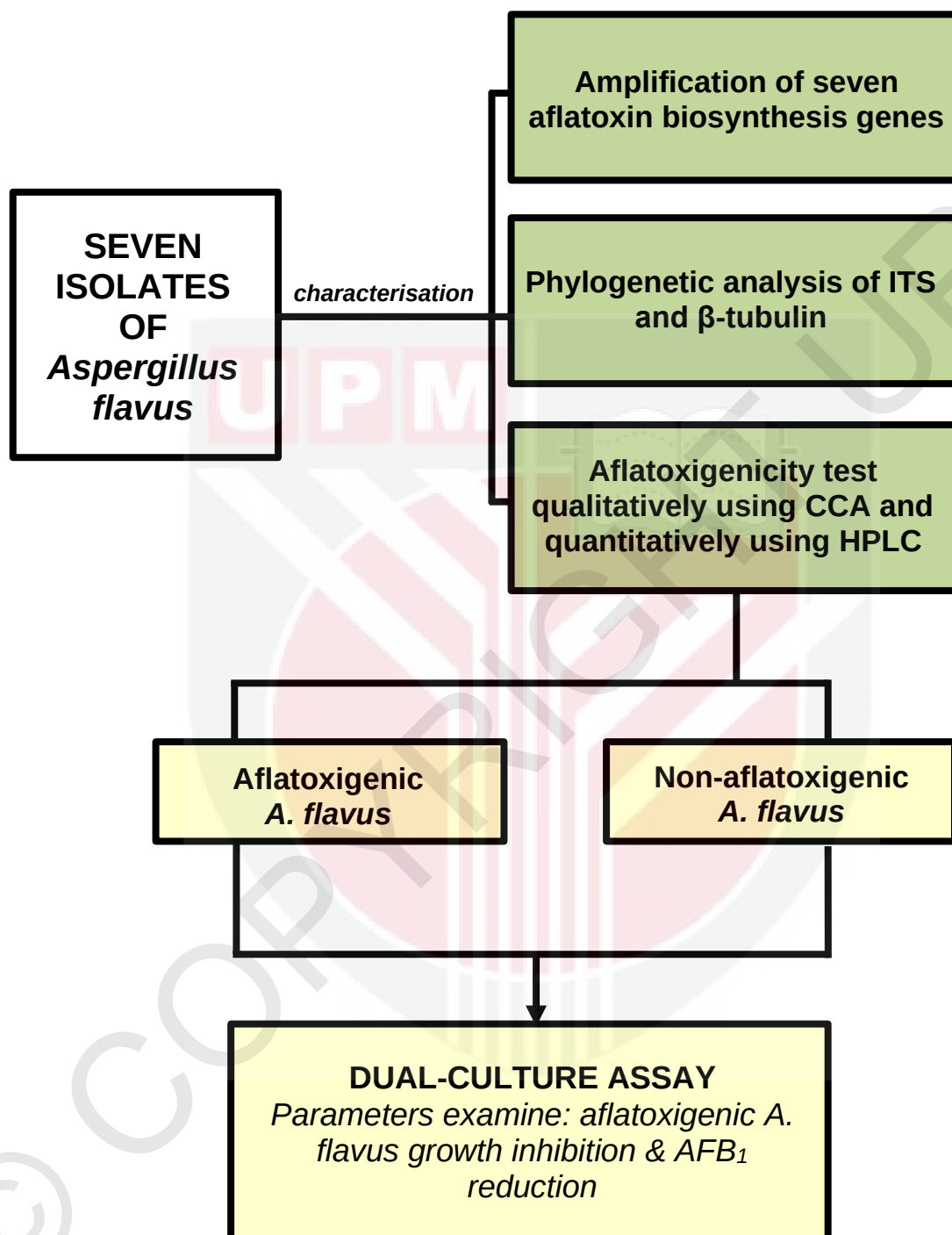


Figure 4.1: Flow diagram of the methodology used in Chapter 4

4.3 Results

4.3.1 Detection of aflatoxin biosynthesis genes and aflatoxin production potentials of indigenous *A. flavus* isolates

In general, following DNA amplification, the presence of all aflatoxin biosynthesis genes within the gene cluster indicates the ability of any *A. flavus* isolate to produce aflatoxins, hence the isolate is termed aflatoxigenic. Conversely, the absence of one or more genes often indicates *vice versa*, and the isolate is termed non-aflatoxigenic. To confirm the aflatoxigenicity of *A. flavus* isolates, they were subjected to PCR analysis, and the targeted aflatoxin biosynthesis genes at early (*pksA* and *nor-1*), middle (*ver-1* and *verA*, including regulatory genes *aflR* and *aflJ*), and late (*omtA*) regions of the cluster were tested. Table 4.4 depicts the amplification of targeted aflatoxin biosynthesis genes of *A. flavus* isolates. The amplification pattern as showed by DNA bands of these genes was provided in Appendix I. Five *A. flavus* isolates, namely Af3SD, Af6KR, Af7KR, Af2SR, and Af4TR exhibited all the targeted genes, and deemed aflatoxigenic. The remaining two *A. flavus* isolates, namely Af1KD and Af5TD, failed to yield amplicons of the targeted genes located at early and middle regions of the gene cluster, and were only able to amplify the targeted gene located at the late region of the cluster (*omtA*), therefore deemed non-aflatoxigenic. A gene in the sugar cluster, *glcA*, is a positive marker for *A. flavus*, and was amplified by all the *A. flavus* isolates tested. In addition, all targeted genes in the type strain (positive control) were also amplified.

Table 4.4: Amplification of targeted aflatoxin biosynthesis gene and one gene in sugar cluster of indigenous *Aspergillus flavus* isolates by PCR

Isolate	Aflatoxin biosynthesis gene cluster							^a Sugar cluster
	Early		Middle			Late		
	pksA	nor-1	aflR	aflJ	ver-1	verA	omtA	glcA
	<i>aflC</i>	<i>aflD</i>	<i>aflR</i>	<i>aflS</i>	<i>aflM</i>	<i>aflN</i>	<i>aflP</i>	
^b NRRL 3357	+	+	+	+	+	+	+	+
Af1KD	–	–	–	–	–	–	+	+
Af3SD	+	+	+	+	+	+	+	+
Af5TD	–	–	–	–	–	–	+	+
Af6KR	+	+	+	+	+	+	+	+
Af7KR	+	+	+	+	+	+	+	+
Af2SR	+	+	+	+	+	+	+	+
Af4TR	+	+	+	+	+	+	+	+

^aA gene in the sugar utilisation cluster. ^bA. *flavus* type strain (positive control). Original aflatoxin gene names are above and in bold letters. New aflatoxin gene names are below and in italic letters. + target gene present, – target gene absent.

Table 4.5 depicts the productions of aflatoxins ($\mu\text{g/g}$) by *A. flavus* isolates obtained from pioneer grain maize farms in Terengganu, Malaysia using HPLC-FLD. As expected, HPLC-FLD data paralleled those of molecular data (Table 4.4). Among the seven tested *A. flavus* isolates, five produced AFB₁ on the media tested (aflatoxigenic), and the other two did not (non-aflatoxigenic). Of the five aflatoxigenic *A. flavus* isolates, only three produced both AFB₁ and AFB₂. No AFG₁ and AFG₂ were detected from all the five aflatoxigenic isolates tested. With regard to aflatoxin concentrations, the productions varied based on the growth media they grew on. Generally, all five aflatoxigenic *A. flavus* isolates produced AFB₁ of more than 10 $\mu\text{g/g}$. AFB₂ was produced much lesser, of less than 1 $\mu\text{g/g}$. CCA analysis, however, showed varying correlation with HPLC-FLD analysis. Three aflatoxigenic *A. flavus* isolates fluoresced on

Table 4.5: Production of aflatoxins ($\mu\text{g/g}$) on different growth media by *Aspergillus flavus* isolates obtained from Kampong Dadong and Rhu Tapai pioneer grain maize farms in Terengganu, Malaysia

Isolate	CCA	PDA ($\mu\text{g/g}$)		MEA ($\mu\text{g/g}$)	
		AFB ₁	AFB ₂	AFB ₁	AFB ₂
^a NRRL 3357	+	4.6 $\pm$ 1.9	n.d.	2.9 $\pm$ 0.5	n.d.
Af1KD	-	n.d.	n.d.	n.d.	n.d.
Af3SD	-	10.3 $\pm$ 3.5	n.d.	15.7 $\pm$ 1.5	n.d.
Af5TD	-	n.d.	n.d.	n.d.	n.d.
Af6KR	+	61 $\pm$ 7.2	0.5 $\pm$ 0.1	37 $\pm$ 4.3	0.1 $\pm$ 0
Af7KR	-	72 $\pm$ 6.9*	0.1 $\pm$ 0†	10 $\pm$ 0.8*	n.d.†
Af2SR	+	62.6 $\pm$ 3.6	0.5 $\pm$ 0.1	39.3 $\pm$ 4.1	0.2 $\pm$ 0.1
Af4TR	-	7.6 $\pm$ 0.8	n.d.	10 $\pm$ 0.7	n.d.

PDA: Potato Dextrose Agar; MEA: Malt Extract Agar. ^aA. *flavus* type strain (positive control). AFB₁: aflatoxin B₁; AFB₂: aflatoxin B₂. +: fluoresce; - not fluoresce. n.d.: not detected. Data are means of triplicate ($n = 3$), and expressed as mean $\pm$ standard error ($\pm$ SE). Asterisk (*) and dagger (†) indicate statistically significance following independent samples t-test. Bold indicate the only isolate having statistically significant mean for each AFB₁ and AFB₂ production on both PDA and MEA.

CCA, and showed parallel result with HPLC-FLD analysis, while two aflatoxigenic *A. flavus* isolates showed negative result on CCA. Comparing aflatoxin production on PDA and MEA, all isolates generally produced the toxins greater on PDA as compared to MEA, except for strain Af3SD and Af4TR. However, only strain Af7KR showed statistically significant different ($p < 0.05$) on the amount of AFB₁ and AFB₂ production between both media.

4.3.2 Phylogenetic analysis

Figures 4.2 and 4.3 depict the phylogenetic relationships between fungal species in *Aspergillus* section *Flavi* based on individual and combined datasets

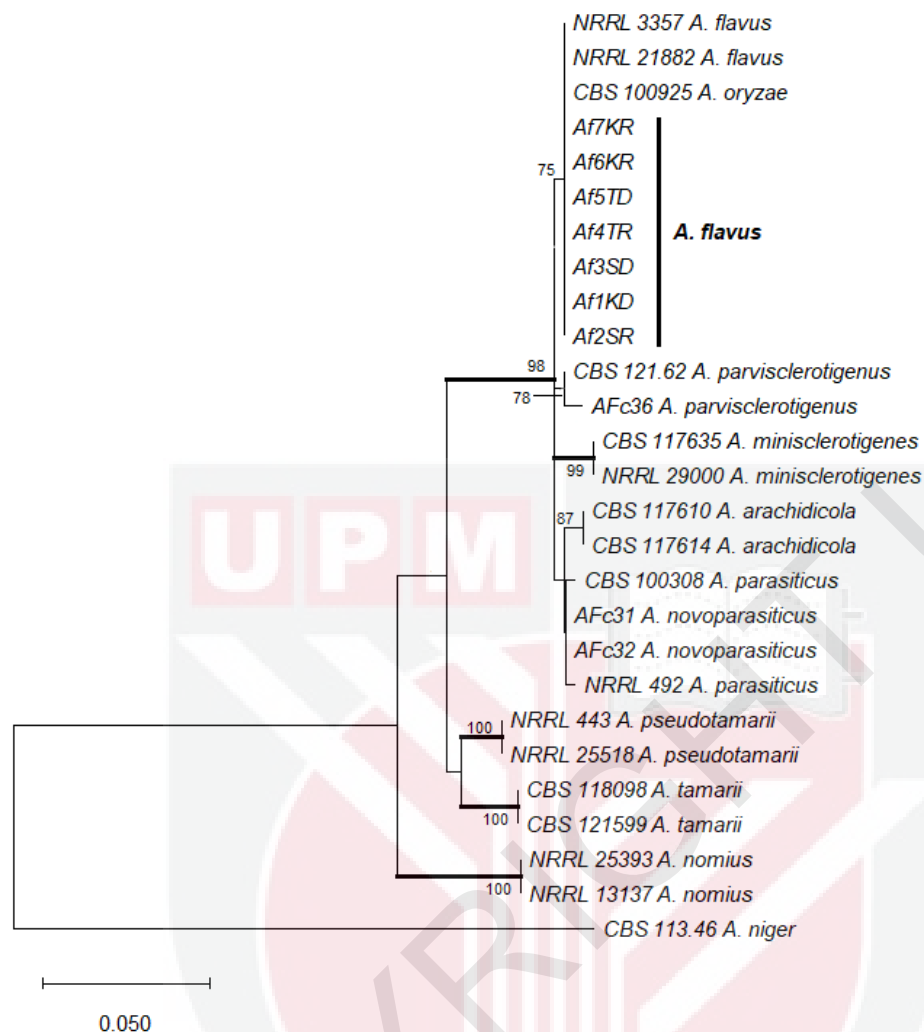


Figure 4.2: Maximum likelihood tree of *Aspergillus* section *Flavi* based on β -tubulin gene. Isolates with the initial code Af labelled as *A. flavus* in black are isolates tested in the present study. Branches in bold are highly supported with bootstrap values of > 90%. Bootstrap values of < 70% are not shown. *A. niger* strain CBS 113.46 is the outgroup taxon. Scale bars are the number of substitutions per site

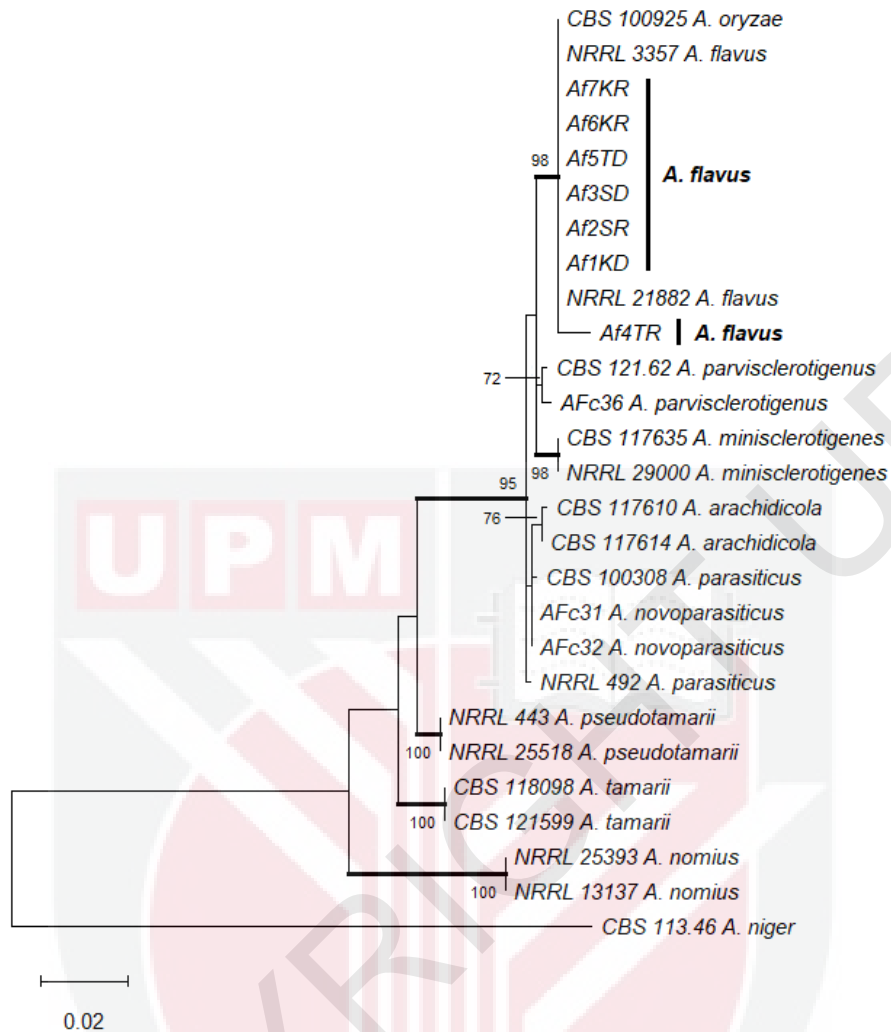


Figure 4.3: Maximum likelihood tree of *Aspergillus* section *Flavi* based on the combined datasets of ITS and β -tubulin. Isolates with the initial code Af labelled as *A. flavus* in black are isolates tested in the present study. Branches in bold are highly supported with bootstrap values of > 90%. Bootstrap values of < 70% are not shown. *A. niger* strain CBS 113.46 is the outgroup taxon. Scale bars are the number of substitutions per site

of ITS and β -tubulin. For the phylogenetic tree of ITS, it was shown in Appendix R. The ITS region gave poor resolution, and could not discriminate most of the species in *Aspergillus* section *Flavi*. This gene clustered together all seven tested sequences with other reference species of *A. flavus*, *A. oryzae*, *A. minisclerotigenes*, and *A. parvisclerotigenus*. Therefore, inference on valid nomenclature for the tested isolates was not possible. The β -tubulin gene provided better resolution, and could resolve most of the species in this section. The exception was for *A. oryzae* that shared the same clade with *A. flavus*, and likewise *A. novoparasiticus* with *A. parasiticus*. The *A. flavus*/*A. oryzae* clade was supported by high bootstrap value (> 70%), and clustered all the seven tested isolates. The combined set also showed similar result as the individual β -tubulin phylogeny, with even higher statistical support (98% bootstrap value) in *A. flavus*/*A. oryzae* clade.

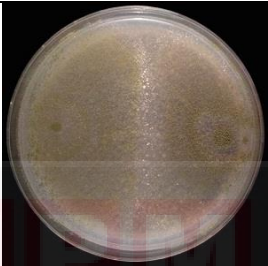
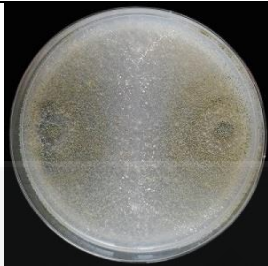
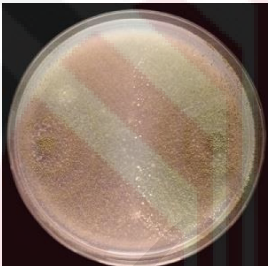
4.3.3 Indigenous non-aflatoxigenic *A. flavus* as potential biocontrol agents to control aflatoxin production

Two indigenous non-aflatoxigenic *A. flavus* isolates identified in the present study were tested against the aflatoxigenic *A. flavus* isolates in dual-culture assay on GMA. Initially, five aflatoxigenic *A. flavus* isolates were inoculated and incubated on GMA for seven days at 30°C to determine the baseline aflatoxin production prior to co-cultivation. However, only two isolates, namely Af6KR and Af7KR, produced AFB₁ (Af6KR, mean = 124.4 ng/g, and Af7KR, mean = 114.9 ng/g), and the AFB₂ could not be quantified from any of the seven tested isolates despite multiple attempts. Therefore, the subsequent

dual-culture assay was conducted with only these two aflatoxigenic *A. flavus* isolates, and the aflatoxin reduction was analysed only for AFB₁.

Table 4.6 depicts the *in vitro* fungal interaction as explained by the interaction score, Index of Dominance (I_D), and the Percentage Inhibition of Radial Growth (PIRG%) of co-cultivated aflatoxigenic and non-aflatoxigenic *A. flavus* isolates which exhibited sparse growth and conidial formation on GMA. Interaction scores and the Index of Dominance use numerical notation to indicate the nature of interaction between the non-aflatoxigenic *A. flavus* and the aflatoxigenic counterparts, as well as their possible mechanism of antagonism (Magan & Lacey, 1984). The non-aflatoxigenic isolates are deemed highly competitive and become dominant when they score a 4/0 or 5/0 against the aflatoxigenic isolates, and not as competitive when they score a 1/1, 2/2, or 3/3. Index of Dominance (I_D) is the sum of interaction scores for each non-aflatoxigenic *A. flavus* isolate. The higher the Index of Dominance, the higher the antagonism potential of an antagonist candidate. While, PIRG (%) determine the non-aflatoxigenic isolates as high ($\geq 80\%$), moderate (60–79%), or weak antagonists ($\leq 59\%$) (Soytong & Quimio, 1989). Both non-aflatoxigenic *A. flavus* isolates displayed weak antagonistic activity (PIRG < 50%). Although non-aflatoxigenic strain Af1KD showed higher I_D as compared to the non-aflatoxigenic strain Af5TD, both were less aggressive against the tested aflatoxigenic *A. flavus* isolates (interaction score; 2/2 and 3/3).

Table 4.6: *In vitro* fungal interaction as explained by interaction score, Index of Dominance (I_D), and Percentage Inhibition of Radial Growth (PIRG, %) of co-cultivated aflatoxigenic (right) and non-aflatoxigenic (left) *Aspergillus flavus* isolates obtained from pioneer grain maize farms in Terengganu, Malaysia on 3% semi-synthetic grain maize agar

Non-aflatoxigenic isolate	Aflatoxigenic isolate		I_D
	Af6KR	Af7KR	
Af1KD			
Interaction score	2/2	3/3	5/5
PIRG	41 ± 4.1	44 ± 2.1	
Af5TD			
Interaction score	2/2	2/2	4/4
PIRG	43 ± 0.6	41 ± 1.2	

Aflatoxigenic *A. flavus* isolates: Af6KR, Af7KR. Non-aflatoxigenic *A. flavus* isolates: Af1KD, Af5TD. The first integer of the interaction score indicates the numerical score for non-aflatoxigenic isolates, while the second integer indicates the numerical score for aflatoxigenic isolates. Index of Dominance (I_D) was the sum of interaction scores for each non-aflatoxigenic *A. flavus* isolate. Data of PIRG (%) are means of four replicates ($n = 4$), and expressed as mean ± standard error (± SE).

Next, AFB₁ reduction by both non-aflatoxigenic *A. flavus* isolates was tested using HPLC-FLD. Figure 4.4 depicts the AFB₁ (ng/g) production by two aflatoxigenic *A. flavus* strains Af6KR and Af7KR on GMA after co-cultivation with non-aflatoxigenic *A. flavus* strains Af1KD and Af5TD. AFB₁ production by aflatoxigenic *A. flavus* strain Af6KR was significantly reduced ($p < 0.05$) by the

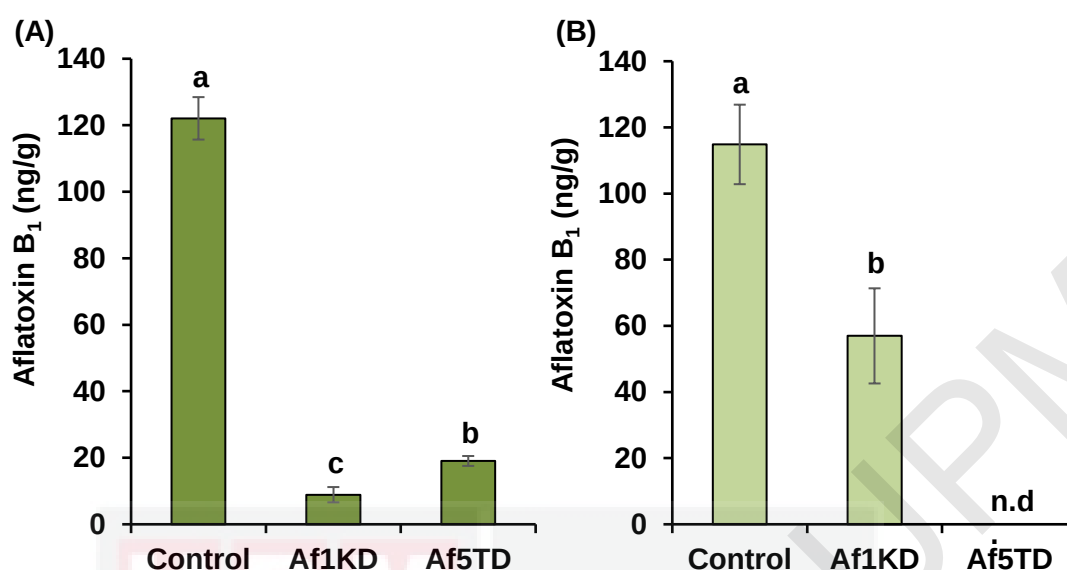


Figure 4.4: Aflatoxin B₁ (AFB₁; ng/g) produced by aflatoxigenic *Aspergillus flavus* strains (A) Af6KR and (B) Af7KR on 3% semi-synthetic grain maize agar after co-cultivation with non-aflatoxigenic *A. flavus* isolates Af1KD and Af5TD obtained from grain maize farms in Terengganu, Malaysia. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

non-aflatoxigenic *A. flavus* strains Af1KD and Af5TD by approximately 93% and 85%, respectively, while AFB₁ production by aflatoxigenic *A. flavus* strain Af7KR was significantly reduced ($p < 0.05$) by the non-aflatoxigenic *A. flavus* strains Af1KD and Af5TD by approximately 50% and 100%, respectively.

4.4 Discussion

Based on the phylogenetic analysis, the tested isolates were confirmed as *A. flavus*. The present Chapter supported the conclusion made in the previous Chapter on their identification as indigenous *A. flavus* isolates from the pioneer grain maize farms in Terengganu, Malaysia. In Chapter 3, morphological and

molecular analyses targeting only ITS region to identify the indigenous *A. flavus* isolates were performed. Although ITS is enough to differentiate between fungal species of different genus and the congeneric species (fungi in the same genus), yet it may not be sufficient to resolve the species identification especially for closely related species such as fungi in *Aspergillus* section *Flavi*. Therefore, secondary barcode is necessary to delineate the species (Lücking *et al.*, 2021; Houbraken *et al.*, 2014). β -tubulin is appropriate for this purpose since it could accurately discriminate cryptic (closely related) species within the section *Flavi* (Kim *et al.*, 2020). In the present study, the β -tubulin and the combined sets resolved the tested isolates to *A. flavus*. *A. oryzae*, which resides in the same clade as *A. flavus*, is considered as the domesticated species of *A. flavus* without the aflatoxin-producing gene, and has been used for koji fermentation of fermented foods, not found in agricultural fields (Frisvad *et al.*, 2019), thus is not the same species as *A. flavus*. This agreed with Norlia *et al.* (2019).

One of the requirements for the registration of new non-aflatoxigenic strain as biocontrol agent is their validated taxonomy and risk assessment analysis including their toxicity studies (Braverman *et al.*, 2010). Therefore, it is important that the strain is validated into its correct species. Since many strains in section *Flavi* could also produce the same metabolites (Norlia *et al.*, 2019) as *A. flavus* (e.g., aflatoxins), it is important to validate this species for proper biocontrol registration. In the case of misidentification, Uka and colleagues (2019) published a work delineating a misidentified strain that had long been known and utilised as *A. flavus*. This strain has been renamed into its closely

related species *A. nomius* (Uka *et al.*, 2019). They also emphasised the importance of having molecular analysis (such as phylogenetic) along with the phenotype-based identification.

Of the two non-aflatoxigenic *A. flavus* isolates identified, both had the same amplification pattern, that is, only one of the seven targeted genes was amplified, which was located at the end of the cluster. This indicated a deletion of the genes at early and middle regions of the cluster, thus suggesting large gene deletions (> 1-kb, Adhikari *et al.*, 2016) across the cluster of 70-kb region. Other studies have shown similar, or even diverse amplification pattern within the aflatoxin gene cluster of *A. flavus* (Adhikari *et al.*, 2016; Mauro *et al.*, 2013; Chang *et al.*, 2005). These patterns may explain the deletion / mutation events occurred that render the isolates unable to produce aflatoxins, thus becoming non-aflatoxigenic.

Although the amplification of the targeted genes in the present study was complementary to that of aflatoxin production data obtained by HPLC-FLD, some studies have found some rather conflicting results (Norlia *et al.*, 2019; Gallo *et al.*, 2012). They deduced that lack of aflatoxin production in the tested *A. flavus* isolates, despite having all targeted genes amplified, were due to some untested genes that most likely carried defects or deletions. This indicated that it is still necessary for a thorough examination of all the 25 genes to reveal the exact deletion pattern that could occur in the aflatoxin gene cluster, and therefore confirm their non-aflatoxigenicity status. Furthermore, among the requirements for the registration of biocontrol agents is the

information on their mechanism of non-aflatoxicity (Grubisha & Cotty, 2015). Besides the gene amplification that explain deletions, detail examination on SNP in the gene cluster can also be performed in future work.

Among the seven tested isolates, five were aflatoxigenic, and four of these were high aflatoxin producers ($> 10,000$ ng/g; Norlia *et al.*, 2018). This contradicts the results reported in Chapter 3 in which only three isolates were deemed as aflatoxigenic based on the quantification from GMA. While using agar-based natural substrates (Yazid *et al.*, 2018) could provide nearly accurate mycotoxigenic production potential of the mycotoxigenic species on the chosen substrates, it might not accurately define the true mycotoxin potential of the mycotoxigenic strain, hence the discrepancy. Previous studies have utilised commercial media such as Yeast Extract Agar (YES) and Czapek Yeast Autolysate (CYA) (Norlia *et al.*, 2018; Rodrigues *et al.*, 2009) to optimise aflatoxin production since these media contain added compounds that fit for this purpose (Yazid *et al.*, 2018). Nevertheless, analysis on GMA in the present study is still necessary to better predict their mycotoxigenic potential on the real maize substrates, as earlier mentioned. Furthermore, in some cases, the use of actual maize kernels may be needed to complimentary the use of maize-based agar (Probst & Cotty, 2012). Therefore, actual maize kernels were used in subsequent Chapter for deeper analyses. Regarding aflatoxin production on PDA and MEA, higher aflatoxin production on the former commercialised media as compared to the latter might be attributed to their media formulation. Specifically, PDA contains dextrose as simple sugar which readily accessible by *A. flavus* that may then utilised this nutrient faster therefore shorten the

period for primary metabolism before entering secondary metabolism (aflatoxin produce during secondary metabolism of mycotoxigenic fungi). This eventually increase the growth cycle of *A. flavus* that resulted in high aflatoxin production (Yazid *et al.*, 2018).

Although the indigenous non-aflatoxigenic *A. flavus* isolates could not strongly inhibit the growth of their aflatoxigenic counterparts, our findings showed that it could nevertheless significantly reduce AFB₁ production *in vitro*. Similar result was also reported by Rahman *et al.* (2022). The AFB₁ might have been highly reduced due to the thigmoregulation/touch inhibition mechanism, in which the reduction occurred because of physical contact of both non-aflatoxigenic and aflatoxigenic *A. flavus* isolates. Specifically, the physical contact mediated by ligand of both non-aflatoxigenic and aflatoxigenic *A. flavus* isolates prevented aflatoxin production by the aflatoxigenic *A. flavus* isolate (Ojiambo *et al.*, 2018; Huang *et al.*, 2011). In the present study, the contact occurred on all co-cultivated plates (Table 4.6) that although all non-aflatoxigenic *A. flavus* isolates mutually inhibited their aflatoxigenic counterparts as indicated by a mycelial separation, the hyphae continued to grow and touch one another in the middle.

From the biocontrol standpoint, effective selection of non-aflatoxigenic isolate as biocontrol candidate requires examination of several criteria, as listed by Moral *et al.* (2020). Among these criteria, we had only determined the mechanism of non-aflatoxigenicity of the selected indigenous *A. flavus* isolates, as well as their efficacy *in vitro* against their aflatoxigenic

counterparts. However, other criteria are also prerequisite prior to their commercialisation and wide applications. These include screening potential isolates for area-wide adaptation, and those obtained from a widely distributed non-aflatoxigenic vegetative compatibility group (VCG) to ensure genetic stability, and therefore limiting possibility of recombination. Besides, the selected isolate as biocontrol agent must also be tested widely and for multiple times in the targeted crop regions. These could be undertaken in the future work.

4.5 Conclusions

Based on molecular amplification of selected aflatoxin biosynthesis genes, aflatoxin analysis using HPLC, and phylogeny, the present Chapter confirmed the identity and the aflatoxigenicity of previously isolated indigenous *A. flavus* from two pioneer grain maize farms in Terengganu, Malaysia. Of the seven tested isolates, two were non-aflatoxigenic based on the amplification of only one (*i.e.*, *omtA*) over seven tested aflatoxin biosynthesis genes, and no production of aflatoxins B₁ and B₂ observed on both PDA and MEA. While, the remaining five isolates were aflatoxigenic based on the presence of all aflatoxin biosynthesis genes tested (*i.e.*, *pksA*, *nor-1*, *ver-1*, *verA*, *aflR*, *aflJ*, *omtA*), and the production of both aflatoxins B₁ and B₂ on the tested media. All isolates were also confirmed as *A. flavus* following phylogenetic analysis of the individual β -tubulin gene and the combined set of ITS and β -tubulin. The antagonistic potential of the two identified non-aflatoxigenic isolates was also obtained. The non-aflatoxigenic *A. flavus* isolates could highly reduce AFB₁

produced by two aflatoxigenic strains Af6KR and Af7KR tested on 3% semi-synthetic GMA, albeit having weak ability to inhibit their growth on the dual-culture assay based on the Percentage Inhibition of Radial Growth (PIRG%), interaction score, and Index of Dominance. The aflatoxigenic *A. flavus* strains Af6KR and Af7KR were further used for antagonistic study of the other potential indigenous antagonists by dual-culture assay in Chapter 5. While, the non-aflatoxigenic *A. flavus* strains Af1KD and Af5KD were further tested for aflatoxin reduction on 3% GMA and grain maize kernels using pathogen:antagonist ratio assay in Chapter 6.

CHAPTER 5

ANTAGONISTIC STUDY OF POTENTIAL BIOLOGICAL CONTROL AGENTS AGAINST ISOLATED INDIGENOUS MYCOTOXIGENIC FUNGI FROM GRAIN MAIZE AGRO-ECOSYSTEM

5.1 Introduction

To combat mycotoxigenic fungi and mycotoxin production, various control approaches have been employed including physical, chemical, and biological, as earlier mentioned. Of these, biocontrol is regarded as an alternative for the harmful usage of chemical pesticides, and the laborious physical control. At present, efforts on the development of new biocontrol agents are targeted on the species/strains obtained from the indigenous environmental niches of the fungal pathogens as they could be more effective than introducing exotic/foreign biocontrol agents. Among the advantages of using indigenous biocontrol agents is that they could reduce the risk of non-target effect, and preserve the ecological balance of the ecosystem (De Clerq *et al.*, 2011). Besides, indigenous biocontrol agents could also adapt better to the niche's microclimates which will assure their survival and efficacy (Moore, 2022).

Although the application of biocontrol agents against mycotoxigenic fungi in other parts of the world is already advanced, the same is still in infancy in Malaysia. Among the primary challenges in developing and applying biocontrol agents is ensuring mycotoxin inhibition instead of mycotoxin stimulation which is common among mycotoxigenic fungal species in the face of habitat/nutrient

competition with the applied biocontrol agents. The objectives of the present Chapter were: (1) to determine the antagonistic potential of biocontrol agent candidates (excluding non-aflatoxigenic *Aspergillus flavus*) against fungal growth of indigenous mycotoxigenic fungi on grain maize agar (*in vitro*) by dual-culture assay, and (2) to determine the antagonistic potential of biocontrol agent candidates (excluding non-aflatoxigenic *A. flavus*) against mycotoxin production of indigenous mycotoxigenic fungi on grain maize agar (*in vitro*) by high performance liquid chromatography. In the present Chapter, all mycotoxigenic isolates that could produce three important mycotoxins which have been classified as carcinogenic and placed in the Group 1 (*Aspergillus flavus* producing AFBs) and Group 2B (*A. niger* producing OTA; and *Fusarium verticillioides* and *F. proliferatum* producing fumonisins) by IARC were used. These mycotoxigenic isolates were the indigenous isolates obtained from Kampong Dadong and Rhu Tapai grain maize farms in Terengganu, Malaysia in Chapter 3, as well as the indigenous aflatoxigenic *A. flavus* that have been validated by molecular and aflatoxigenicity test in Chapter 4.

5.2 Materials and methods

5.2.1 Mycotoxigenic fungi

Seven mycotoxigenic fungal isolates were assessed in the present Chapter; two *A. flavus* isolates producing AFB₁ (strains Af6KR and Af7KR), two *A. niger* isolates producing OTA (strains An1KD and An3KD), two *Fusarium verticillioides* isolates producing FB₁ and FB₂ (strains Fv21KD and Fv92SR),

and one *F. proliferatum* isolate producing FB₁ and FB₂ (strain Fp9DD). These isolates were among the highest mycotoxin-producer assessed in Chapter 3 and 4. All fungal isolates were maintained on PDA, and incubated at 30°C for seven days prior to the dual-culture assay.

5.2.2 *In silico* selection of antagonist fungi

Among 30 fungal species isolated and identified from the pioneer grain maize farms in Terengganu, Malaysia (Chapter 3), only 22 species were further screened and assessed *in silico* using established scientific databases (Google Scholar, ScienceDirect, Scopus) and published reports to select for suitable potential biocontrol agents, and to avoid the inclusion of risky isolates in the development and commercialisation of biocontrol agents. The remaining eight species that were not included in this screening were all mycotoxigenic fungi (five species), and three other fungal species, namely *Penicillium charlesii*, *P. daleae*, and *Talaromyces radicus*, that could not be revive even after multiple attempts.

The complete stepwise screening procedures in the development of a novel biocontrol agents were outlined by Köhl *et al.* (2011). Inclusion screening criteria included reported antagonistic activity against pathogenic fungi or mycotoxigenic fungi. Exclusion screening criteria included mycotoxigenic fungi and pathogenic fungi infecting animals, humans, or plants. The search keywords included “mycotoxin”, “mycotoxigenic fungi”, “corn/maize pathogen”, “corn/maize disease”, “animal pathogen”, “plant pathogen”, “human

pathogen”, “antagonist”, and “biological control/biocontrol”. For maize pathogens, a reference list by Munkvold (2017) was also used. Following *in silico* screening, fungal species that matched with the selection criteria were selected and used in the dual-culture assay against the mycotoxigenic fungal species.

5.2.3 Preparation of grain maize agar as semi-synthetic growth medium for dual-culture assay

A 3% semi-synthetic GMA was prepared as described in sub-Section 3.2.4.

5.2.4 Dual-culture assay

Dual-culture assay of both mycotoxigenic fungi and antagonist candidates and the Percentage Inhibition of Radial Growth (PIRG, %) were performed as described in sub-Section 4.2.4, and the types of interaction, also the Index of Dominance (I_D) were scored as described in Table 4.3.

5.2.5 Quantification of aflatoxin B₁ inhibition using high performance liquid chromatography

Aflatoxin B₁ extraction and quantification from the GMA were performed as described in sub-Section 3.2.5.1.

5.2.6 Quantification of ochratoxin A inhibition using high performance liquid chromatography

Ochratoxin A extraction and quantification from the GMA were performed described in sub-Section 3.2.5.2.

5.2.7 Quantification of fumonisin inhibition using high performance liquid chromatography

Fumonisin B₁ and B₂ extraction, derivatisation, and quantification from the GMA were performed as described in sub-Section 3.2.5.3.

Preparation of solvents for mobile phases, mycotoxin separation, and data processing were performed as described in sub-Section 3.2.5. The percentage of mycotoxin inhibition was calculated based on the Equation 5.1.

$$\% \text{Mycotoxin Inhibition} = \frac{B^0 - B^1}{B^0} \times 100$$

Equation 5.1: Formula for percentage of mycotoxin inhibition

Where, B⁰ was the concentration of mycotoxin in control plate, and B¹ was the concentration of mycotoxin in treatment plate (Rahman *et al.*, 2022). Data of % inhibition were included in Appendix T.

5.2.8 Statistical analysis

All experiments were conducted in four replicates ($n = 4$). Data of PIRG (%) were then averaged and presented as mean $\pm$ SE (standard error). Data of multi-mycotoxin production following dual-culture assay were tested for normality based on Kolmogorov-Smirnov and Shapiro-Wilk. All data violated the normality assumption (Appendix S), even after transformation. Therefore, non-parametric Kruskal-Wallis was employed to compare the distribution between groups for each mycotoxigenic isolates tested. All analyses were statistically significant at $p < 0.05$. The subsequent *post hoc* Dunn's multiple comparisons were conducted, and each resulting p -values were corrected with Benjamini-Hochberg False Discover Rate (FDR). The mycotoxin concentration between pairs were considered as statistically significant based on the corrected p -value at < 0.05 . Data of multi-mycotoxin were reported in median with interquartile range (IQR) as error bars. The statistical software SPSS version 29.0 was used to perform the analysis.

Figure 5.1 summarise the methodology used in the present Chapter.

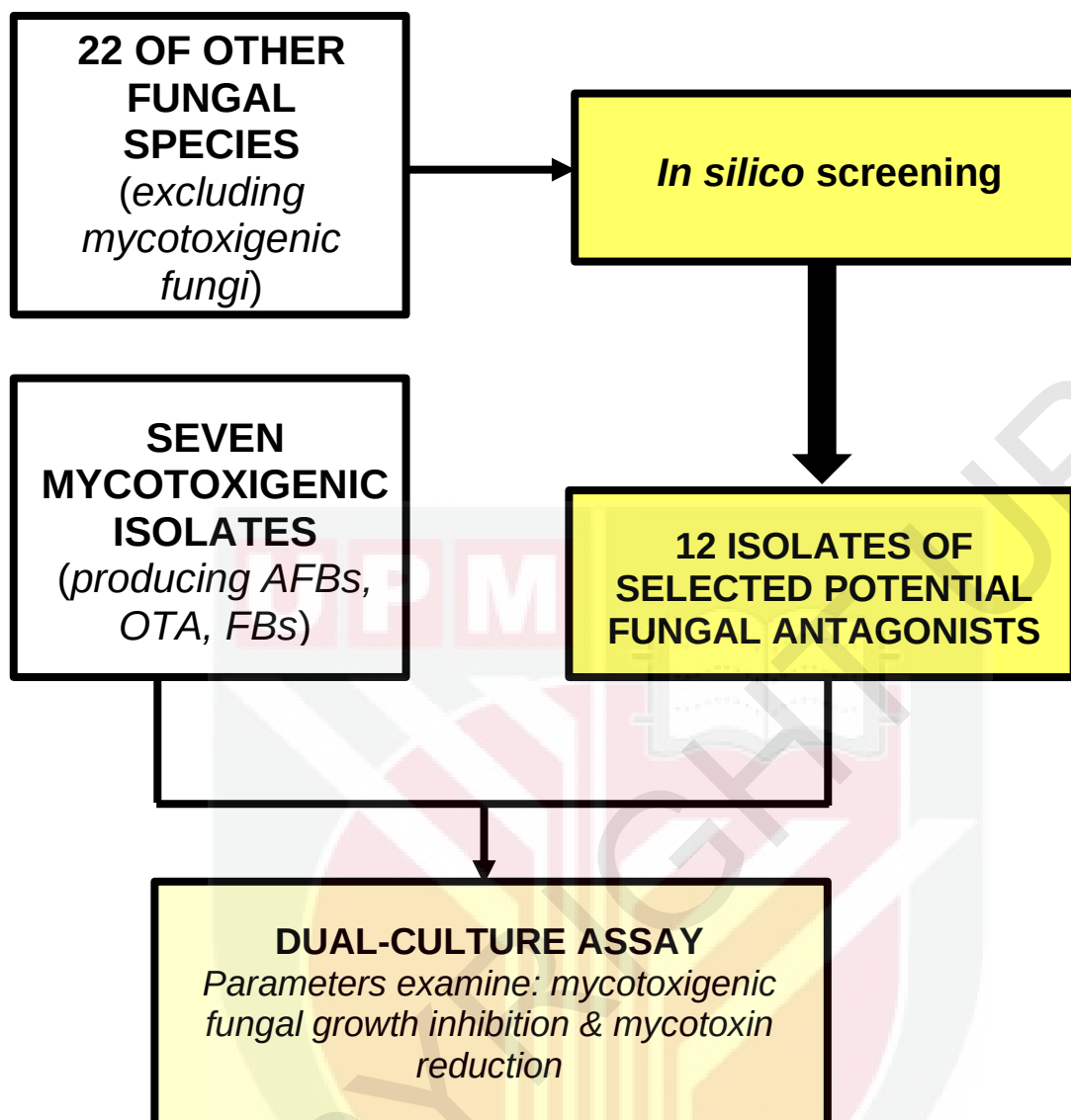


Figure 5.1: Flow diagram of the methodology used in Chapter 5

5.3 Results

5.3.1 Antagonist candidates

Table 5.1 shows the fungal isolates obtained from grain maize farms in Terengganu, Malaysia, and the rationale for their selection or rejection based on *in silico* screening as antagonist candidates for dual-culture assay. Only eight species (12 isolates) matched with the selection criteria *i.e.*, neither a plant, human, or animal pathogen, nor a major mycotoxigenic fungal species. These antagonist candidates were *Bjerkandera adusta* Ba42DD, *Penicillium janthinellum* strains Pj46SD and Pj81SR, *Schizophyllum commune* Sc40DD, *Trametes cubensis* Tc28DD, *Trichoderma asperelloides* Ta39KR, *T. asperellum* Ta31KR, *T. harzianum* strains Th33SR, Th20SR, Th24KD, and Th36SR, and *T. yunnanense* Ty34KD. Unlike *A. flavus*, all 12 isolates were not a species having both mycotoxigenic and non-mycotoxigenic strains. Besides, these 12 isolates are not major mycotoxin producer. Major mycotoxigenic fungal producer in this context is define as species that often contaminating economically relevant food and feed products (Pitt, 2000), and their mycotoxins can be carcinogenic for which regulations have been prescribed all over the world (Magan *et al.*, 2020; Ostry *et al.* 2017). Therefore, the identification and amplification of their mycotoxin biosynthetic genes in the present study is not necessary.

Table 5.1: Indigenous fungal isolates obtained from grain maize farms in Terengganu, Malaysia, and rationale for selection or rejection from *in silico* screening as antagonist candidates for dual-culture assay

Isolate ^a	GenBank accession no.	No of isolate ^c	Isolate code ^c	Rationale for selection or rejection	Reference
<i>Aspergillus aculeatus</i>	MW542998			Potential mycotoxigenic ^d : secalonic acid	Lamboni et al. (2016)
<i>Bjerkandera adusta</i>	MW543001	1	Ba42DD	Potential antagonist	Feng et al. (2021)
<i>Curvularia</i> sp.	MW543002			Maize pathogen	Munkvold (2017)
<i>Fusarium longipes</i>	MW543004			Maize pathogen	Han et al. (2021)
<i>Lasiodiplodia theobromae</i>	MW543007			Maize pathogen	Munkvold (2017)
<i>Neosartorya fischeri</i> ^e	MW543008			Human pathogen	Sugui et al. (2015)
<i>Penicillium</i> sp.	MW543009			Potential maize pathogen	Munkvold (2017)
<i>Penicillium</i> sp.	MW543014			Potential maize pathogen	Munkvold (2017)
<i>P. citreonigrum</i>	MW543011			Potential mycotoxigenic ^d : citreoviridin	Mohammadi Shad et al. (2022)
<i>P. janthinellum</i>	MW543013	2	Pj46SD, Pj81SR	Potential antagonist	Liu et al. (2021)
<i>P. polonicum</i>	MW543015			Plant pathogen, Potential mycotoxigenic ^d : penicillic acid	Bradshaw et al. (2021), Frisvad et al. (2004)
<i>P. rubens</i>	MW543016			Potential maize pathogen	Munkvold (2017)
<i>Phomopsis</i> sp.	MW543017			Plant pathogen	Udayanga et al. (2011)
<i>Pyrthoderma noxium</i>	MW543018			Plant pathogen	Leung et al. (2020)

Table 5.1: Continued

<i>Schizophyllum commune</i>	MW543019	1	Sc40DD	Potential antagonist	Puig & Cumagun (2019)
<i>Talaromyces islandicus</i>	MW543020			Potential mycotoxigenic ^d : cyclochlorotine	Schafhauser <i>et al.</i> (2016)
<i>T. purpureogenus</i>	MW543022			Potential mycotoxigenic ^d : luteoskyrin	Yilmaz <i>et al.</i> (2012)
<i>Trametes cubensis</i>	MW543023	1	Tc28DD	None^f	-
<i>Trichoderma asperelloides</i>	MW543024	1	Ta39KR	Potential antagonist	Ruangwong <i>et al.</i> (2021)
<i>T. asperellum</i>	MW543025	1	Ta31KR	Potential antagonist	Wang <i>et al.</i> (2021)
			Th33SR, Th20SR, Th24KD, Th36SR		
<i>T. harzianum</i>	MW543026	4		Potential antagonist	Allaga <i>et al.</i> (2021)
<i>T. yunnanense</i>	MW543027	1	Ty34KD	Potential antagonist	Karmakar <i>et al.</i> (2021)

^aFungal species in bold were selected as antagonist candidates for the dual-culture assay.

^bComplete GenBank accession no. have been reported in Chapter 3.

^cThe number of isolates and the isolate code were only presented for the selected antagonist candidates.

^dMycotoxin produced by the mycotoxigenic fungal species listed in the International Agency for Research on Cancer (IARC) in Groups 1 – 3, or which has been reported toxic to human (citreoviridin).

^e*N. fischeri* is the closely related species to *Aspergillus fumigatus*, a common human pathogen.

^fNo reported data as a plant, human, or animal pathogen, or a mycotoxigenic fungal species.

5.3.2 Growth inhibition of mycotoxigenic fungal isolates co-cultivated with antagonist candidates in dual-culture assay

The growth inhibition of the mycotoxigenic fungal isolates co-cultivated with the antagonist candidates were quantified by (a) Percentage Inhibition of Radial Growth (PIRG; %), and (b) Index of Dominance (I_D). Table 5.2 shows the PIRG (%) of seven mycotoxigenic fungal isolates co-cultivated with antagonist candidates. The PIRG (%) shows varying trends of radial growth inhibition of the mycotoxigenic fungal isolates, which can be divided into three classes: high, medium, or weak antagonists (Soytong & Quimio, 1989). Overall, high antagonistic activity ($\geq 80\%$) was shown by all *Trichoderma* spp. against all mycotoxigenic fungal isolates, except for *T. harzianum* Th33SR against *A. flavus*, of which the PIRG was moderate (60–79%). Besides, *B. adusta* Ba42DD also showed high antagonistic activity against *A. flavus*, while *T. cubensis* Tc28DD showed high antagonistic activity against *F. verticillioides* and *F. proliferatum*. Further, *B. adusta* Ba42DD and *S. commune* Sc40DD showed moderate antagonistic activity against both *F. verticillioides* and *F. proliferatum*, while *P. janthinellum* Pj46SD and Pj81SR showed moderate antagonistic activity against *A. flavus* Af6KR. Others were mostly weak antagonists ($\leq 59\%$).

Table 5.2: Percentage Inhibition of Radial Growth (PIRG, %) of seven mycotoxigenic fungal isolates co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia

Antagonist candidate	Percentage Inhibition of Radial Growth (PIRG, %)						
	Af6KR	Af7KR	An1KD	An3KD	Fp9DD	Fv21KD	Fv92SR
Ba42DD	83 ± 3.3	91 ± 1.4	33 ± 1.3	40 ± 3.9	78 ± 1.1	74 ± 6.8	62 ± 1.2
Pj46SD	67 ± 4.6	46 ± 5.2	0.7 ± 0.7	10 ± 8.1	38 ± 1.2	36 ± 1.0	45 ± 0.4
Pj81SR	64 ± 1.7	63 ± 1.3	12 ± 1.7	19 ± 1.9	38 ± 2.3	42 ± 1.8	46 ± 1.0
Sc40DD	52 ± 2.4	59 ± 1.9	34 ± 2.2	37 ± 2.3	63 ± 3.5	65 ± 1.3	71 ± 1.0
Tc28DD	45 ± 7.0	44 ± 1.3	26 ± 10.8	27 ± 8.2	87 ± 5.3	83 ± 2.5	92 ± 1.1
Ta39KR	100 ± 0	100 ± 0	100 ± 0	97 ± 3.4	100 ± 0	97 ± 2.8	99 ± 1.2
Ta31KR	98 ± 1.7	99 ± 1.1	90 ± 3.5	96 ± 4.0	100 ± 0	94 ± 1.3	98 ± 1.6
Th20SR	91 ± 3.0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Th24KD	87 ± 3.0	88 ± 2.3	100 ± 0	100 ± 0	99 ± 1.3	100 ± 0	100 ± 0
Th33SR	73 ± 3.8	92 ± 4.1	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Th36SR	85 ± 1.6	91 ± 2.2	94 ± 5.5	96 ± 4.0	100 ± 0	100 ± 0	100 ± 0
Ty34KD	87 ± 2.2	81 ± 1.1	85 ± 1.5	83 ± 0.7	93 ± 2.8	99 ± 0.4	100 ± 0

Data are means of four replicates ($n = 4$), and expressed as mean $\pm$ standard error ($\pm$ SE). Mycotoxigenic fungal isolates: Af6KR, Af7KR – *Aspergillus flavus*; An1KD, An3KD – *A. niger*; Fp9DD – *Fusarium proliferatum* strain; Fv21KD, Fv92SR – *F. verticillioides*. Antagonist candidates: Ba42DD – *Bjerkandera adusta*; Pj46SD, Pj81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*.

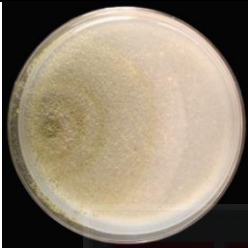
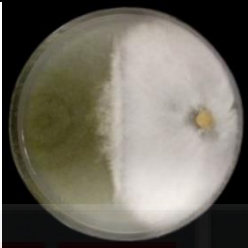
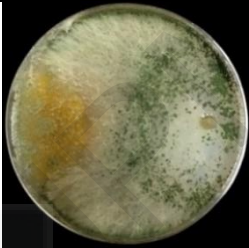
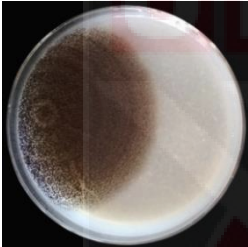
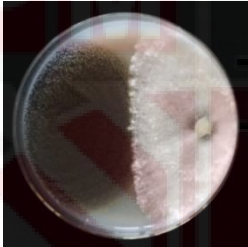
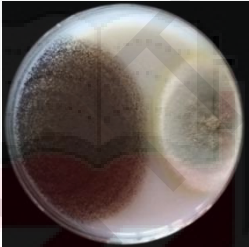
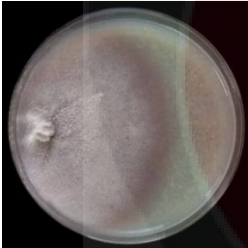
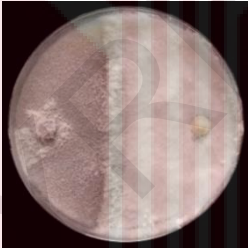
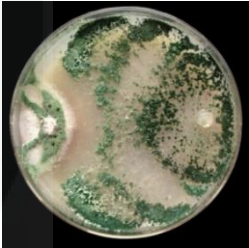
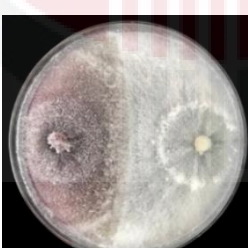
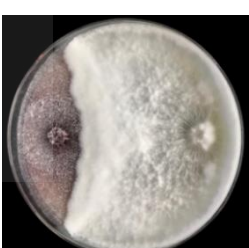
Table 5.3 shows the interaction scores and Index of Dominance (I_D) between seven mycotoxigenic fungal isolates and antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Meanwhile, Table 5.4 shows the visual fungal interaction *in vitro* of co-cultivated antagonists and mycotoxigenic isolates on 3% GMA and isolated from the grain maize farms in Terengganu, Malaysia. Interaction scores and the subsequent I_D utilise numerical notation to indicate the nature of interaction between the antagonist candidates and the mycotoxigenic isolates, as well as the possible mechanism

Table 5.3: Interaction scores and Index of Dominance (I_D) of seven mycotoxigenic fungal isolates co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia

Antagonist candidate	Mycotoxigenic fungal isolate							I_D
	Af6KR	Af7KR	An1KD	An3KD	Fp9DD	Fv21KD	Fv92SR	
Ba42DD	4/0	4/0	1/1	1/1	4/0	4/0	4/0	22/2
Pj46SD	1/1	2/2	2/2	2/2	2/2	2/2	2/2	13/13
Pj81SR	1/1	2/2	2/2	2/2	2/2	2/2	2/2	13/13
Sc40DD	1/1	1/1	1/1	1/1	1/1	1/1	4/0	10/6
Tc28DD	4/0	4/0	1/1	1/1	4/0	4/0	4/0	22/2
Ta39KR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0
Ta31KR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0
Th20SR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0
Th24KD	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0
Th33SR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0
Th36SR	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0
Ty34KD	4/0	4/0	4/0	4/0	4/0	4/0	4/0	28/0

Mycotoxigenic fungal isolates: Af6KR, Af7KR – *Aspergillus flavus*; An1KD, An3KD – *A. niger*; Fp9DD – *Fusarium proliferatum* strain; Fv21KD, Fv92SR – *F. verticillioides*. Antagonist candidates: Ba42DD – *Bjerkandera adusta*; Pj46SD, Pj81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. The first integer of the interaction score indicates the numerical score for potential antagonists, while the second integer indicates the numerical score for mycotoxigenic fungi. Bold I_D value indicates the highest I_D scores exhibited by the antagonist candidates.

Table 5.4: Visual fungal interaction *in vitro* of co-cultivated antagonists (right) and mycotoxigenic isolates (left) on 3% grain maize agar and isolated from grain maize farms in Terengganu, Malaysia

Mycotoxigenic isolate (Control)	Interaction score		
	1/1 Antagonist	2/2 Antagonist	4/0 Antagonist
 <i>Aspergillus flavus</i>	 Sc40DD	 Pj46SD	 Th24KD
 <i>A. niger</i>	 Sc40DD	 Pj81SR	 Ty34KD
 <i>Fusarium proliferatum</i>	 Sc40DD	 Pj81SR	 Ta39KR
 <i>F. verticillioides</i>	 Sc40DD	 Pj46SD	 Tc28DD

Antagonist candidates: Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Th24KD – *T. harzianum*; Ty34KD – *T. yunnanense*.

of antagonism (Magan & Lacey, 1984). Antagonists are deemed highly competitive and become dominant when they score a 4/0 or 5/0 against the mycotoxigenic species, and not as competitive when they score a 1/1, 2/2, or 3/3 (sub-Section 4.2.4, Table 4.3). The 4/0 score indicates strong inhibition of the antagonist against the mycotoxigenic species “upon contact” which could mean that the former is a mycoparasite, and the possible mechanism of antagonism is the production of cell wall-degrading enzymes that could inhibit the mycotoxigenic fungal growth. The 5/0 score indicates strong inhibition of the antagonist against the mycotoxigenic species “at a distance” which could mean that the possible mechanism of antagonism is the production of antifungal compounds by the antagonist that could inhibit the pathogenic growth. Taken together (*i.e.*, cumulative of different interaction scores from similar antagonist against mycotoxigenic species), these interaction scores provide the Index of Dominance (I_D) in which the higher the I_D , the higher the antagonism potential of an antagonist candidate. Overall, *Trichoderma* spp. exhibited the highest I_D , while *S. commune* Sc40DD exhibited the lowest. The antagonist candidates *Trichoderma* spp. against all tested mycotoxigenic fungal isolates, *B. adusta* Ba42DD against *A. flavus*, *F. proliferatum*, and *F. verticillioides* isolates, and *T. cubensis* Tc28DD against *A. flavus*, *F. proliferatum*, and *F. verticillioides* isolates all showed strong antagonism upon contact (4/0), while *S. commune* Sc40DD was only able to strongly antagonise one mycotoxigenic fungal isolate (*F. verticillioides* Fv92SR). Other antagonist candidates were less aggressive upon contact (< 2 mm zone of inhibition; *P. janthinellum* Pj46SD and Pj81SR), or did not show antagonism at all (1/1).

None of the antagonist candidates could inhibit mycotoxigenic fungal isolates at a distance (5/0).

5.3.3 Multi-mycotoxin reduction of mycotoxigenic fungal isolates co-cultivated with antagonist candidates in dual-culture assay

Following the dual-culture assay, all antagonist candidates were tested for their ability to reduce mycotoxins produced by each of the tested mycotoxigenic fungal isolates using HPLC. Overall, all antagonist candidates did not show consistent pattern of mycotoxin reduction. Although most of the antagonist candidates could reduce the tested mycotoxins, some candidates could only reduce the mycotoxin in moderate amounts, while some even induced/stimulated mycotoxin production, which is a poor trait for a potential biocontrol agent. Therefore, the best antagonist candidates were selected based on their ability to reduce the highest amount of individual mycotoxin produced by all the tested mycotoxigenic fungal isolates. For example, the best antagonist candidate against two tested aflatoxigenic *A. flavus* isolates should be able to reduce AFB₁ in both isolates, instead of in only one isolate.

Although AFB₂ was produced and detected by one isolate of *A. flavus* on the 3% GMA (Chapter 3), this aflatoxin analogue could not be quantified from any of the seven tested isolates, despite multiple attempts. Thus, *A. flavus* strains Af6KR and Af7KR were only examined for the AFB₁ reduction. Figure 5.2 shows the AFB₁ production (ng/g) by two aflatoxigenic *A. flavus* isolates co-

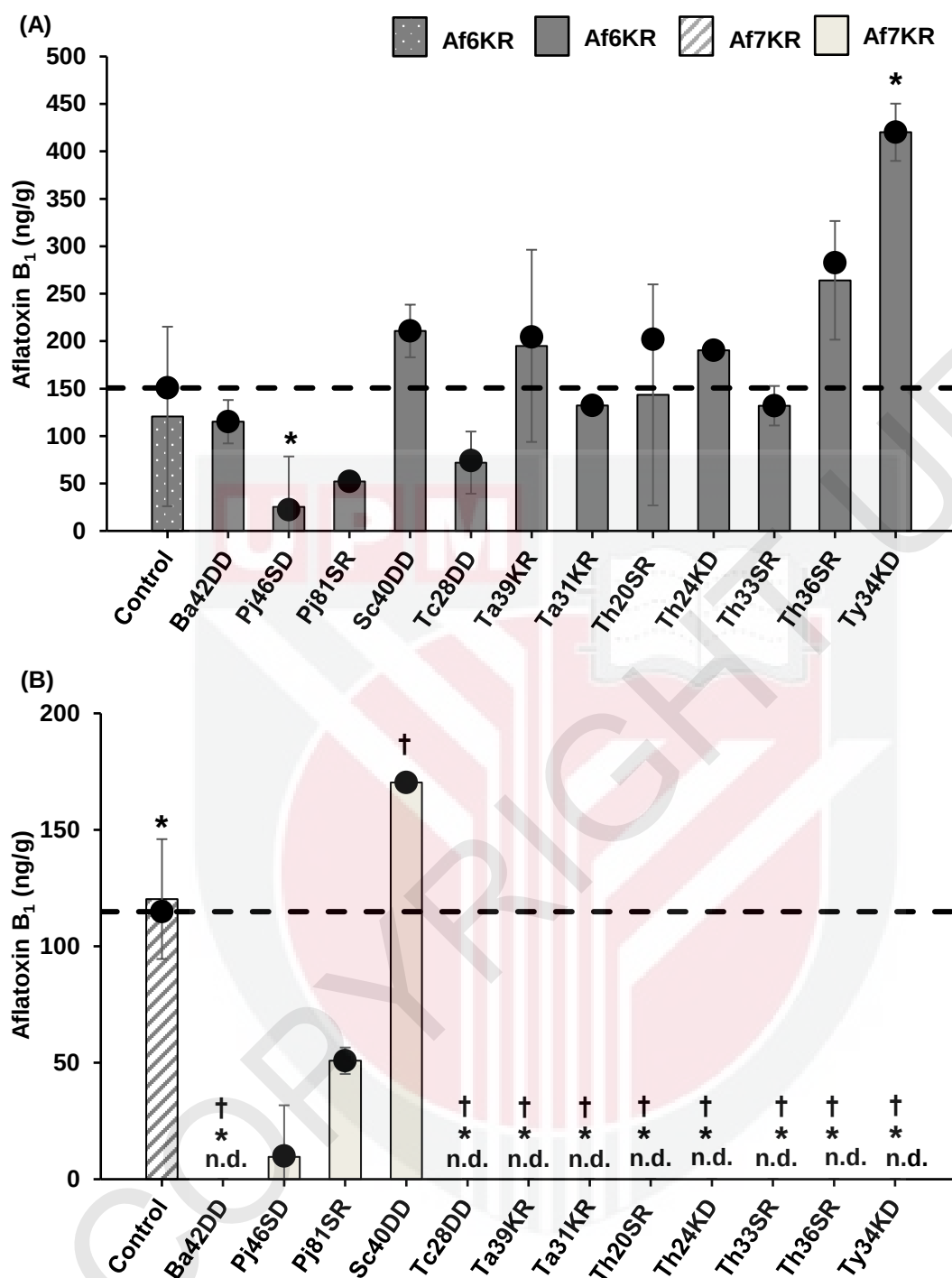


Figure 5.2: Aflatoxin B₁ (AFB₁; ng/g) produced by *Aspergillus flavus* strains (A) Af6KR and (B) Af7KR co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. Data are median $\pm$ IQR. Black circles indicate the means of AFB₁ concentration of both control (mycotoxigenic isolate) and treatments. Dashed line is the cut-off point (based on the control value) by which AFB₁ is considered as inhibited (black circles below line) or

stimulated (black circles above line) by the antagonists. Asterisk (*) and dagger (†) indicate statistical significance at a corrected p -value < 0.05 following *post hoc* Dunn's multiple comparisons with Benjamini-Hochberg FDR correction. Symbol for bars in (A) – *statistically significant between each other. Symbol for bars in (B) – *statistically significant as compared to control, †statistically significant as compared to Sc40DD. n.d. – not detected

cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. No significant difference was observed between the production of AFB₁ by Af6KR co-cultivated with all antagonists against control. Nevertheless, several antagonist candidates could reduce the AFB₁ production by strain Af6KR by more than 50% (Appendix T). Against *A. flavus* strain Af7KR, most antagonists could significantly reduce the AFB₁ production by approximately 100%. For both tested aflatoxigenic *A. flavus* isolates, three antagonist candidates that could reduce the highest amount of AFB₁ were *P. janthinellum* Pj46SD (median; 50.6 and 9.6 ng/g), *T. cubensis* Tc28DD (median; 72 and 0 ng/g), and *B. adusta* Ba42DD (median; 115.1 and 0 ng/g) by approximately 75% and 89%, 50% and 100%, and 24% and 100% (Appendix T), respectively. In contrast, *S. commune* Sc40DD and almost all *Trichoderma* spp. stimulated AFB₁ production by the *A. flavus* isolates.

Figure 5.3 shows the OTA production (ng/g) by two *A. niger* isolates co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Similarly, no significant difference was observed between OTA production of all co-cultivated *A. niger* An1KD with antagonists, against control. Nevertheless, six antagonist isolates could reduce OTA production by An1KD at $> 80\%$ (Appendix T). In contrast, OTA production by *A. niger* strain An3KD was significantly reduced by five *Trichoderma* spp. The

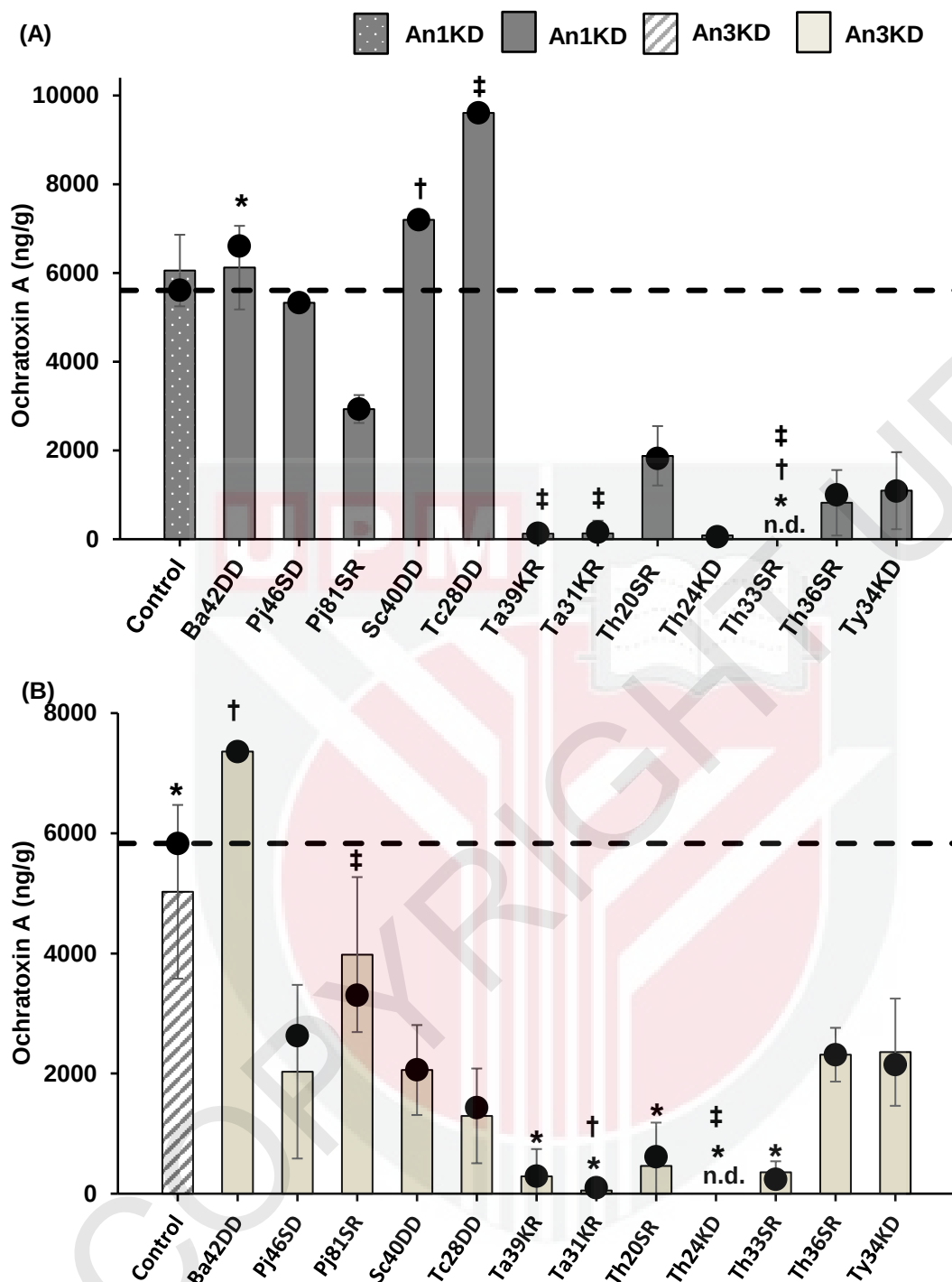


Figure 5.3: Ochratoxin A (OTA; ng/g) produced by *Aspergillus niger* strains (A) An1KD and (B) An3KD co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. Data are median ± IQR. Black circles indicate the means of OTA concentration of both control (mycotoxigenic isolate) and treatments. Dashed line is the cut-off point (based on the control value) by which OTA is considered as inhibited (black circles below line) or

stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), and diesis (§) indicate statistical significance at a corrected *p*-value < 0.05 following *post hoc* Dunn's multiple comparisons with Benjamini-Hochberg FDR correction. Symbol for bars in (A) – *statistically significant between each other, †statistically significant between each other, §statistically significant as compared to Tc28DD. Symbol for bars in (B) – *statistically significant as compared to control, †statistically significant between each other, §statistically significant between each other. n.d. – not detected

three best antagonist candidates that could reduce the highest amount of OTA were *T. harzianum* Th24KD (median; 83.2 and 0 ng/g), *T. harzianum* Th33SR (median; 0 and 356.8 ng/g), and *T. asperellum* Ta31KR (median; 130.3 and 53.7 ng/g) by approximately 98% and 100%, 100% and 95%, and 97% and 98% (Appendix T), respectively. In contrast, the basidiomycete *B. adusta* Ba42DD, *S. commune* Sc40DD, and *T. cubensis* Tc28DD stimulated OTA production by the *A. niger* isolates.

Figures 5.4 and 5.5 show the FB₁ and FB₂ production (µg/g) by two *F. verticillioides* isolates co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Against *F. verticillioides* strain Fv21KD, three *T. harzianum* isolates could significantly reduce FB₁ and FB₂ production. These antagonists were *T. harzianum* strain Th20SR that could significantly reduce both FB₁ and FB₂, strain Th24KD that could significantly reduce FB₁, and strain Th33SR that could significantly reduce FB₂. Besides, the FB₁ concentration produced by Fv21KD against *T. harzianum* Th20SR and Th24KD were significantly lower than those against the antagonists *P. janthinellum* isolates and *T. cubensis* Tc28DD. Against *F. verticillioides* strain Fv92SR, almost all antagonists could significantly reduce the FB₂ production. However, no significant difference was observed between the FB₁ production

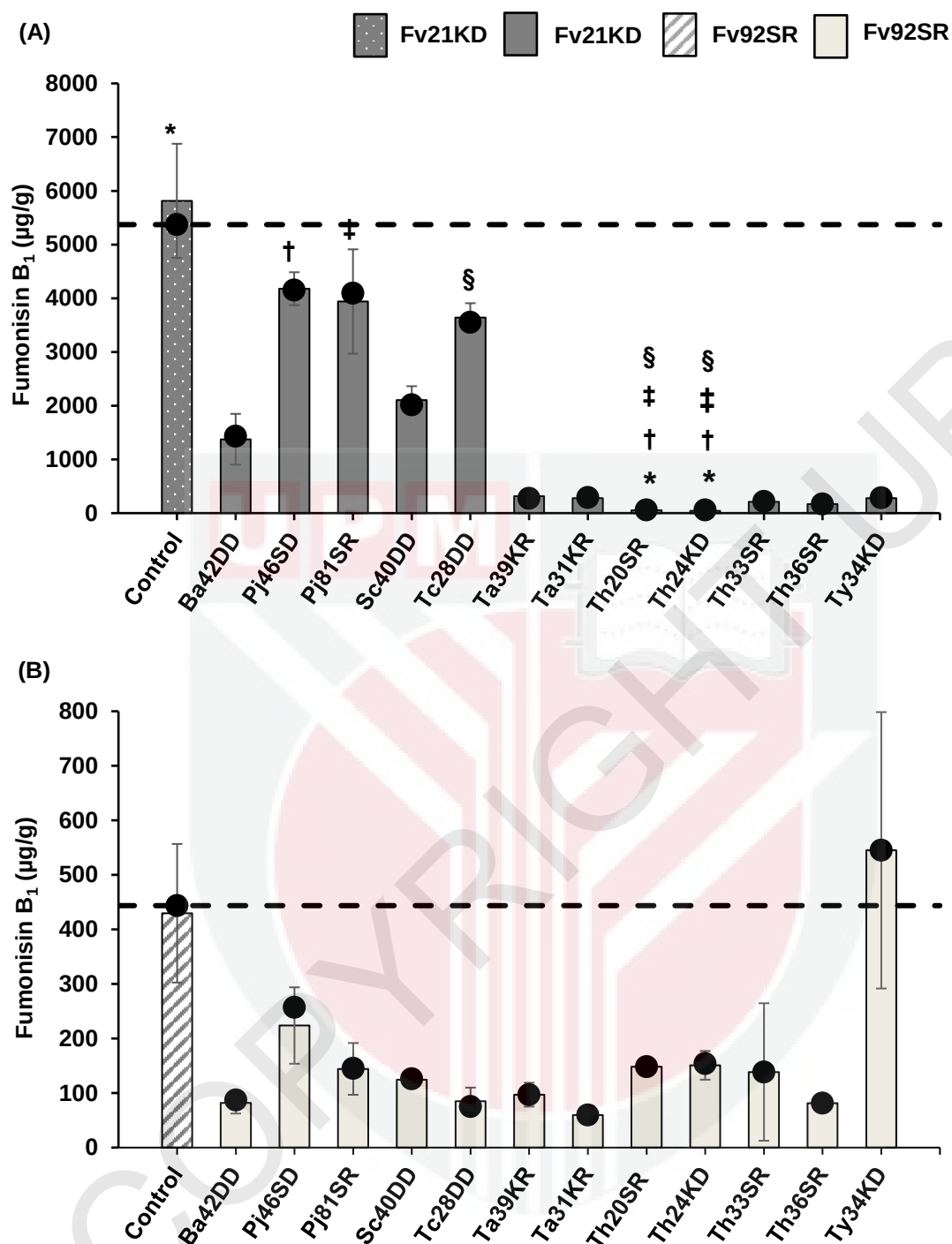


Figure 5.4: Fumonisin B₁ (FB₁; µg/g) produced by *Fusarium verticillioides* strains (A) Fv21KD and (B) Fv92SR co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. Data are median ± IQR. Black circles indicate the means of FB₁ concentration of both control (mycotoxigenic isolate) and treatments. Dashed line is the cut-off point (based on the control value) by which FB₁ is considered as inhibited (black circles below line) or

stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), diesis (§), and silcrow (§) indicate statistical significance at a corrected p -value < 0.05 following *post hoc* Dunn's multiple comparisons following Benjamini-Hochberg FDR correction. Symbol for bars in (A) – *statistically significant as compared to control, †statistically significant as compared to Pj46SD, ‡statistically significant as compared to Pj81SR, §statistically significant as compared to Tc28DD. Bars in (B) are not statistically significant to each other. n.d. – not detected



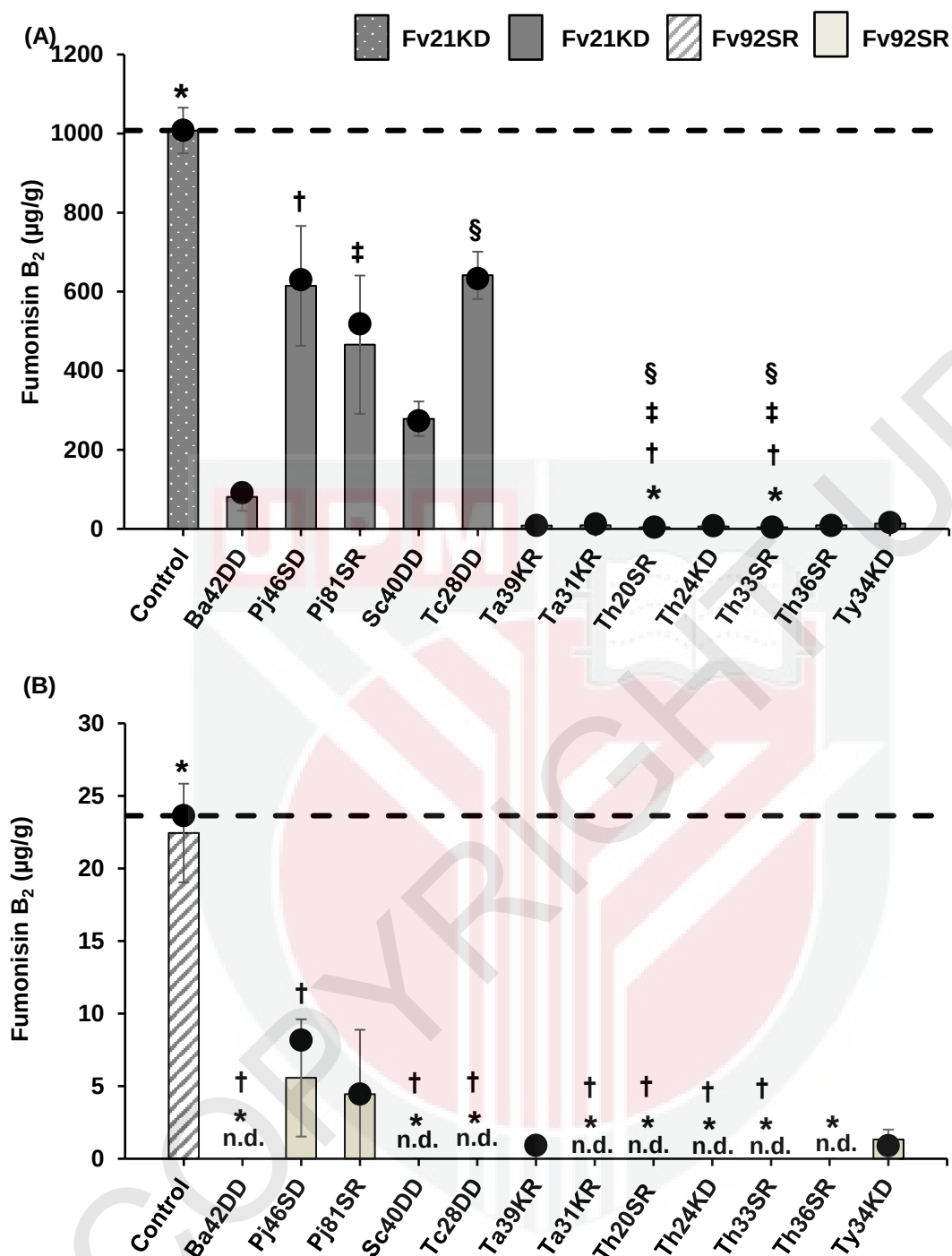


Figure 5.5: Fumonisin B₂ (FB₂; µg/g) produced by *Fusarium verticillioides* strains (A) Fv21KD and (B) Fv92SR co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. Data are median ± IQR. Black circles indicate the means of FB₂ concentration of both control (mycotoxigenic isolate) and treatments. Dashed line is the cut-off point (based on the control value) by which FB₂ is considered as inhibited (black circles below line) or

stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), diesis (§), and silcrow (§) indicate statistical significance at a corrected *p*-value < 0.05 following *post hoc* Dunn's multiple comparisons with Benjamini-Hochberg FDR correction. Symbol for bars in (A) – *statistically significant as compared to control, †statistically significant as compared to Pj46SD, ‡statistically significant as compared to Pj81SR, §statistically significant as compared to Tc28DD. Symbol for bars in (B) – *statistically significant as compared to control, †statistically significant as compared to Pj46SD. n.d. – not detected

by Fv92SR control strain with all other isolates co-cultivated with antagonist candidates. Nevertheless, these antagonists could reduce FB₂ production by Fv92SR to approximately 86% (Appendix T). *T. asperellum* Ta31KR, *T. harzianum* Th36SR, and *T. asperelloides* Ta39KR could reduce the highest amount of FB₁ production (median; 278.1 and 59.4; 166.2 and 81.2; 317.5 and 97 µg/g, respectively) by *F. verticillioides* by approximately 94% and 86%, 86% and 81%, and 94% and 78%, respectively. *T. harzianum* Th20SR, Th33SR, and Th24KD could reduce FB₂ production by *F. verticillioides* at almost 100% (median; 3.8 and 0; 3.8 and 0; 5.9 and 0 µg/g, respectively). In contrast, *T. yunnanense* Ty34KD stimulated FB₁ production by the *F. verticillioides*.

Figure 5.6 shows the FB₁ and FB₂ production (µg/g) by *F. proliferatum* co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia. Four *Trichoderma* spp. could significantly reduce FB₁ and FB₂ produced by the mycotoxigenic isolate. These antagonists were *T. harzianum* Th20SR that could reduce both FB₁ and FB₂, *T. asperelloides* Ta39KR that could reduce FB₁, and *T. harzianum* strains Th24KD and Th36SR that could reduce FB₂. *T. harzianum* Th20SR could also significantly reduce FB₂ by *F. proliferatum* as compared to *S. commune* Sc40DD. The fungal

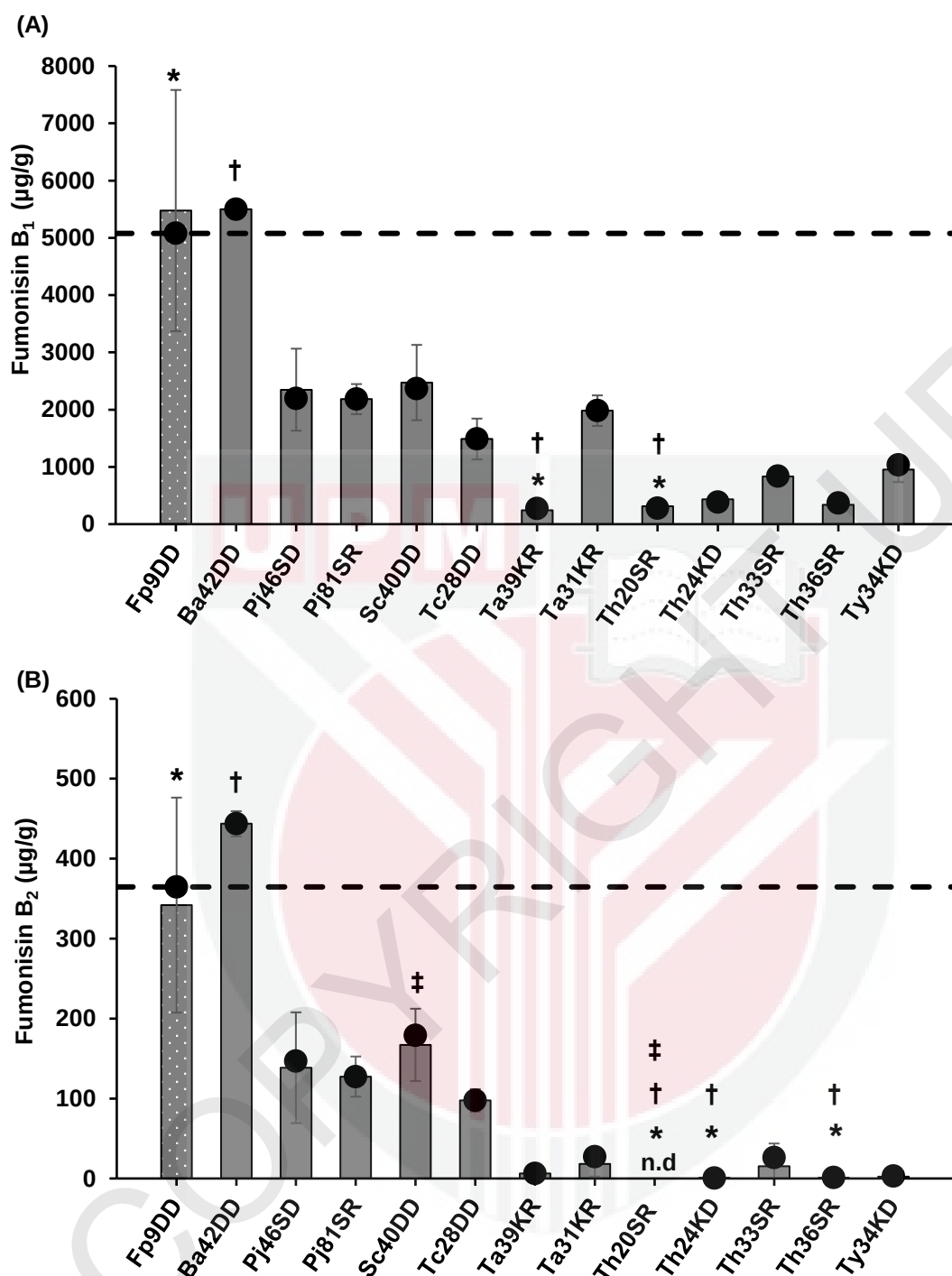


Figure 5.6: (A) Fumonisin B₁ (FB₁, µg/g) and (B) fumonisin B₂ (FB₂, µg/g) produced by *Fusarium proliferatum* strain Fp9DD co-cultivated with antagonist candidates isolated from grain maize farms in Terengganu, Malaysia. Ba42DD – *Bjerkandera adusta*; Pj46SD, PJ81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. Data are median $\pm$ IQR. Black circles indicate the means of FB₁ and FB₂ concentration of both control (mycotoxigenic isolate) and treatments. Dashed line is the cut-off point (based on the control value) by which FB₁ and FB₂ are considered as inhibited (black circles below

line) or stimulated (black circles above line) by the antagonists. Asterisk (*), dagger (†), and diesis (§) indicate statistical significance at a corrected p -value < 0.05 following *post hoc* Dunn's multiple comparisons with Benjamini-Hochberg FDR correction. Symbol for bars in (A) – *statistically significant as compared to control, †statistically significant as compared to Ba42DD. Symbol for bars in (B) – *statistically significant as compared to control, †statistically significant as compared to Ba42DD, ‡statistically significant between each other. n.d. – not detected

antagonists that reduced FB₁ produced by *F. proliferatum* in high amount were *T. asperelloides* Ta39KR (median; 244.2 µg/g), *T. harzianum* Th20SR (median; 314.2 µg/g), and Th36SR (median; 337.9 µg/g), by approximately 94%, 94%, and 92%. The antagonist candidates that could reduce FB₂ in high amount were *T. harzianum* Th20SR (median; 0 µg/g), Th36SR (median; 0.9 µg/g), and Th24KD (median; 1 µg/g), by approximately 100%, 99%, and 99%, respectively (Appendix T). In contrast, *B. adusta* Ba42DD stimulated the fumonisin production by *F. proliferatum*.

5.4 Discussion

In the present Chapter, fungal isolates indigenous to maize grain maize farms in Terengganu, Malaysia that were neither pathogenic nor mycotoxigenic were screened for their potentials to inhibit mycotoxigenic fungi and reduce mycotoxin production *in vitro* using the dual-culture assay on GMA. In developing potential biocontrol agents for commercialisation, these selection criteria are of utmost importance, other than the economic value of the crop on which the biocontrol agents are to be applied (Köhl *et al.*, 2011). For this approach, a Malaysian guideline, that is the “Guidelines for Biopesticide Registration” GP 7/2016 (data requirements for microbial registration)

(Department of Agriculture, 2016), was used as the basis for the selection of suitable antagonists. The guideline was promulgated in reference to international guidelines such as those prescribed by the Food and Agricultural Organisation (2012) "Guidance for Harmonising Pesticide Regulatory Management in Southeast Asia".

During the *in silico* screening, it was found that some fungal isolates assessed in the present Chapter might behave as pathogens to plants, humans, or animals, but also exhibited beneficial characteristics that could be exploited by the industry (e.g., production of degrading enzyme for organic pollutant, or synthesis of silver nanoparticles for biotechnological applications; Kang *et al.*, 2019; Arun *et al.*, 2014). This is especially the case for the basidiomycete isolates *B. adusta* and *S. commune* which are wood-rot fungi, and have been implicated with systemic mycoses such as allergic reaction in humans (Chowdhary *et al.*, 2014). In fact, it is not uncommon for certain fungi to exhibit dual-nature of existence; both beneficial and detrimental. Despite the pathogenic nature of these species, some publications have also reported their efficacy as biocontrol agents either *in vitro* (Burnevica *et al.*, 2022; Choo *et al.*, 2021; Naidu *et al.*, 2016) or *in planta* (Chen *et al.*, 2022; Feng *et al.*, 2021). Therefore, it was deduced that both species may behave as pathogen to a narrow host range, and that their pathogenicity could be minimised by good agricultural practices (GAP). If their advantages as a strong biocontrol agents could outweigh their disadvantages as a pathogen, their elimination in the earlier antagonist screening will hinder the development of novel and effective biocontrol agents against mycotoxigenic fungal species. Of course, one of the

strictest criteria for the development of effective biocontrol agents against mycotoxigenic fungal species in maize agro-ecosystems was that no fungal isolates that have been proven as maize pathogen (of which *B. adusta* and *S. commune* are not) or mycotoxigenic species should be included in further screening by the dual-culture assay.

Based on the growth inhibition data, *Trichoderma* spp. could serve as excellent antagonist candidates. Of these, the antagonism potential of *T. harzianum* isolates was the highest. Besides *Trichoderma* spp., *B. adusta* and *T. cubensis* could also serve as excellent antagonist candidates, particularly against *A. flavus*, *F. verticillioides* and *F. proliferatum*. In terms of mycotoxin reduction, varying patterns were observed. However, *T. asperelloides* was observed to reduce OTA and both fumonisins by at least > 70% (Appendix T), thus showing the ability of a single antagonist candidate against a broad range of mycotoxins. To the best of our knowledge, no report is available on the antagonism potential of *T. asperelloides* against *A. niger*, *F. verticillioides*, *F. proliferatum*, OTA, and fumonisins. For the other *Trichoderma* isolates, the antagonism potentials of *T. yunnanense* against mycotoxigenic fungal growth and mycotoxin production was also not reported elsewhere. In addition, the data on the antagonism potentials against mycotoxigenic fungal species and mycotoxin production by non-*Trichoderma* isolates such as *P. janthinellum* and *T. cubensis* demonstrated in the present Chapter, are largely unknown. Therefore, these isolates could become novel antagonist candidates that may be exploited in the development of effective biocontrol agents against mycotoxigenic species and mycotoxin production.

The results presented herein were consistent with the other studies that have been conducted on the efficacy of fungal antagonists against mycotoxigenic fungal growth and the subsequent mycotoxin production. For example, *B. adusta* has been reported as a good fungal agent that could reduce AFB₁ *in vitro* by approximately 100%. The toxin removal was attributed to the binding on cell wall and cell lysates along with exopolysaccharides of *B. adusta* (Choo *et al.*, 2021). Besides, the efficacy of *Trichoderma* spp. had also been documented. Gajera & Vakharia (2010) reported high inhibition of *T. harzianum* against *A. niger* growth on PDA, while, Valero *et al.* (2006) reported high inhibition of *T. harzianum* against OTA of the sister species, *A. carbonarius*, on synthetic nutrient medium. *T. asperellum*, *T. harzianum*, and *T. viride* were also effective against the growth of *A. niger* in onion bulb (Prajapati & Patil, 2017). For *Fusarium* spp., *T. asperellum* could reduce *F. verticillioides* growth by approximately 100% *in vitro*, and could also significantly reduce the average damage of fungal disease in maize plant (Cuervo-Parra *et al.*, 2022). Meanwhile, *T. asperellum* and *T. harzianum* could reduce FB₁ and FB₂ production *in vitro* by >60% and >80%, respectively (Tian *et al.*, 2020). *T. harzianum* has been reported to induce growth reduction of *F. proliferatum* by approximately 30% on meal maize agar, as well as almost total reduction of FB₁ on both maize agar and maize kernel (Rojo *et al.*, 2007).

Trichoderma spp. are well-established as biocontrol agents against phytopathogens. They are mycoparasitic fungi that could produce a cascade of extracellular proteins and cell wall-degrading enzymes as antagonism mechanisms against pathogens. *T. harzianum* could parasitise *A. flavus* by

forming coils around the latter's hyphae, followed by degradation and lysis of the latter's hyphae (Kifle *et al.*, 2017). Similar mechanism was also reported for *Trichoderma* spp. against *A. niger* (Gajera & Vakharia, 2010), *F. verticillioides*, and *F. proliferatum* (Yassin *et al.*, 2021). Besides, *T. harzianum* could also produce volatile inhibitory compounds and/or compete for nutrients (glucose) during the interaction with *F. verticillioides*. The mechanism of action of the basidiomycete *B. adusta* against pathogens is mycoparasitism and the production of enzymes such as catalase, superoxide dismutase, and peroxidase (Feng *et al.*, 2021). *S. commune* is also mycoparasitic, and could produce the enzyme laccase (Puig & Cumagun, 2019; Takemoto *et al.*, 2010). Since very little is known on *T. cubensis* as biocontrol agent, the knowledge on its antagonism characteristic is, therefore, still new, and is open for biocontrol agent development opportunity.

Despite the excellent pathogenic growth inhibition and mycotoxin reduction exhibited by *Trichoderma* spp., *B. adusta*, and *T. cubensis*, certain tested antagonist candidates could also induce/stimulate mycotoxin production in the present Chapter. The stimulation of mycotoxins following the co-cultivation with antagonist candidates, although undesirable, is a normal defence response exerted by the mycotoxigenic fungal species as a result of stress and competition for niche and/or nutrient. According to Künzler (2018), chemical defence, such as the production of chemical metabolites, is the main defence strategy of fungi. Therefore, it is assumed that the aflatoxin, OTA, and fumonisin stimulation upon competition with the antagonist candidates (*B. adusta*, *Trichoderma* spp., *T. cubensis*, *S. commune*) observed in the present

Chapter was the result of their defence mechanism. The mechanisms upon which defence occurs might be explained by several hypotheses: (1) physical contact between the mycotoxigenic species and the antagonist (Schroeckh *et al.* 2009), (2) as a reaction to the mycoparasitic behaviour of antagonist (Pellan *et al.*, 2021), and (3) the induction of transcription factor of genes in biosynthetic pathways of mycotoxins (Künzler, 2018; Yin *et al.*, 2012).

5.5 Conclusions

In conclusion, several selected indigenous fungal antagonists showed antagonistic activity against the growth and mycotoxin production by selected indigenous mycotoxigenic isolates from the pioneer grain maize farms in Terengganu, Malaysia. All *Trichoderma* isolates exhibited high antagonistic activity against the growth of all the tested mycotoxigenic fungal isolates. Besides, *B. adusta* Ba42DD and *T. cubensis* Tc28DD could also inhibit the mycotoxigenic fungal growth to a certain extent. High AFB₁ reduction was shown by *P. janthinellum* Pj46SD, *T. cubensis* Tc28DD, and *B. adusta* Ba42DD. OTA was highly reduced by *T. harzianum* Th20SR, Th24KD and Th33SR, and *T. asperellum* Ta31KR. FB₁ and FB₂ produced by *F. verticillioides* was highly reduced *T. harzianum* Th20SR, Th24KD, Th33SR, and Th36SR, *T. asperelloides* Ta39KR, and *T. asperellum* Ta31KR. FB₁ and FB₂ produced by *F. proliferatum* was highly reduced by *T. harzianum* Th20SR, Th24KD, and Th36SR and *T. asperelloides* Ta39KR. Three isolates were promising as novel antagonists, namely *P. janthinellum*, *T. cubensis*, and *T. asperelloides*. Four antagonists, namely *P. janthinellum* Pj46SD, *T.*

asperelloides Ta39KR, *T. asperellum* Ta31KR, and *T. harzianum* Th20SR that exhibited good antagonism against the fungal growth and mycotoxin production were further used for mycotoxin reduction on 3% GMA and grain maize kernels using pathogen:antagonist ratio assay in Chapter 6.



CHAPTER 6

EFFICACY OF DIFFERENT PATHOGEN:ANTAGONIST INOCULUM RATIOS ON MULTI-MYCOTOXIN REDUCTION BY SELECTED FUNGAL ANTAGONISTS INDIGENOUS TO GRAIN MAIZE

6.1 Introduction

As earlier mentioned, biocontrol utilises living organisms (or microorganisms) to reduce or suppress population density of the targeted pathogens to below their damaging level (van Lenteren *et al.*, 2018). Therefore, biocontrol strategy heavily relies on the combative strategy of the biocontrol agents. For biocontrol of phytopathogens, often antagonist with competitive edge will dominate the weaker pathogen in the targeted niche, thus restricting their growth and population density. However, in the case of mycotoxigenic fungi, apart from controlling fungal growth, effective antagonist should also be able to reduce or suppress the mycotoxin production in the targeted crops. Reason being, in some cases, partial or inefficient control of fungal growth will stimulate mycotoxin production (Magan *et al.*, 2020), which results in higher mycotoxin accumulation in crops. Therefore, selection of antagonist for mycotoxin control should consider this outcome before any decisions are made for downstream processing. For successful control against mycotoxins, the antagonist inoculum should be at the threshold level that is both efficient and economical (Magan *et al.*, 2020; Samsudin & Magan, 2016).

One *in vitro* approach to determine the effective antagonist inoculum threshold for fungal and mycotoxin reduction is by varying the inoculum ratios of both

antagonist and pathogen (Chang & Hua, 2007; Mohale *et al.*, 2013; Samsudin & Magan, 2016; Samsudin *et al.*, 2016). From this assay, it is expected that at each inoculum level, a good antagonist with competitive edge will dominate the weaker mycotoxigenic isolate, thus reducing or suppressing the mycotoxin production (Chang & Hua, 2007; Samsudin & Magan, 2016).

In Chapters 4 and 5, dual-culture assay was performed to screen for potential antagonists against the indigenous mycotoxigenic fungi and their subsequent mycotoxin production. Therefore, the present Chapter aimed to determine the most effective antagonist in reducing the production of AFs, OTA, and FBs using different pathogen:antagonist inoculum ratios on GMA and grain maize kernels. The objectives of the present Chapter were: (1) to examine multi-mycotoxin reduction of mycotoxigenic fungi by selected potential biocontrol agents using different pathogen:antagonist inoculum ratios on GMA, and (2) to examine multi-mycotoxin reduction of mycotoxigenic fungi by selected potential biocontrol agents using different pathogen:antagonist inoculum ratios on grain maize kernels. These *in vitro* and maize plant sample (although dead, but still retained actual maize nutrients) would provide excellent baseline before field trial on actual maize plant (*in planta/in situ*).

6.2 Materials and methods

6.2.1 Fungal isolates

Six fungal antagonists were used in the present study against four mycotoxigenic isolates producing AFs, OTA, and FBs (*i.e.*, *Aspergillus flavus* Af6KR, *A. niger* An1KD, *Fusarium verticillioides* Fv21KD, and *F. proliferatum* Fp9DD) (Table 6.1). These antagonists were: non-aflatoxigenic *A. flavus* strains Af1KD and Af5KD, *Penicillium janthinellum* Pj46SD, *Trichoderma asperelloides* Ta39KR, *T. asperellum* Ta31KR, and *T. harzianum* Th20SR. Among six antagonists, two were the non-aflatoxigenic *A. flavus* strains Af1KD and Af5KD from Chapter 4, while four were selected from a pool of 12 potential isolates assessed in Chapter 5. The selection was done based on their ability to highly reduce the respective mycotoxin produced by mycotoxigenic isolates in the dual-culture assay, and exhibited good growth inhibition (*i.e.*, 41% – 100%). Besides, all antagonists must also be able to produce spores to a concentration of $\geq 10^6$ spores/mL. Among three best antagonists against AFB₁, only *P. janthinellum* Pj46SD was selected for the present study, because the other two, *T. cubensis* Tc28DD and *B. adusta* Ba42DD, could not produce spores to $\geq 10^6$ spores/mL. Against OTA, and FBs produced by *F. verticillioides* Fv21KD, three best antagonists of different *Trichoderma* species were randomly selected (*i.e.*, *T. asperelloides* Ta39KR, *T. asperellum* Ta31KR, and *T. harzianum* Th20SR). Against FBs produced by *F. proliferatum* Fp9DD, two best antagonists of different *Trichoderma* species were randomly selected (*i.e.*, *T. asperelloides* Ta39KR and *T. harzianum* Th20SR). For OTA and FBs,

Table 6.1: Mycotoxigenic fungi and their respective fungal antagonists used for the pathogen:antagonist inoculum ratio assay

Mycotoxigenic fungi	Fungal antagonist	^a Mycotoxin reduction (%)
<i>Aspergillus flavus</i> Af6KR (producing aflatoxins B ₁ and B ₂)	Non-aflatoxigenic <i>A. flavus</i> Af1KD	AFB ₁ – ^b 93
	Non-aflatoxigenic <i>A. flavus</i> Af5TD	AFB ₁ – ^b 85
	<i>Penicillium janthinellum</i> Pj46SD	AFB ₁ – 75
<i>A. niger</i> An1KD (producing ochratoxin A)	<i>Trichoderma asperelloides</i> Ta39KR	OTA – 97
	<i>T. asperellum</i> Ta31KR	OTA – 97
	<i>T. harzianum</i> Th20SR	OTA – 68
<i>Fusarium verticillioides</i> Fv21KD (producing fumonisins B ₁ and B ₂)	<i>T. asperelloides</i> Ta39KR	FB ₁ – 95, FB ₂ – 99
	<i>T. asperellum</i> Ta31KR	FB ₁ – 95, FB ₂ – 99
	<i>T. harzianum</i> Th20SR	FB ₁ – 99, FB ₂ – 100
<i>F. proliferatum</i> Fp9DD (producing fumonisins B ₁ and B ₂)	<i>T. asperelloides</i> Ta39KR	FB ₁ – 95, FB ₂ – 98
	<i>T. harzianum</i> Th20SR	FB ₁ – 95, FB ₂ – 100

^bData of mycotoxin reduction were obtained from the dual-culture assay in Chapter 5.

^bData of AFB₁ reduction by the non-aflatoxigenic *A. flavus* were obtained from the dual-culture assay in Chapter 4.

similar strains of *Trichoderma* strains, including the newly-found novel antagonist *T. asperelloides* were selected to identify if any of the *Trichoderma* spp. could reduce both mycotoxins using the pathogen:antagonist assays.

6.2.2 Preparation of grain maize agar and grain maize kernels

For preparation of grain maize agar, a 3% semi-synthetic GMA was prepared as described in sub-Section 3.2.4.

For preparation of grain maize kernels, healthy, non-defect grain maize kernels of hybrid GWG 888 were used. This hybrid is planted for commercialisation of grain maize in Malaysia. Firstly, the kernels were surface-sterilised to remove the surface microbial contaminants while maintaining its integrity and germinative capacity (Davoudpour *et al.*, 2020) by stirring in 70% ethanol (v/v) for three minutes, followed by two times stirring in 10% sodium hypochlorite (v/v) for three minutes, and two times washing with sterile distilled water for three minutes, before left to dry on sterile paper towel and in sterile condition (Davoudpour *et al.*, 2020; Lanubile *et al.*, 2021). Following surface-sterilisation, five kernels were inoculated on PDA in triplicates, and incubated for seven days at 30°C to evaluate the efficacy of surface-sterilisation and kernel germinative capacity. All kernels were free from fungal growth, and they could germinate following incubation. Next, approximately 10 g of sterile grain maize kernels were placed in sterile 90 mm Ø Petri plates for further analyses.

6.2.3 Preparation and inoculation of different pathogen:antagonist inoculum ratios for mycotoxin analysis

Fungal spores were harvested from seven-day old axenic culture on PDA by washing with 10 mL 0.01% (v/v) sterile Tween 80 solution. The concentration of each fungal suspension was then estimated using a Neubauer

haemocytometer, and subsequently adjusted to a concentration of approximately 10^6 spores/mL by 10-fold serial dilution. Thereafter, the mixed-culture assay was performed by preparing pathogen:antagonist inoculum ratios – 75:25, 50:50, 25:75 pathogen:antagonist, and two controls – 100:0 (positive control) and 0:100 (negative control) pathogen:antagonist. Approximately 100 μ L and 1,000 μ L each of the inoculum ratios were inoculated on GMA, and the sterilised grain maize kernels, respectively, before evenly spread-plated and mixed well (Samsudin & Magan, 2016; Samsudin *et al.*, 2016). All treatments and controls were incubated at 30°C in the dark for 10 days for mycotoxigenic *Aspergillus* spp., and 14 days for mycotoxigenic *Fusarium* spp.

6.2.4 Mycotoxin analyses

For mycotoxin extraction from GMA, hyphal plugs were obtained as described in subsection 3.2.5.

For mycotoxin extraction from the fungal-colonised grain maize kernels, the kernels were first milled using Waring blender into fine powder, and transferred into a 50-mL Falcon tube (Fisher Scientific, New Hampshire, USA). Preparation of solvents for mobile phases, mycotoxin separation, and data processing were performed as described in sub-Section 3.2.5.

6.2.4.1 Aflatoxin extraction and quantification

For GMA, aflatoxin extraction was performed as described in sub-Section 3.2.5.1.

For the fungal-colonised grain maize kernels, extraction was performed following manufacturer's instruction with slight modification. Approximately, 5 g of ground grain maize kernel was mixed with 5 g of salt and 25 mL of 70% methanol (v/v) before homogenised at high speed for two minutes. Next, the extract was filtered through fluted filter paper (Vicom, Milford, USA) into a clean vessel. Next, 10 mL of filtered extract was diluted with 20 mL purified water before filtered through glass microfibre filter GF/A (Whatman®, Maidstone, UK). Approximately, 15 mL of the filtered extract (equivalent to 1 g original sample) was then passed through *AflaTest®WB* immunoaffinity column (Vicom, Milford, USA) for clean-up, before pipetting 1 mL of absolute methanol through the column to collect eluate containing aflatoxins. The eluate was then diluted with 1 mL of purified water before HPLC injection. Aflatoxin separation and quantification were performed as described in sub-Section 3.2.5.1.

6.2.4.2 Ochratoxin A extraction and quantification

For GMA, OTA extraction was performed as described in sub-Section 3.2.5.2.

For the fungal-colonised grain maize kernels, extraction was performed following manufacturer's instruction with slight modification. Approximately, 5

g of ground grain maize kernel was mixed with 5 g of salt and 50 mL of 80% methanol (v/v), before homogenised at high speed for one minute. Next, the extract was filtered through fluted filter paper into a clean vessel. Thereafter, 10 mL of filtered extract was diluted with 40 mL of purified water before filtered through microfibre filter (Whatman®, Maidstone, UK). Approximately, 10 mL of the filtered extract (equivalent to 0.2 g original sample) was then passed through *OchraTestTMWB* immunoaffinity column (Vicom, Milford, USA) for clean-up, before pipetting 1.5 mL of absolute methanol through the column to collect eluate containing OTA. The eluate was then diluted with 1.5 mL of purified water before HPLC injection. OTA separation and quantification were performed as described in sub-Section 3.2.5.2.

6.2.4.3 Fumonisin extraction, derivatisation, and quantification

For GMA, fumonisin extraction was performed as described in sub-Section 3.2.5.3.

For the fungal-colonised grain maize kernels, extraction was done following manufacturer's instruction with slight modification. Approximately, 5 g of ground grain maize kernel was mixed with 12.5 mL of extraction solvent (acetonitrile:methanol:water 25:25:50, v/v/v), before shaking with orbital shaker for 20 minutes, and centrifuged at 2,500 *g* for 10 minutes. The extract was then filtered through Whatman No. 4 filter paper (12 cm Ø, Whatman®, Maidstone, UK) into a clean vessel. The extraction was repeated twice with another 12.5 mL of the extraction solvent. Thereafter, 5 mL of filtered extract

was diluted with 20 mL of PBS solution, before filtered through glass microfibre filter GF/A (Whatman®, Maidstone, UK). Approximately, 10 mL of the filtered extract (equivalent to 0.4 g original sample) was passed through FumoniTest™WB immunoaffinity column (Vicom, Milford, USA) for clean-up, before pipetting 1.5 mL of absolute methanol through the column to collect eluate containing fumonisin. All extract from GMA and grain maize kernels were dried by evaporating in a stream of nitrogen, and re-dissolved in the same mobile phase for HPLC analysis. Fumonisin derivatisation, separation, and quantification were performed as described in sub-Section 3.2.5.3.

6.2.4.4 Method validation

Based on criteria prescribed by the International Union of Pure and Applied Chemistry (IUPAC, Thompson *et al.*, 2002), linearity (R^2), limit of detection (LOD), limit of quantification (LOQ), and precision were assessed for mycotoxin detection on the grain maize kernels. Following quantification, linear calibration curves of each tested mycotoxin was constructed by plotting the peak area obtained from HPLC analysis against the concentrations of each standard solutions to calculate the actual mycotoxin concentration. The standard solutions were prepared and diluted in different solvents: AFB₁ (0.5 – 100 ng/mL), AFB₂ (0.15 – 30 ng/mL) and OTA (2.5 – 100 ng/mL) in methanol, while FB₁ and FB₂ (0.1 – 25 µg/mL) in acetonitrile:water. For AFB₁, AFB₂, and OTA used to estimate the concentrations in the kernels, the working standard solutions were diluted with purified water before injection into HPLC. Next, linear regression was constructed to obtain the correlation coefficient (R^2). The

LOD and LOQ of each analysis were estimated using the formula; $LOD = 3\sigma/s$, and $LOQ = 10\sigma/s$, where σ was the standard deviation of blank responses, and s was the slope of the calibration curve (Shrivastava & Gupta, 2011). Precision is the relative standard deviation (RSD, %) of mycotoxin standards obtained by injection in triplicates ($n = 3$) on the same day (intra-day), and on different days (inter-day). HPLC method validation also included percentage recovery and specificity/selectivity for detection of mycotoxins in kernels. Percentage recovery (%) was determined by spiking the blank samples of grain maize kernels with known concentrations of mycotoxin in triplicates ($n = 3$). Specificity/selectivity was determined by comparing the retention times of spiked grain maize kernels with known concentrations of mycotoxin standards. Table 6.2 depicts the linearity, limit of detection (LOD), and limit of quantification (LOQ), selectivity/specificity and recovery (%) for the mycotoxin analyses on grain maize kernels. Table 6.3 depicts the precision and recovery (%) for all the mycotoxins analysed.

Table 6.2: Linearity, limit of detection (LOD), limit of quantification (LOQ), and selectivity/specificity for mycotoxin analyses on grain maize kernels

^a Analyte	Linearity		^b LOD	^b LOQ	^c Specificity/selectivity	
	Linear equation	R^2			Standard	Spiked Samples
^d AFB ₁	$y = 422580x$	0.995	1.9496	5.9078	22.99 ± 0.43	22.59 ± 0.22
^d AFB ₂	$y = 294053x$	0.991	0.1098	0.3328	19.96 ± 0.29	19.64 ± 0.12
^d OTA	$y = 25801x$	0.996	0.2211	0.7370	8.38 ± 0.06	8.41 ± 0.01
FB ₁	$y = 27740456x$	0.990	0.0002	0.0005	9.11 ± 0.23	9.30 ± 0.63
FB ₂	$y = 17798487.9x$	0.990	0.0003	0.0009	18.75 ± 0.64	18.51 ± 1.18

^aAFB₁ – aflatoxin B₁; AFB₂ – aflatoxin B₂; OTA – ochratoxin A; FB₁ – fumonisin B₁; FB₂ fumonisin B₂. ^bLOD and LOQ of AFB₁, AFB₂, and OTA were in ng/mL, while the LOD and LOQ of FB₁ and FB₂ were in µg/mL. ^cThe specificity/selectivity were reported in mean $\pm$ SD. ^dAFB₁, AFB₂, OTA – the working standard solutions were diluted with purified water before HPLC injection.

Table 6.3: Precision (based on the relative standard deviation of peak area, %RSD) and recovery (%) of all mycotoxins analysed

^a Analyte	Concentration (ng/mL)	Precision (%RSD)		Recovery (%)
		Intra-day	Inter-day	
AFB ₁	25	0.20	2.91	105.58
	75	2.39	4.21	84.91
AFB ₂	25	0.34	7.65	90.65
	75	1.12	3.74	83.72
OTA	25	1.05	3.42	84.42
	100	0.50	2.13	101.01
FB ₁	100	5.71	5.66	91.21
	250	2.63	11.35	96.57
FB ₂	100	2.89	2.95	91.39
	250	2.41	13.10	104.36

^aAFB₁ – aflatoxin B₁; AFB₂ – aflatoxin B₂; OTA – ochratoxin A; FB₁ – fumonisin B₁; FB₂ – fumonisin B₂.

6.2.5 Statistical analysis

All experiments were conducted in four replicates ($n = 4$). Measurements were averaged and reported as mean $\pm$ SE. Next, data were checked for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. The statistical analysis was done using one-way analysis of variance (ANOVA) with 95% confidence interval, and p -value of < 0.05 was accepted as significant difference. A *post hoc* Tukey's Honest Significant Difference (Tukey's HSD) were conducted with p -value of < 0.05 to compare the significant difference between groups. The statistical software SPSS v. 29.0 was used to perform all statistical analyses.

Figure 6.1 summarise the methodology used in the present Chapter.

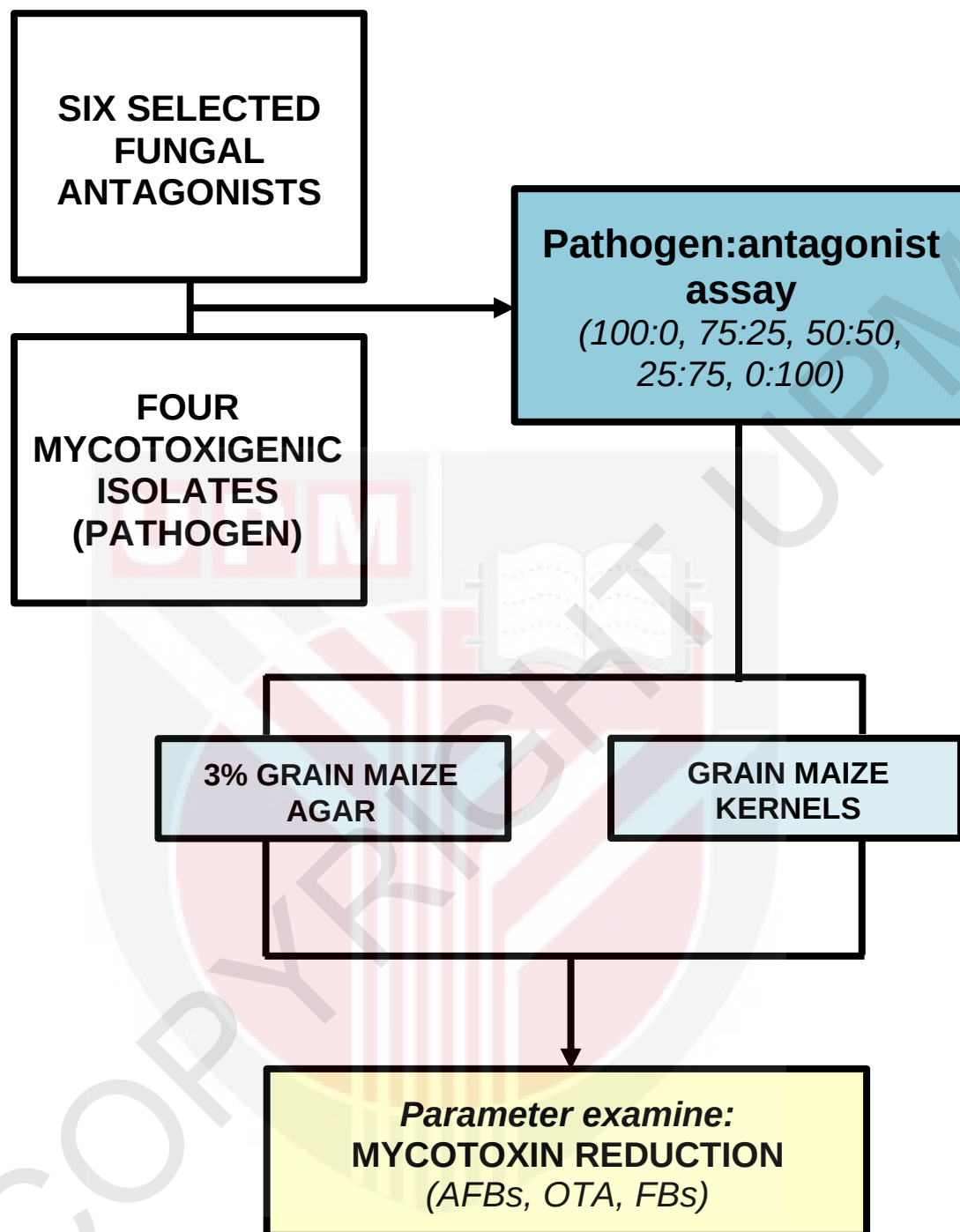


Figure 6.1: Flow diagram of the methodology used in Chapter 6

6.3 Results

6.3.1 Aflatoxin reduction

Figures 6.2 and 6.3 depict the AFB₁ and AFB₂ production by *A. flavus* in mixed culture of different pathogen:antagonist inoculum ratios, and cultivated on GMA and grain maize kernels. In the previous Chapter, aflatoxigenic strain Af6KR could produce both AFB₁ and AFB₂. However, in the present Chapter AFB₂ was not detected on GMA, presumably because the production was below the detection limit. Therefore, AFB₂ was examined only on the grain maize kernels. Overall, all antagonists gave significant ($p < 0.05$) reduction of aflatoxins on GMA, but not so much ($p > 0.05$) on the kernels. No coherent inhibition pattern of aflatoxin was obtained when observed simultaneously across all substrates, and at each inoculum level.

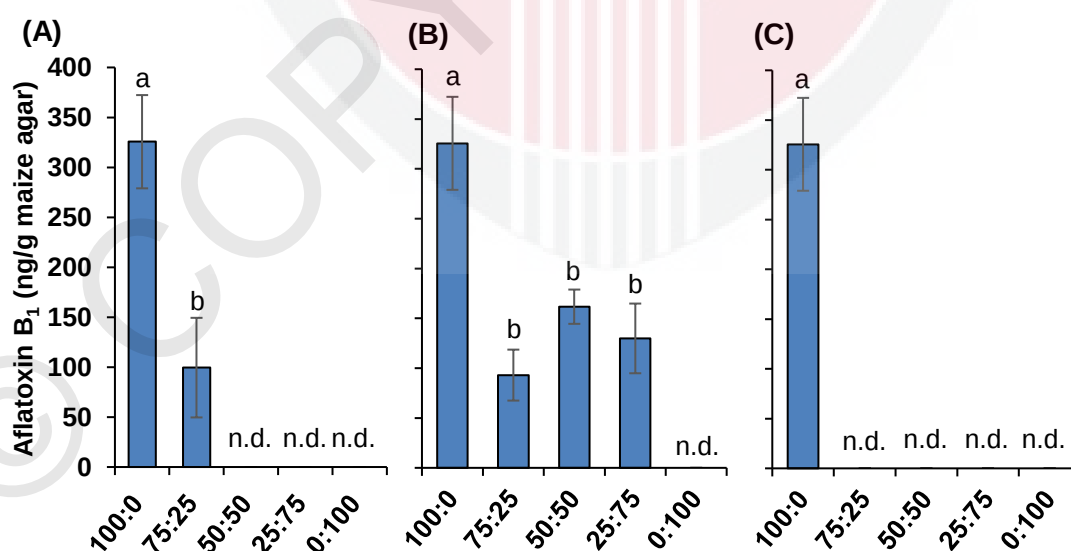


Figure 6.2: Aflatoxin B₁ (AFB₁; ng/g) production by *Aspergillus flavus* in mixed culture of different pathogen:antagonist inoculum ratios and cultivated on 3% grain maize agar.

(A) *A. flavus* Af6KR:non-aflatoxigenic *A. flavus* Af1KD

(B) *A. flavus* Af6KR:non-aflatoxigenic *A. flavus* Af5TD

(C) *A. flavus* Af6KR:*Penicillium janthinellum* Pj46SD

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

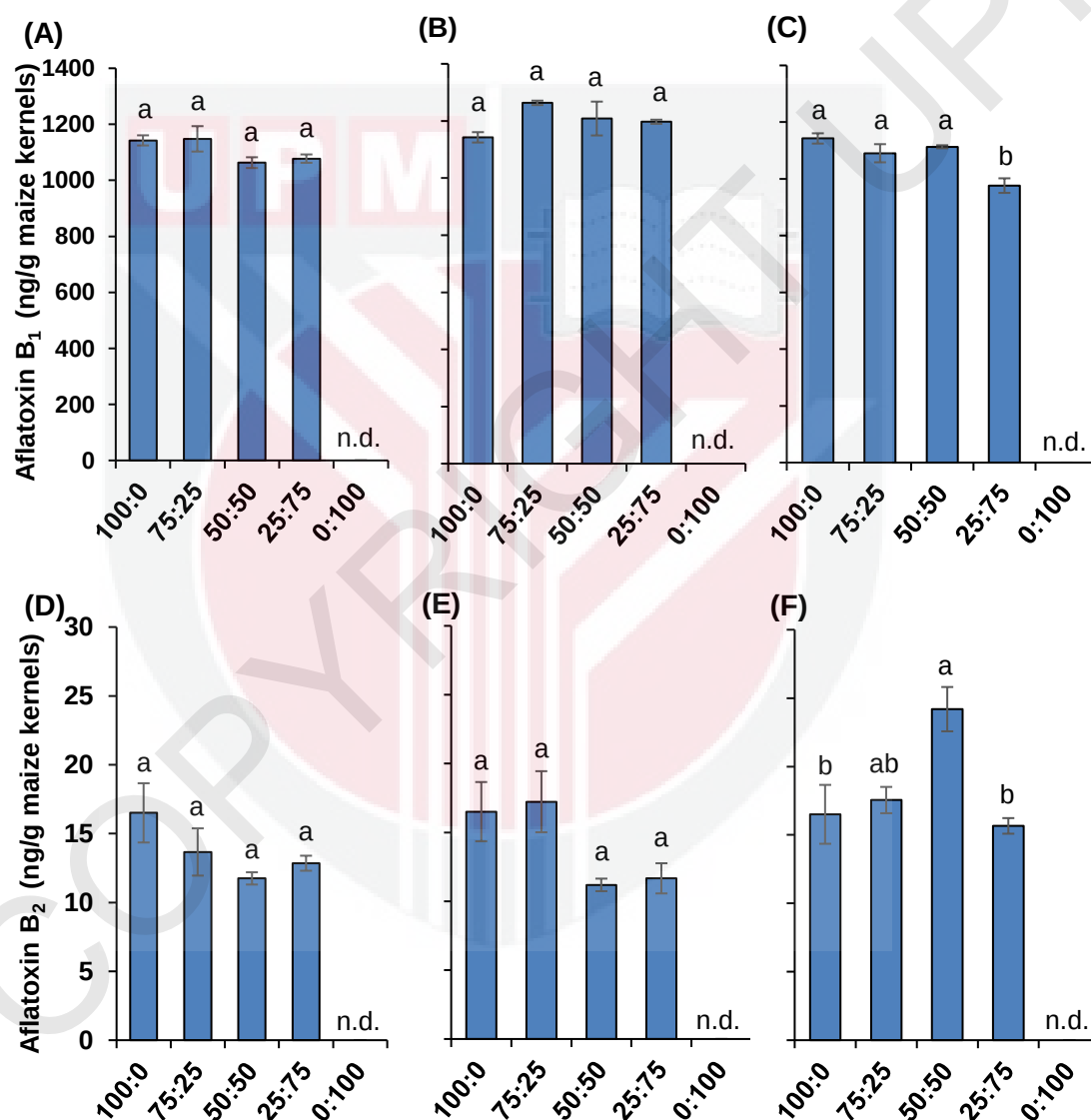


Figure 6.3: Aflatoxin B₁ (AFB₁; ng/g) and aflatoxin B₂ (AFB₂; ng/g) production by *Aspergillus flavus* in mixed culture of different pathogen:antagonist inoculum ratios and cultivated on grain maize kernels.

(A, D) *A. flavus* Af6KR:non-aflatoxigenic *A. flavus* Af1KD

(B, E) *A. flavus* Af6KR:non-aflatoxigenic *A. flavus* Af5TD

(C, F) *A. flavus* Af6KR:*Penicillium janthinellum* Pj46SD

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

The best control was shown by non-aflatoxigenic *A. flavus* Af1KD, that could exhibit significant ($p < 0.05$) reduction of AFB₁ on GMA at all ratios tested. On grain maize kernels and relative to the other antagonists, the reduction of both AFB₁ and AFB₂ by non-aflatoxigenic *A. flavus* Af1KD were the highest. *P. janthinellum* Pj46SD was second as it could significantly ($p < 0.05$) reduce AFB₁ on GMA and grain maize kernels, but not as effective in reducing AFB₂ on grain maize kernels. Besides, non-aflatoxigenic *A. flavus* Af1KD required lower inoculum level for aflatoxin reduction (i.e., 50%, 50:50), than *P. janthinellum* Pj46SD (i.e., 75%, 25:75). For non-aflatoxigenic *A. flavus* Af5TD, the highest and significant ($p < 0.05$) AFB₁ reduction was shown at 75:25 (71%) on GMA. On grain maize kernels, non-aflatoxigenic *A. flavus* Af5TD only stimulated aflatoxins except for AFB₂ at ratio 50:50 and 25:75 (i.e., 32 and 29% reduction, respectively). Aflatoxin stimulation varied on grain maize kernels depending on ratios. Generally, stimulation occurred at all ratios, but more apparent when the aflatoxigenic isolate were subjected to low (i.e., 75:25) or the same inoculum level (i.e., 50:50) with the antagonists. For non-aflatoxigenic *A. flavus* Af1KD, stimulation of AFB₁ was observed at low inoculum of antagonist (i.e., 75:25). For *P. janthinellum* Pj46SD, stimulation of AFB₂ was observed at 75:25 and 50:50. For non-aflatoxigenic *A. flavus* Af5TD, stimulation of AFB₁ and AFB₂ was observed at all ratios tested, and at 75:25, respectively. As expected, negative control (i.e., 0:100; 0% pathogen and 100% antagonist) yielded no aflatoxin production.

6.3.2 Ochratoxin A reduction

Figure 6.4 depicts the OTA production by *A. niger* in mixed culture of different pathogen:antagonist inoculum ratios, and cultivated on GMA. The baseline OTA was only produced on GMA, and not on grain maize kernels. Therefore, only those data were presented herein. Overall, all *Trichoderma* spp. tested as antagonists in the present Chapter could significantly ($p < 0.05$) reduce OTA on GMA, by approximately 100%, at all ratios tested with no stimulation in OTA production was observed. All antagonists also exhibited low threshold level for efficient control of OTA which was at 75:25. As expected, negative control (*i.e.*, 0:100; 0% pathogen and 100% antagonist) yielded no OTA production.

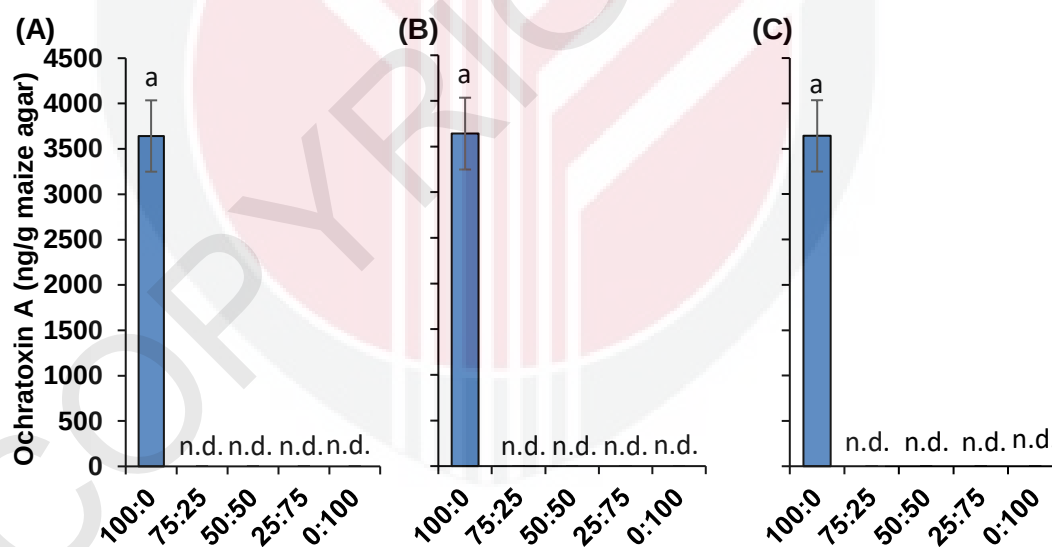


Figure 6.4: Ochratoxin A (OTA; ng/g) production by *Aspergillus niger* in mixed culture of different pathogen:antagonist inoculum ratios and cultivated on 3% grain maize agar.

(A) *A. niger* An1KD:*Trichoderma asperelloides* Ta39KR

(B) *A. niger* An1KD:*T. asperellum* Ta31KR

(C) *A. niger* An1KD:*T. harzianum* Th20SR

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between

groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d.
– not detected

6.3.3 Fumonisin reduction

Both *F. verticillioides* and *F. proliferatum* could produce FB₁ and FB₂. However, FB₂ was not detected on GMA, and only detected on grain maize kernels. Therefore, only those data were reported herein. Figures 6.5 and 6.6 depict the FB₁ and FB₂ production by *F. verticillioides* in mixed culture of different pathogen:antagonist inoculum ratios, and cultivated on GMA and grain maize kernels. Overall, all tested *Trichoderma* antagonists could significantly ($p < 0.05$) reduce FB₁ and FB₂ on both GMA and grain maize kernels, except for *T. harzianum* Th20SR that stimulated production of both fumonisins on the kernels at ratio 25:75 (*i.e.*, high antagonist inoculum). The best control was shown by *T. asperellum* and *T. asperelloides*. *T. asperellum* exhibited steady fumonisin inhibition (*i.e.*, complete inhibition or continual decrease) at all ratios tested, and on both tested substrates. *T. asperelloides*, on the other hand, yielded varying inhibition effects across all ratios tested, and on grain maize kernels. All tested antagonists exhibited threshold inoculum of control for fumonisins at 75:25 (*i.e.*, low antagonist inoculum).

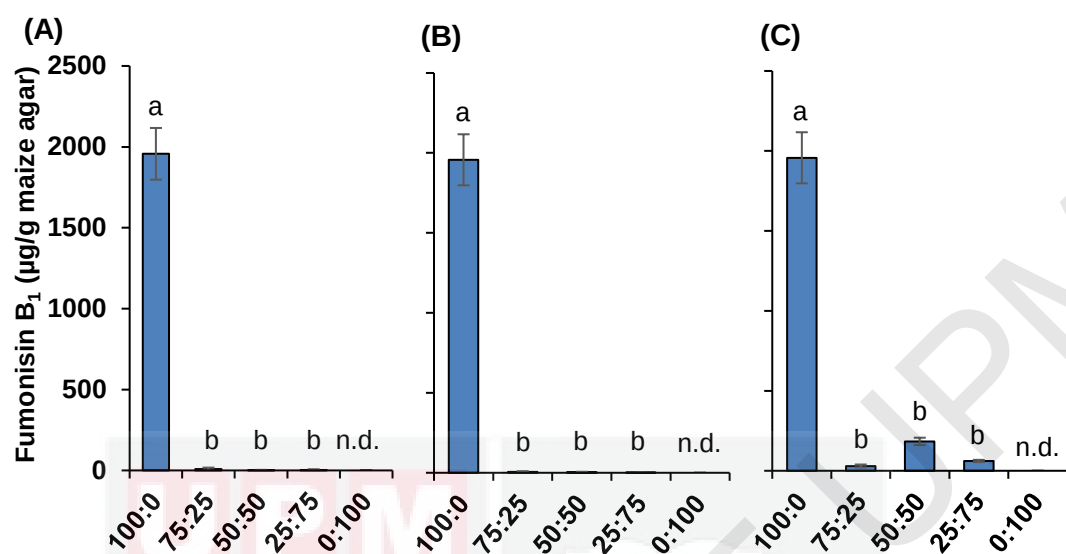


Figure 6.5: Fumonisin B₁ (FB₁; µg/g) production by *Fusarium verticillioides* in mixed culture of different pathogen:antagonist inoculum ratios and cultivated on 3% grain maize agar.

(A) *F. verticillioides* Fv21KD:*Trichoderma asperelloides* Ta39KR

(B) *F. verticillioides* Fv21KD:*T. asperellum* Ta31KR

(C) *F. verticillioides* Fv21KD:*T. harzianum* Th20SR

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

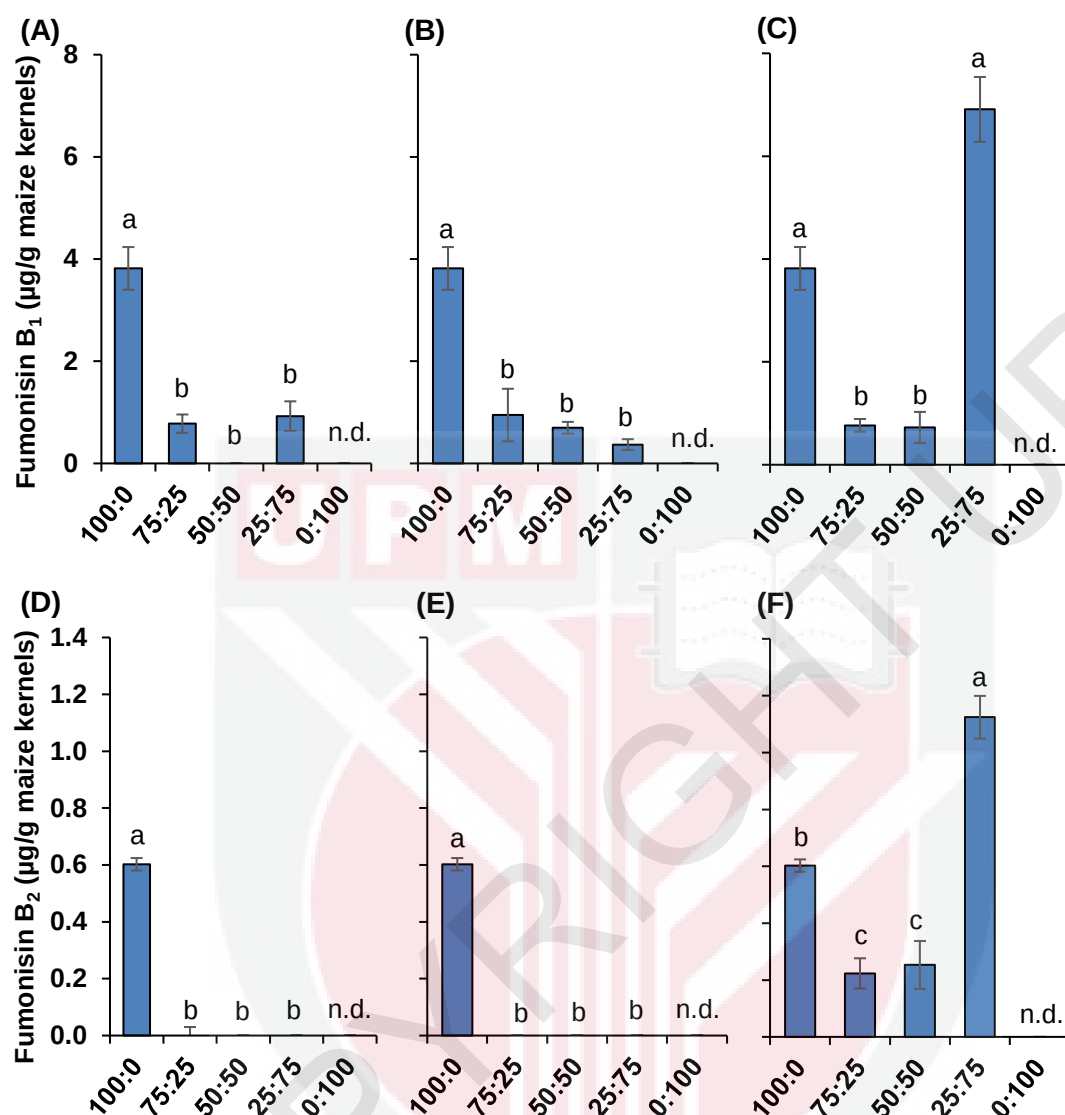


Figure 6.6: Fumonisin B₁ (FB₁; µg/g) and fumonisin B₂ (FB₂; µg/g) production by *Fusarium verticillioides* in mixed culture of different pathogen:antagonist inoculum ratios and cultivated on grain maize kernels.

(A, D) *F. verticillioides* Fv21KD:*Trichoderma asperelloides* Ta39KR

(B, E) *F. verticillioides* Fv21KD:*T. asperellum* Ta31KR

(C, F) *F. verticillioides* Fv21KD:*T. harzianum* Th20SR

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

Figures 6.7 and 6.8 depict the FB₁ and FB₂ production by *F. proliferatum* in mixed culture of different pathogen:antagonist inoculum ratios, and cultivated on GMA and grain maize kernels. Overall, *T. asperelloides* exhibited the best control with the highest (*i.e.*, 96% to 100%) and significant ($p < 0.05$) fumonisin reduction at all tested ratios, and on both tested substrates. For *T. harzianum* Th20SR, the reduction of FB₁ was significant ($p < 0.05$) at all tested ratios on both tested substrates. *T. harzianum* inhibited FB₁ by more than 90% on GMA at all ratios tested. On grain maize kernels, *T. harzianum* showed almost complete inhibition (99%) of FB₁ at 75:25 (*i.e.*, low antagonist inoculum). However, it exhibited medium fumonisin reduction, of approximately 65% and 75% FB₁, and 55% and 71% FB₂, at 50:50 and 25:75, respectively. Both antagonists exhibited significant control even at low inoculum level (*i.e.*, 75:25).

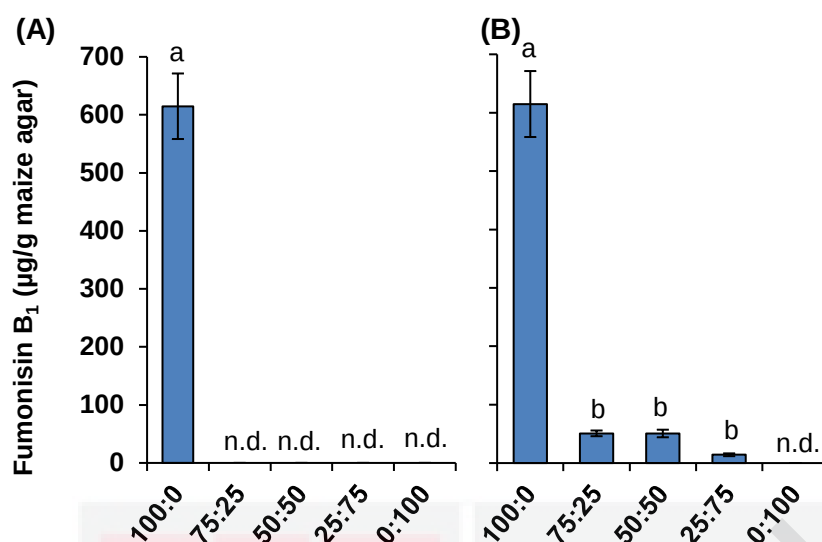


Figure 6.7: Fumonisin B₁ (FB₁; µg/g) production by *Fusarium proliferatum* in mixed culture of different pathogen:antagonist inoculum ratios and cultivated on 3% grain maize agar.

(A) *F. proliferatum* Fp9DD:*Trichoderma asperelloides* Ta39KR

(B) *F. proliferatum* Fp9DD:*T. harzianum* Th20SR

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean ± SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

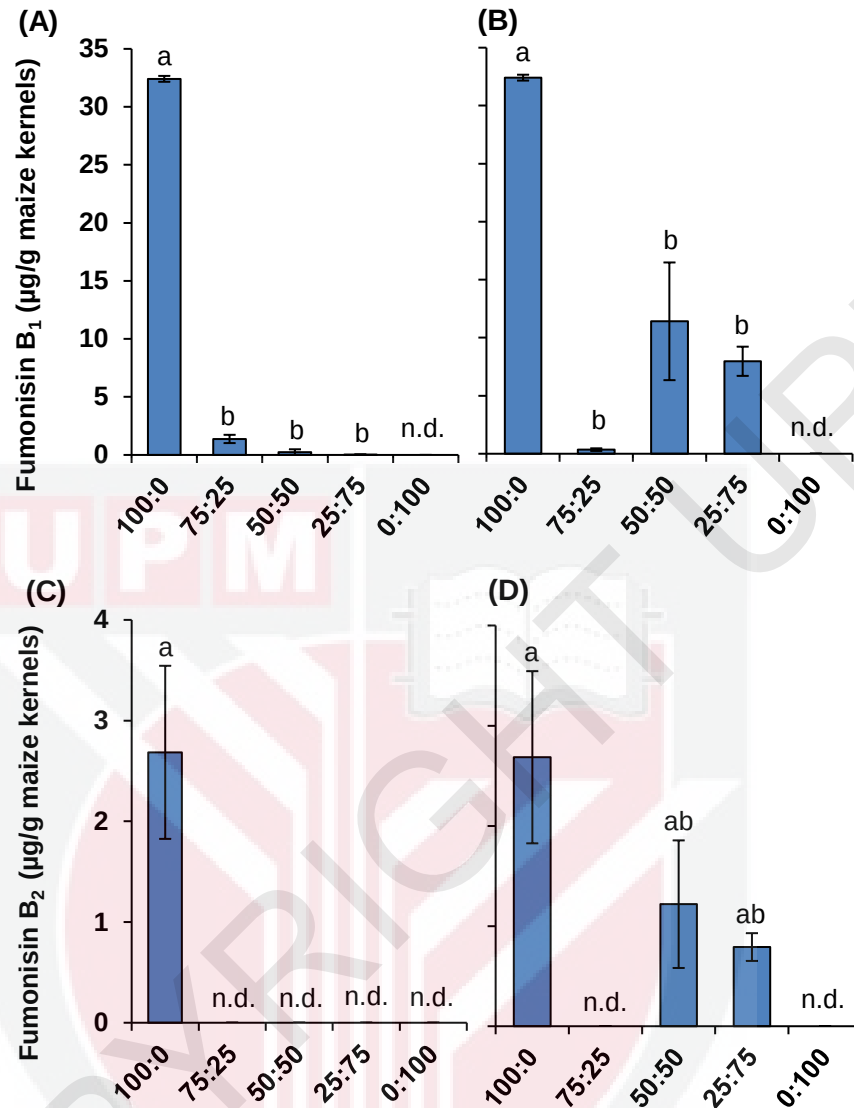


Figure 6.8: Fumonisin B₁ (FB₁; $\mu\text{g/g}$) and fumonisin B₂ (FB₂; $\mu\text{g/g}$) production by *Fusarium proliferatum* in mixed culture of different pathogen:antagonist inoculum ratios on grain maize kernels.

(A, C) *F. proliferatum* Fp9DD:*Trichoderma asperelloides* Ta39KR

(B, D) *F. proliferatum* Fp9DD:*T. harzianum* Th20SR

Ratios 100:0 and 0:100 serve as positive and negative controls, respectively. Data are mean $\pm$ SE of four replicates ($n = 4$). Different lowercase letters indicate significant difference ($p < 0.05$) between groups using Tukey's Honest Significant Difference (Tukey's HSD). n.d. – not detected

6.4 Discussion

The present Chapter demonstrated that each strain of the tested fungal antagonists showed different efficacy for mycotoxin control following the pathogen:antagonist inoculum ratios on both GMA and grain maize kernels. This is particularly apparent with the antagonists against aflatoxins, as both tested non-aflatoxigenic *A. flavus* isolates could either reduce the aflatoxin production albeit in small amount on both substrates (*i.e.*, strain Af1KD), or induced them (*i.e.*, strain Af5TD). For *Trichoderma* spp. against OTA and FBs, various pattern of reduction was also observed. Both *T. asperellum* and *T. asperelloides* could almost completely reduce OTA and fumonisins regardless of the inoculum ratios, while *T. harzianum* could not.

Previous studies utilising inoculum ratios have shown various pattern of mycotoxin reduction by different fungal strains (Samsudin & Magan 2016; Mohale *et al.*, 2013) on milled-maize agar (MMA) and maize kernels. In fact, this was more apparent when different environmental factors (*i.e.*, temperature and water activity) were incorporated in these studies. This indicated that virulence of each antagonistic strains could be largely varied (Hajek & Eilenberg, 2018). Similarly, this also indicated that each individual antagonistic strains may adopt specific mechanism of action that could provoke and elicit different combative strategies against the mycotoxigenic isolates on the same substrates (Boddy, 2016; Samsudin *et al.*, 2016).

In the present Chapter, the non-aflatoxigenic *A. flavus* isolates strain Af5TD was a weaker antagonist as compared to strain Af1KD. This contradicts to the result in Chapter 4, whereas dual-culture assay of both tested non-aflatoxigenic isolates exhibited considerable reduction of aflatoxins (even with low growth inhibition), and this is attributed to their “touch inhibition” strategy (Huang *et al.*, 2011). Based on the result in the present Chapter, it is therefore assumed that strain Af5TD may in fact have weaker competitive ability against the aflatoxigenic *A. flavus* Af6KR on both tested substrates, as compared to strain Af1KD. It is also assumed that physical contact is simply not enough to significantly reduce aflatoxins by both tested non-aflatoxigenic isolates, rather other mechanisms also occurred. This was observed when the proportion of strain Af1KD increased relative to the aflatoxigenic isolate and exhibited greater aflatoxin reduction primarily on GMA. This indicates direct replacement (*i.e.*, competitive exclusion) strategy by outcompeting the aflatoxigenic counterparts and occupying the niche/substrate faster (Sweany *et al.*, 2022). This was also observed *in vitro* by Wicklow *et al.* (2003), and corroborated by Abbas *et al.* (2011) in a field study. The other mechanism of action adopted by strain Af1KD could be the production of inhibitory substance (Sweany *et al.*, 2022). However, this was not ascertained in the present study, and might open an opportunity for future studies.

Comparing the aflatoxin reduction on the grain maize kernels, the non-aflatoxigenic isolates could not considerably reduce aflatoxins on the kernels. This might be attributed to the infectivity of these isolates to colonise and proliferate in grain maize kernels. It was found that *A. flavus* isolates which

obtained from maize are more readily infecting maize even at low inoculum load than those isolated from soil (Sweany, 2019). This is because the maize-isolated strain is commonly from L-morphotype (*i.e.*, large sclerotia) that contain genes for strain adaptability in the maize phyllosphere. Specifically, L-morphotype contains gene that encodes for cutinase at the inversion breakpoint of chromosome 8 which supports nutrient acquisition in the kernel; and also enriched in protein with LysM domain which supports colonisation in the kernel while evading plant host's defence response. However, the S-morphotype (*i.e.*, small sclerotia) commonly found in soils lack these genes and proteins, therefore is less adaptable to the kernels (Ohkura *et al.*, 2018). Both aflatoxigenic isolates assessed in the present Chapter was isolated from maize, and therefore might be better at infecting the kernels. The non-aflatoxigenic Af5TD, however, was isolated from tassels. So, the primary inoculum might have originally come from the soil/debris, and infected the tassels through air (*i.e.*, horizontal transmission of airborne conidia). Having said that, the non-aflatoxigenic Af1KD was in fact isolated from maize, but still grew poorly on GMA and grain maize kernels, as evidenced by the low reduction of aflatoxins when high inoculum ratio was applied. This non-aflatoxigenic isolate might simply be a weaker strain that produce lesser conidia as inoculum for infection in maize, therefore having low competitiveness (Sweany, 2019). Since fungal competitiveness is an important trait in the development of effective biocontrol agents, both non-aflatoxigenic isolates assessed herein might not be suitable as antagonists for application in the phyllosphere (*i.e.*, above-ground surface of a plant), but may be more

suitable for application in the rhizosphere (*i.e.*, below-ground surface of a plant; *e.g.*, soil treatment). However, this needs to be further validated in future work.

Interestingly, *P. janthinellum* Pj46SD exhibited better control of aflatoxins, at least against AFB₁, when comparing to the non-aflatoxigenic *A. flavus* antagonist candidates. Indeed, *P. janthinellum* (synonym *P. simplicissimum*) is promising as beneficial fungal antagonist. It was reported that this fungus is able to produce indole-diterpenoids (Guo *et al.*, 2019), and various enzymes and secondary metabolites that may act as antifungal agents, such as chitinase and cellulase (Jayasimha & Henderson, 2007; Di Giambattista *et al.*, 2001). Besides, *P. janthinellum* could also reduce fungal growth and crop diseases caused by phytopathogens both *in vitro* and *in planta*. For example, *in vitro* study of *P. janthinellum* against *Phytophthora parasitica* causing black shank in tobacco showed that *P. janthinellum* could highly inhibit the growth of this fungal pathogen (Wang *et al.*, 2022). A greenhouse study also showed *P. janthinellum* as effective against *Puccinia striiformis* f. sp. *tritici* causing yellow rust in leaf of wheat. The mechanism of action of this beneficial antagonist against *P. striiformis* is explained by mycoparasitic behaviour against the pathogen's urediniospores (Esmail *et al.*, 2022). However, the role of this antagonist for mycotoxin inhibition, particularly aflatoxins, is largely unknown, and was demonstrated for the first time in the present study. Based on the results reported herein, opportunity exist to explore *P. janthinellum* as efficient biocontrol agent against aflatoxins.

Ochratoxin A was not produced by *A. niger* An1KD on the grain maize kernels despite multiple attempts. Although OTA had been reported as contaminants in maize samples (Topi *et al.*, 2023; Majeed *et al.*, 2013), and *A. niger* is not an uncommon pathogen of maize since it has previously been isolated from maize kernels (Goher *et al.*, 2020), their occurrence on maize is lesser as compared to the other favourable hosts such as coffee (Leitão 2019), grape (Freire *et al.* 2020; Zhang *et al.*, 2016), or spices (Safaei *et al.* 2023). It is then assumed that this *A. niger* isolate could still produce OTA on the tested kernel substrate, but the production was too low to be detected using HPLC. Besides, the present study assessed OTA after incubation for only 10 days which could not have been sufficient for its production. *A. niger* may requires longer incubation time, of more than 10 days for OTA production on maize kernels (Han *et al.*, 2019).

Mycotoxin stimulation observed in the present Chapter might have been attributed to the defence mechanism of the mycotoxigenic fungi in the face of antagonists' presence/attack (Künzler, 2018). Fungal competition for nutrient eventually leads to nutrient depletion (*i.e.*, initiation of stress), which in turn leads to mycotoxin stimulation to provide competitive advantage of the mycotoxigenic fungi against their competing enemies (Magan & Aldred, 2007). Other than competition for nutrients, physical contact/attack and mycoparasitic behaviours of the antagonists could also trigger mycotoxin stimulation by the mycotoxigenic fungi (Pellan *et al.*, 2021; Künzler, 2018; Schroeckh *et al.*, 2009). Mycotoxin stimulation has also been reported elsewhere (Mohale *et al.*, 2013).

Trichoderma spp. are mycoparasites and aggressive colonisers (Dutta *et al.* 2023; Tian *et al.*, 2020). Therefore, it is not surprising that most *Trichoderma* spp. in the present study highly reduced both OTA and fumonisins regardless of the ratios and substrate tested. This suggested that, particularly for *T. asperelloides* and *T. asperellum*, both species adopted competitive exclusion strategy against the mycotoxigenic isolates, possibly by colonising the substrates faster, and incorporating wide defence and attack strategies, including mycophagy and the production of enzyme and inhibitory compounds (e.g., volatiles and non-volatiles) (Pellan *et al.*, 2021), thereafter reducing the accumulation of mycotoxin produced by the mycotoxigenic isolates. Often, a single *Trichoderma* sp. could exhibit multiple combative strategies at the same time against their enemy (Dutta *et al.*, 2023). However, the predominant strategy can be different between each *Trichoderma* spp. (Dutta *et al.*, 2023). Therefore, further analysis on the mechanism of antagonism adopted by all *Trichoderma* spp. in the present study is necessary. Apart from reducing the growth/population density of mycotoxigenic fungi that resulted in mycotoxin reduction, *Trichoderma* spp. could also bind, degrade, or transform mycotoxins into their glycosylated form or low toxic compounds (Tian *et al.*, 2020), even without fungal growth reduction (Pellan *et al.*, 2021),

For future work, this *in vitro* study could be extended to include environmental factors to increase practicality for field application which naturally has different temperature and moisture conditions. This could also change the dominant species and/or the subsequent reduction of mycotoxin. Besides, future studies could also focus on assessing wider inoculum ratio range (e.g., 10, 20, 30...,

instead of 25, 50, 75 assessed in the present study) to determine which one is most effective in controlling mycotoxins in the field, as well as the most economical in application.

6.5 Conclusions

Among 14 isolates of potential antagonists found in Chapter 4 (*i.e.*, two isolates of non-aflatoxigenic *A. flavus*) and Chapter 5 (*i.e.*, 12 isolates of different fungal species), only six were selected for the pathogen:antagonist assays on GMA and grain maize kernels in the present Chapter. Three factors form the basis of this selection, they should: (1) highly reduce the respective mycotoxin produced by mycotoxigenic isolates in the dual-culture assay, (2) exhibit good growth inhibition, and (3) could produce spores to a concentration of $\geq 10^6$ spores/mL. Following pathogen:antagonist assay, several antagonists were found promising in reducing mycotoxins produced by the tested mycotoxigenic fungi *in vitro* and on the grain maize kernels. For AFB₁ and AFB₂, the non-aflatoxigenic strain Af1KD was the most effective, though not as significant, and *P. janthinellum* Pj46SD was the second best. For OTA, all tested *Trichoderma* spp. (*T. asperelloides* Ta39KR, *T. asperellum* Ta31KR, and *T. harzianum* Th20SR) were effective. However, since the baseline OTA could not be detected on the grain maize kernels, selection for the best antagonist for OTA reduction could not be strongly concluded. For FB₁ and FB₂ produced by *F. verticillioides*, both antagonists *T. asperelloides* Ta39KR and *T. asperellum* Ta31KR were effective. While, for FB₁ and FB₂ produced by *F. proliferatum*, the antagonist *T. asperelloides* Ta39KR was the most effective.

Among all *Trichoderma* spp. tested against OTA and FBs, *T. asperelloides* Ta39KR was the most effective in reducing FBs and OTA produced by *F. verticillioides*, *F. proliferatum*, and *A. niger*, respectively.



CHAPTER 7

SUMMARY, GENERAL CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE RESEARCH

7.1 Summary

To biologically control the proliferation of mycotoxigenic fungi and their subsequent mycotoxin production pre-harvest, the application of indigenous/local biocontrol agents could be more effective than the introduction of exotic/foreign ones, since indigenous agents are better adapted to the environment onto which they would be applied, and may have lower risk of becoming invasive species, or worse targeting other beneficial organisms in the ecosystem. Although the use of biological control in other parts of the world is prevalent, in Malaysia, however, the research and development of biocontrol against mycotoxigenic fungi and mycotoxin contamination in the agro-ecosystem is still in infancy. The main aim of the present study was, therefore, to identify novel fungal biocontrol agents against mycotoxigenic fungi and their mycotoxins. Grain maize was selected as the tested commodity since it has been highly associated with contamination by mycotoxigenic fungi and mycotoxins. Furthermore, Malaysia is embarking on mass-cultivation of grain maize to reduce the dependency on grain maize intended for local ruminant and poultry industries. Sampling of these fungal species was done at the pioneer grain maize farms in Terengganu, Malaysia. Since grain maize plantations in Malaysia is still new and growing, knowledge on the risk of fungal contamination and the potential mycotoxin accumulation in these farms, and

in local grain maize agro-ecosystems in general, is largely unknown. By understanding the fungal community structure, and the potential risk that could be caused by these fungal species, effective control measures particularly biocontrol can be developed. To achieve this aim, the present study was divided into four objectives that were arranged in four separate chapters.

In Chapter 3 (Objective 1), the fungal community structure (*i.e.*, mycodiversity) including that of the mycotoxigenic species was established for the two pioneer grain maize farms in Terengganu, Malaysia. As expected, both grain maize farms were predominated by the mycotoxigenic species *Fusarium verticillioides*, which also has been regarded as one of the notorious contaminants in maize. Since almost all tested *F. verticillioides* could produce fumonisins on 3% semi-synthetic GMA, the risk of fumonisin contamination in the local grain maize farm could be high and alarming. Other mycotoxigenic isolates obtained from both farms were *F. proliferatum* which also produces fumonisins, *Aspergillus flavus* which produces aflatoxins, *A. niger* which produces OTA, and *F. incarnatum* which produces HT-2, T-2, and ZEN. These mycotoxigenic species will certainly produce mycotoxins in the grain maize agro-ecosystem upon favourable conditions such as Malaysia tropical climate which is hot and humid all year round. Besides, other potential biocontrol agents such as *Trichoderma* spp. and non-aflatoxigenic *A. flavus* were also isolated.

In Chapter 4 (Objective 2), seven isolated *A. flavus* that were tentatively deemed as aflatoxigenic and non-aflatoxigenic following cultivation on the 3%

GMA in Chapter 3 were further validated for their aflatoxigenicity molecularly by detecting seven aflatoxin biosynthesis genes, and chemically by quantifying aflatoxins on PDA and MEA. This was to ensure that the non-aflatoxigenic isolate selected for biocontrol development was truly non-aflatoxigenic. Among the seven isolates, five were aflatoxigenic (*i.e.*, strain Af2SR, Af3SD, Af4TR, Af6KR, and Af7KR), as compared to only three isolates detected on the GMA in Chapter 3. This molecular identification method also showed that the remaining two non-aflatoxigenic isolates (*i.e.*, strain Af1KD and Af5TD) as having large gene deletions along the aflatoxin biosynthetic pathway, thus having very low chance of reverting back to aflatoxigenic nature. The identity of the seven isolates were also taxonomically resolved by phylogeny of the species-specific β -tubulin datasets. When the non-aflatoxigenic isolates were co-cultured with the aflatoxigenic counterparts using the dual-culture assay on 3% GMA, both non-aflatoxigenic isolates (*i.e.*, strain Af6KR and Af7KR) showed high antagonism by reducing 50-100% AFB₁ production.

In Chapter 5 (Objective 3), the mycotoxigenic isolates that could produce the three important mycotoxins which have been classified as carcinogenic and placed in the Group 1 (aflatoxins by *A. flavus*) and Group 2B (OTA by *A. niger*, fumonisins by *F. verticillioides* and *F. proliferatum*) by IARC were further tested by dual-culture with the potential antagonists *in vitro* on GMA. Potential antagonists for the dual-culture assay were selected by *in silico* screening the remaining 22 fungal isolates after excluding the mycotoxigenic isolates to assess the potential risk of each isolate towards humans, animals, and plants. This was done to avoid introducing fungal isolates that would become invasive

or pathogenic onto the ecosystem. Isolates that had no or very low reported risk against humans, animals, and plants, or more importantly, were non-mycotoxigenic, were selected for the dual-culture assay. As expected, the mycoparasite *Trichoderma* spp. could highly reduce growth of all the tested mycotoxigenic species. *Trichoderma* spp. were the most effective in reducing OTA and fumonisins. Besides, *T. asperelloides* Ta39KR was found for the first time to inhibit OTA and fumonisin production. Some *Trichoderma* spp., unfortunately, stimulated mycotoxin production instead of inhibiting them. For aflatoxins, the most effective isolates were those that have not been reported for its antagonism against this mycotoxin: *P. janthinellum* Pj46SD and *T. cubensis* Tc28DD.

In Chapter 6 (Objective 4), the selected antagonists in Chapter 5 were further tested for the reduction of the three major mycotoxins and their producer fungi using different pathogen:antagonist inoculum ratios on grain maize agar and grain maize kernels. This type of experimental setting has been applied in many studies to determine the competitiveness between two organisms. By using this assay, antagonist that exhibited the highest or significant reduction of the tested mycotoxin in all ratios and on both tested substrates, was considered as having competitive edge against the tested mycotoxigenic isolates and their mycotoxin production. Antagonists that could significantly reduce the assessed mycotoxins even in their low proportion/ratio of spores in the pathogen:antagonist mixed culture may not only be effective as an agent for control, but may also only require lower downstream production and application costs. Non-aflatoxigenic *A. flavus* Af1KD was the most effective

against aflatoxins by aflatoxigenic *A. flavus*. *P. janthinellum* Pj46SD was the second most effective against this toxin. Further, *T. asperelloides* Ta39KR was the most effective in reducing fumonisins produced by *F. verticillioides* and *F. proliferatum*, and OTA, at least on the maize agar.

7.2 General conclusions

All the set objectives of the present study were duly achieved. The fungal community structure in both pioneer grain maize farms in Terengganu, Malaysia was established, and the mycotoxigenic potential of the isolated mycotoxigenic fungal species were determined in Objective 1. Next, the non-aflatoxigenic *A. flavus* isolates were successfully distinguished from their aflatoxigenic counterparts by molecular analyses and aflatoxigenicity test, and their antagonism against growth and aflatoxin production by their aflatoxigenic counterparts was established *in vitro* in Objective 2. Next, the antagonism *in vitro* of the other potential biocontrol agents indigenous to the grain maize farms against growth and mycotoxin production by the indigenous mycotoxigenic fungal isolates were elucidated by the dual-culture assay and HPLC analyses in Objective 3. The most effective biocontrol isolates against all the tested mycotoxins (aflatoxins, OTA, fumonisins) under different pathogen:antagonist inoculum ratio were identified in Objective 4. Two novel antagonists were found against the selected mycotoxins, namely *P. janthinellum* Pj46SD and *T. asperelloides* Ta39KR. Besides, other indigenous potential antagonists also had potential as good biocontrol agents against mycotoxigenic fungi and mycotoxins. *T. asperelloides* Ta39KR also shown

antagonistic ability against three mycotoxigenic species producing two mycotoxins. My overall original contribution to this study and the field of work was by providing background information on the mycodiversity of both mycotoxigenic and non-mycotoxigenic fungi in pioneer grain maize agro-ecosystem, and new knowledge on the potential of indigenous biocontrol candidates from Malaysia in controlling mycotoxigenic fungi and mycotoxin production in Malaysian agro-ecosystem, particularly the booming mass-cultivation of grain maize.

7.3 Recommendations for future research

Several future works are therefore recommended:

1. Wider spatial and temporal sampling of fungi in grain maize agro-ecosystems to establish more thorough information on the fungal community that exist in the pioneer grain maize farms.
2. Using molecular method such as Next Generation Sequencing (NGS) to obtain a large-scale and accurate information on the community structure that reside within both farms. NGS is rapid and could also identify the viable but non-culturable fungal strains which are otherwise difficult to be isolated using the traditional culture method.
3. Incorporating ecophysiological conditions such as temperature, water activity, carbon dioxide concentrations, and other appropriate abiotic factors that represent the real-field conditions, to accurately determine the resilient indigenous antagonists that can survive and remain effective under the harsh field environment.

4. The selected antagonists can be further tested in greenhouse and *in planta* to determine and confirm their efficacy.
5. The selected antagonists could also be tested against other mycotoxins, mycotoxigenic fungi, and other fungal pathogens, and on other crop commodities.
6. The selected antagonists could be formulated into cocktails, and tested against a wide range of mycotoxigenic fungi and their mycotoxins.
7. The selected antagonists can be formulated for commercialisation, and different formulations and adjuvants could be tested for better delivery and efficacy in the field.

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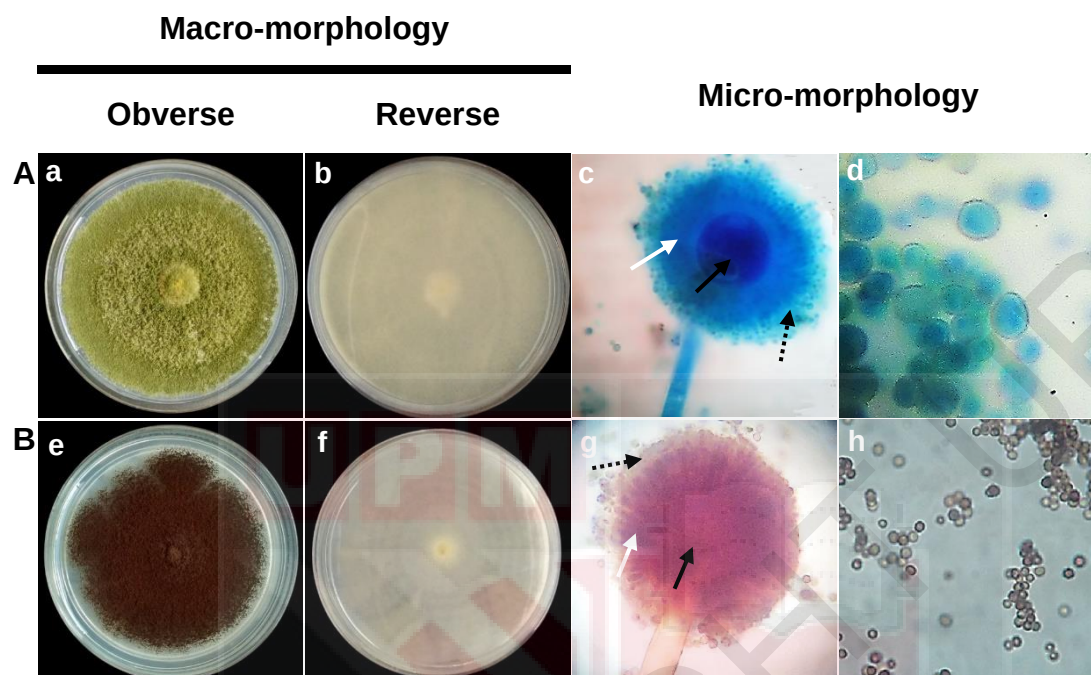


APPENDICES

Appendix A: Samples of debris, kernel, soil, and tassel obtained from grain maize farms in Kampong Dadong and Rhu Tapai, in Terengganu, Malaysia

	Debris	Kernel	Soil	Tassel
Kampong Dadong				
Rhu Tapai				

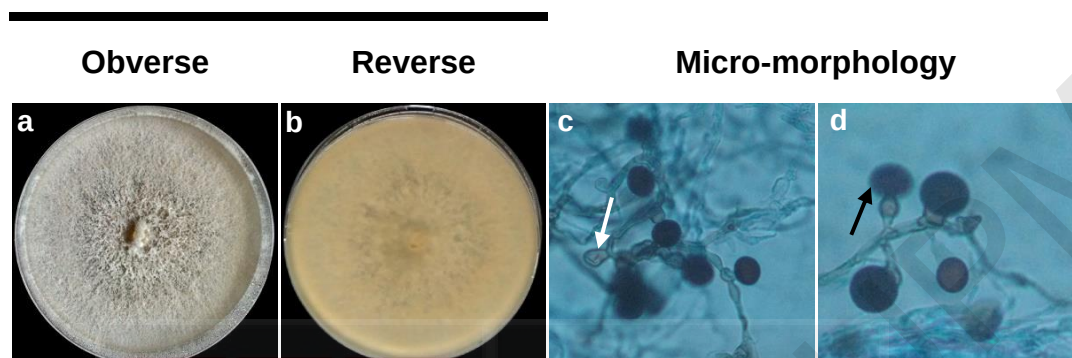
Appendix B: Morphology of *Aspergillus* spp. on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days



(A) *A. flavus*, (B) *A. niger*. *A. flavus*: (a) Colony appears plane and velutinous, with some central conspicuous and floccose mycelia, and yellow-green conidia, (b) colony reverse is in pale brown, (c) vesicle spherical (black arrow), bearing both metulae (white arrow) and phialides (dashed arrow), (d) conidia are spherical and sub-spheroidal. *A. niger*: (e) Colony texture is plane, velutinous, of low, with dark brown conidia, (f) colony reverse is in pale yellow, (g) vesicles spherical (black arrow), bearing metulae (white arrow) and phialides (dashed arrow), (h) conidia are spherical and brown. Adapted from Pitt & Hocking (2009).

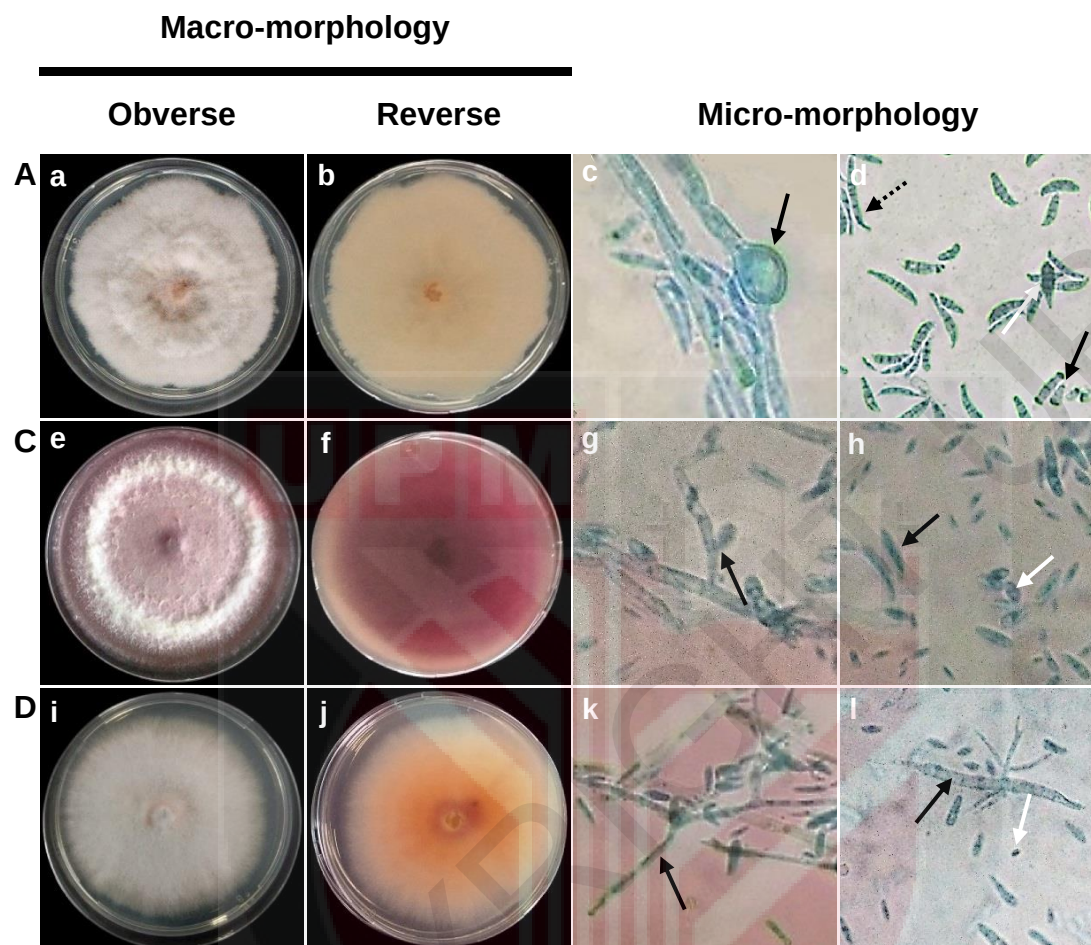
Appendix C: Morphology of *Bjerkandera adusta* on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days

Macro-morphology



(a) Colony is cottony and off-white, (b) colony reverse is pale brown, (c) generative hyphae (monomitic hyphal system) with clamp connection (white arrow), (d) spores are ellipsoidal in shape. Adapted from Jung *et al.* (2014) and Watanabe (2010).

Appendix D: Morphology of *Fusarium* spp. on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days

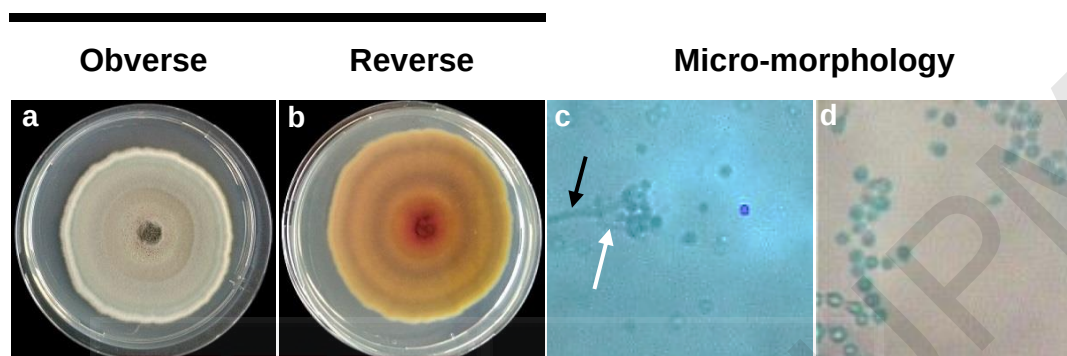


(A) *F. incarnatum*, (B) *F. longipes*, (C) *F. proliferatum*, (D) *F. verticillioides*.

***F. incarnatum*:** (a) Colony is of white, dense, floccose mycelium, (b) colony reverse is in pale salmon, (c) chlamyospore is globose and solitary in the hyphae (black arrow), (d) microconidia are pyriform (pear-shaped) with one septate (black arrow), mesoconidia in fusoid with three septate (white arrow), macroconidia are slender with curved and tapered apical cell, and foot-shaped basal cell. ***F. proliferatum*:** (e) Colony is in purple-violet with age, (f) colony producing violet pigment, (g) polyphialides conidiogenous cells, (h) macroconidia are slender, 3 – 5 septate, and straight with curved apical cell and poorly developed basal cell, microconidia are pyriform with no septate. ***F. verticillioides*:** (i) Colony is in white mycelia but develop violet pigments with age, (j), colony pigment is in grayish orange, (k) monophialides conidiogenous cells, and can appear like a 'rabbit ear' (black arrow), (l) macroconidia are long and slender, slightly falcate (black arrow), microconidia are club-shaped with no septate (white arrow). Adapted from Leslie & Summerell (2008).

Appendix E: Morphology of *Penicillium janthinellum* on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days

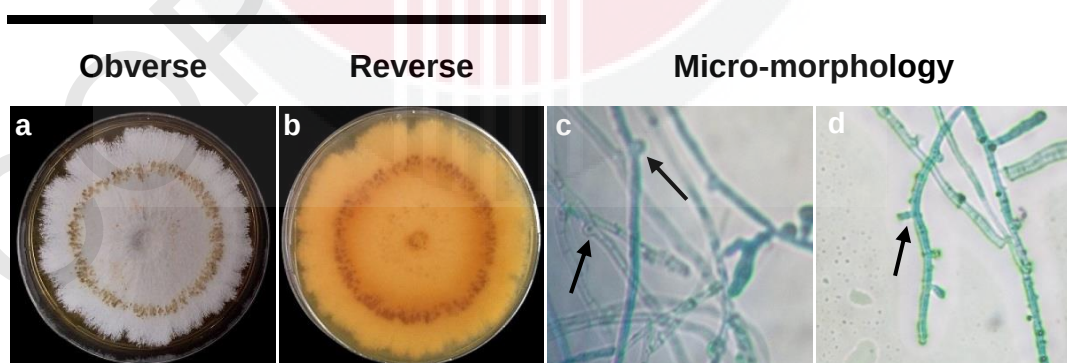
Macro-morphology



(a) Colony obverse appears dense with multiple colours of white, grey green, and buff, (b) colony reverse is pale, brownish, with bright red centre, (c) conidiophore having slender stipe (black arrow) and monoverticillate (non-branched; white arrow), (d) conidia are spherical to ellipsoidal. Adapted from Pitt & Hocking (2009).

Appendix F: Morphology of *Schizophyllum commune* on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days

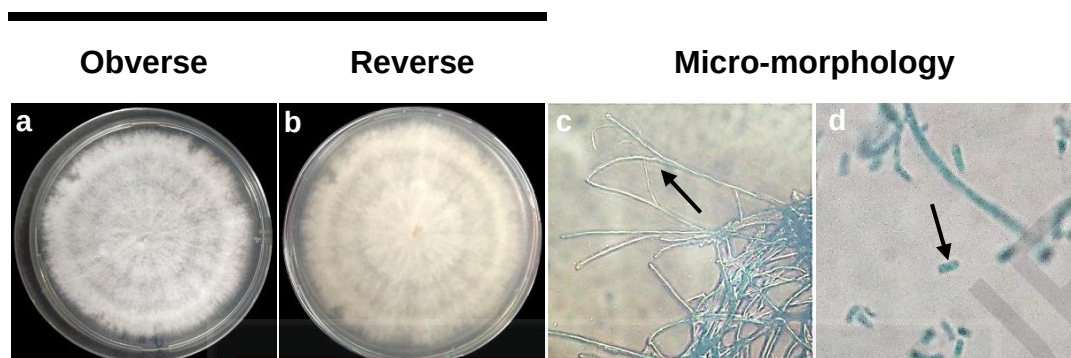
Macro-morphology



(a) Colony is white and woolly, (b) colony reverse in yellow-brown, (c) septate hyphae with clamp connections (black arrows), (d) hyphae bearing spicules (black arrow). Adapted from Hanafusa *et al.* (2016).

Appendix G: Morphology of *Trametes cubensis* on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days

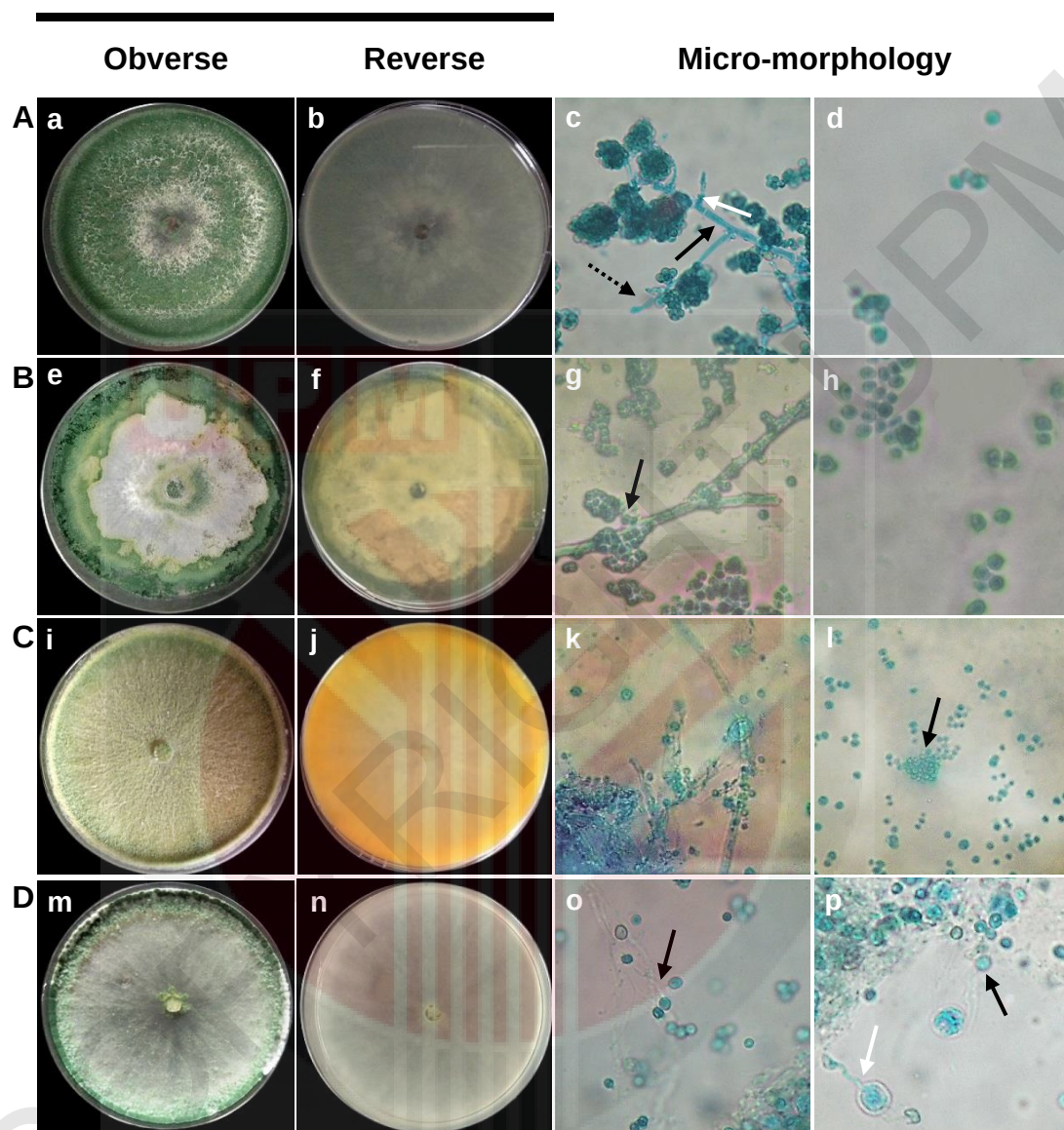
Macro-morphology



(a) Colony obverse is white, (b) colony reverse is white, (c) hyphal system are trimitic (have generative, skeletal, and branched hyphae; generative hyphae in black arrow) and hyaline, (d) basidiospores elongate and cylindrical (black arrow). Adapted from Ediriweera *et al.* (2021).

Appendix H: Morphology of *Trichoderma* spp. on Potato Dextrose Agar (PDA) after incubation at 30°C for seven days

Macro-morphology



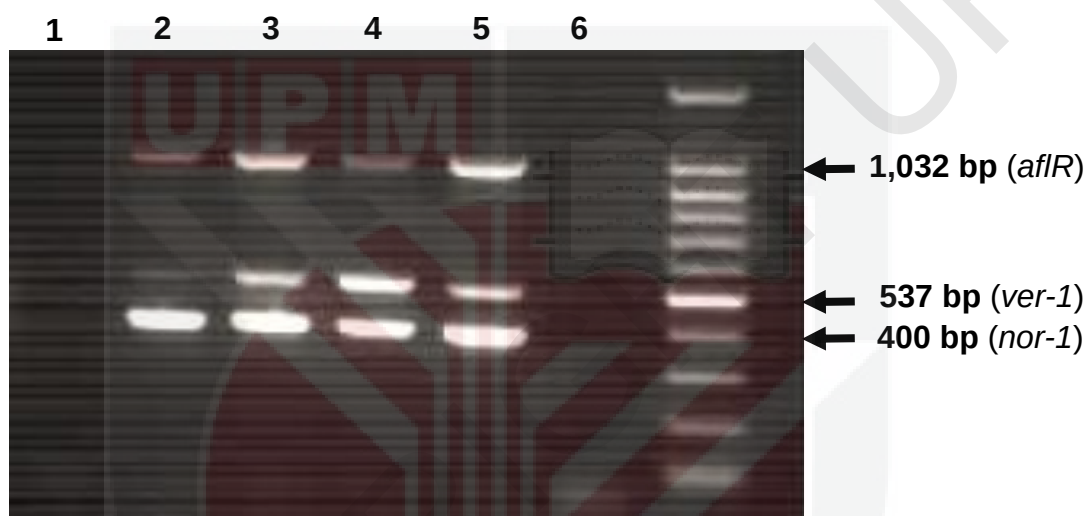
(A) *T. asperelloides*, (B) *T. asperellum*, (C) *T. harzianum*, (D) *T. yunnanense*. *T. asperelloides*: (a) Colony is downy and turned pale to dark green with age, and often form concentric rings, (b) colony reverse is greyish with no pigment, (c) conidiophores are branched (black arrow), with secondary branches paired and asymmetric (white arrow) and bearing a single phialide or cruciate whorl of two to three phialides (dashed arrow), (d) conidia are subglobose. *T. asperellum*: (e) Colony is dark green with age and often form concentric rings, (f) colony reverse is greyish, (g) conidiophores are branched with secondary branches are in paired (black arrow) bearing a cruciate whorl of two to three phialides, (h) conidia are subglobose to ellipsoidal. *T. harzianum*: (i) Colony is white to yellow mycelium with yellow green conidia developing over the whole colony, (j) colony reverse is yellowish, (k) conidiophores branches and bearing phialides irregularly, (l) conidia are singly,

often in clusters (black arrow), globose, sub-globose, or ellipsoidal in shape. ***T. yunnanense***: (m) Colony is cottony, conidia can form in hemispherical pustule (those in green-white on top of the mycelium and near periphery) that either discrete or confluent and grow continuously over the colony centre, (n) colony reverse is transparent/pale, (o) conidiophores branched commonly unilateral with primary branches bearing a single phialide, phialides appressed (black arrow), (p) conidia are ellipsoidal (black arrow), chlamydospores are sub-globose and at terminal (white arrow). Adapted from Samuels *et al.* (2010) and Pitt & Hocking (2009).

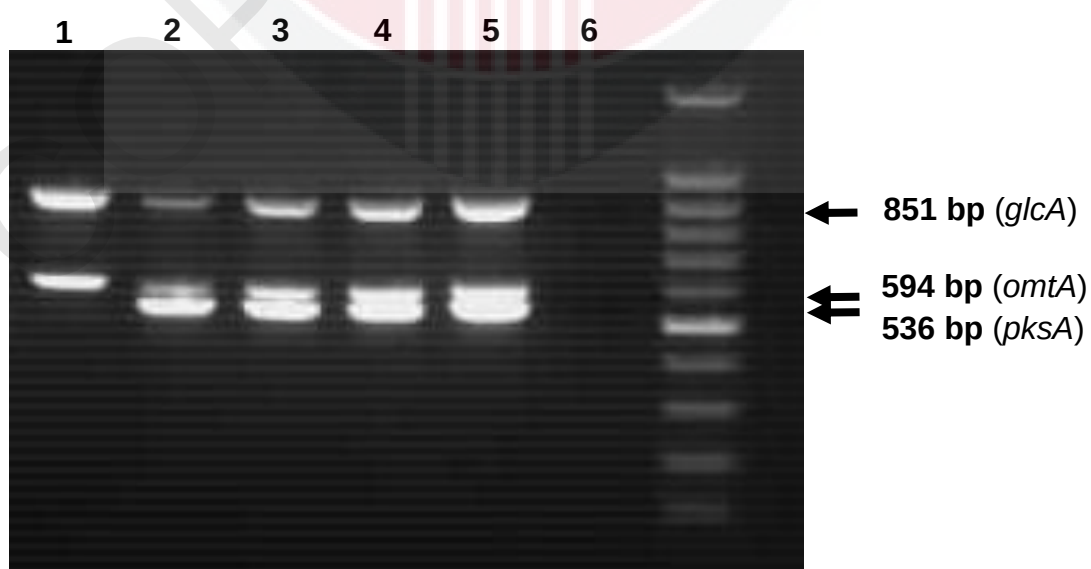


Appendix I: Representative DNA band (amplicon) of seven tested aflatoxin biosynthesis genes (i.e., *aflR*, *nor-1*, *ver-1*, *pksA*, *omtA*, *aflJ*, *verA*) and one sugar cluster gene (i.e., *glcA*) following amplification using polymerase chain reaction (PCR). The band size was estimated using GelPilot 100 bp Plus DNA ladder. Lane 1 – Af1KD, Lane 2 – Af4TR, Lane 3 – Af6KR, Lane 4 – Af7KR, Lane 5 – positive control (*Aspergillus flavus* NRRL 3357), Lane 6 – negative control (PCR reagents without DNA template)

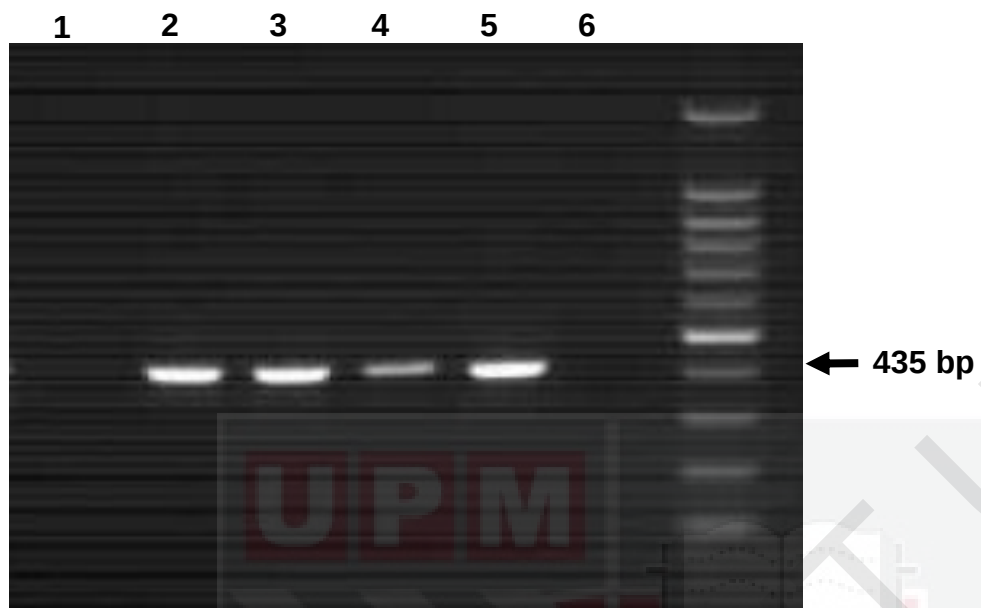
(A) *aflR*, *nor-1*, and *ver-1*



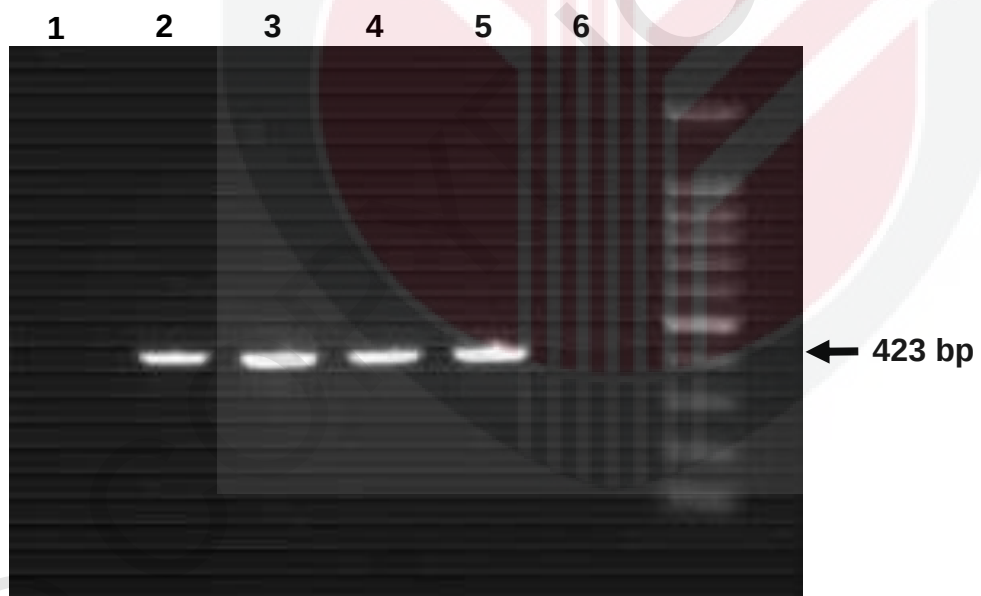
(B) *pksA*, *omtA*, and *glcA*



(C) *afIJ*



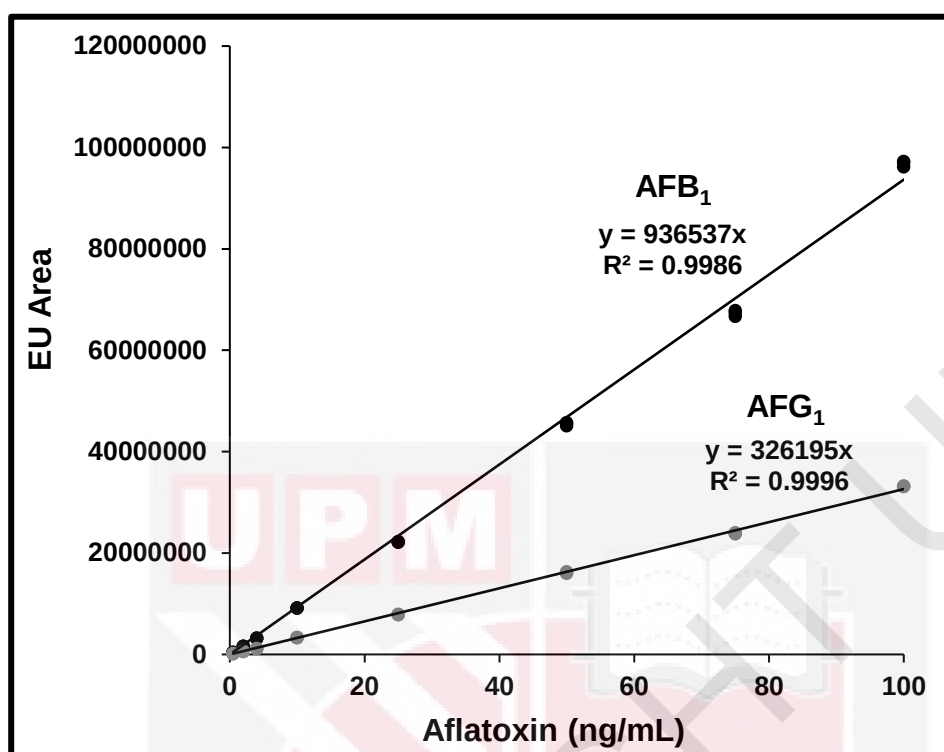
(D) *verA*



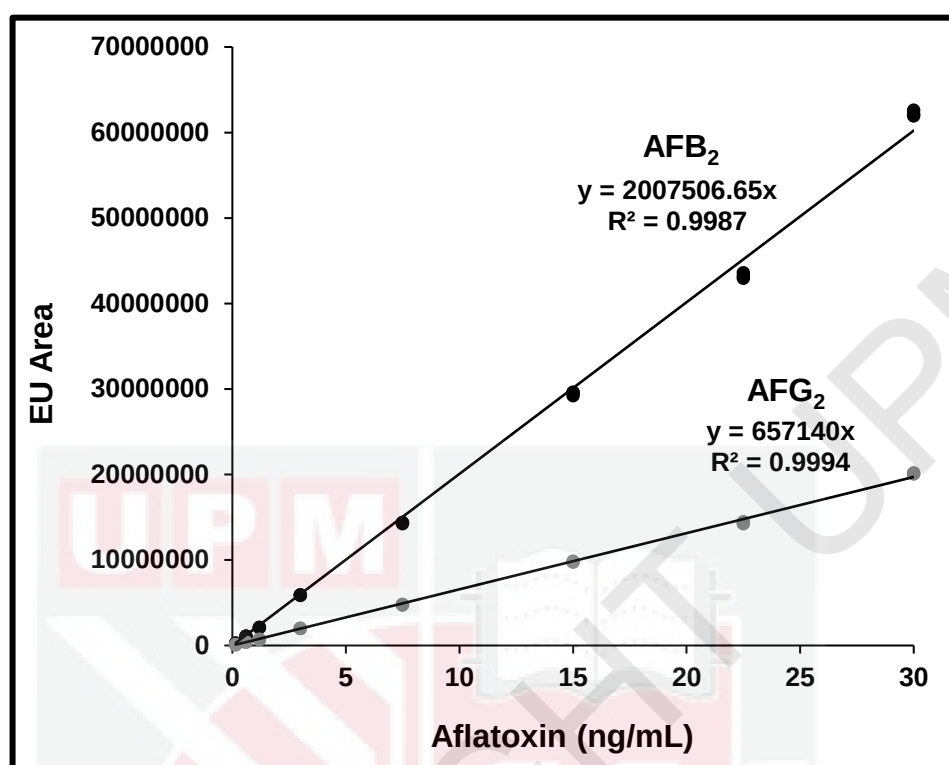
Appendix J: List of mycotoxin standards used for mycotoxin quantification by high performance liquid chromatography

Mycotoxin	Purity (%)	Concentration (µg/mL)	Stock solvent
Aflatoxins			
<i>Mix standard</i>	AFB ₁ - 99	AFB ₁ - 1	methanol
AFB ₁	AFB ₂ - 99	AFB ₂ - 0.3	
AFB ₂	AFG ₁ - 99.7	AFG ₁ - 1	
AFG ₁	AFG ₂ - 98.5	AFG ₂ - 0.3	
AFG ₂			
Deoxynivalenol	99.5	103.3	acetonitrile
Fumonisin			
<i>Mix standard</i>	FB ₁ - ≥97	FB ₁ - 50	acetonitrile
FB ₁	FB ₂ - ≥94	FB ₂ - 50	
FB ₂			
HT-2	≥98	100	acetonitrile
T-2	≥98	100	acetonitrile
Ochratoxin A	99.5	50.2	benzene:acetic acid (99:1)
Zearalenone	≥98	100	acetonitrile

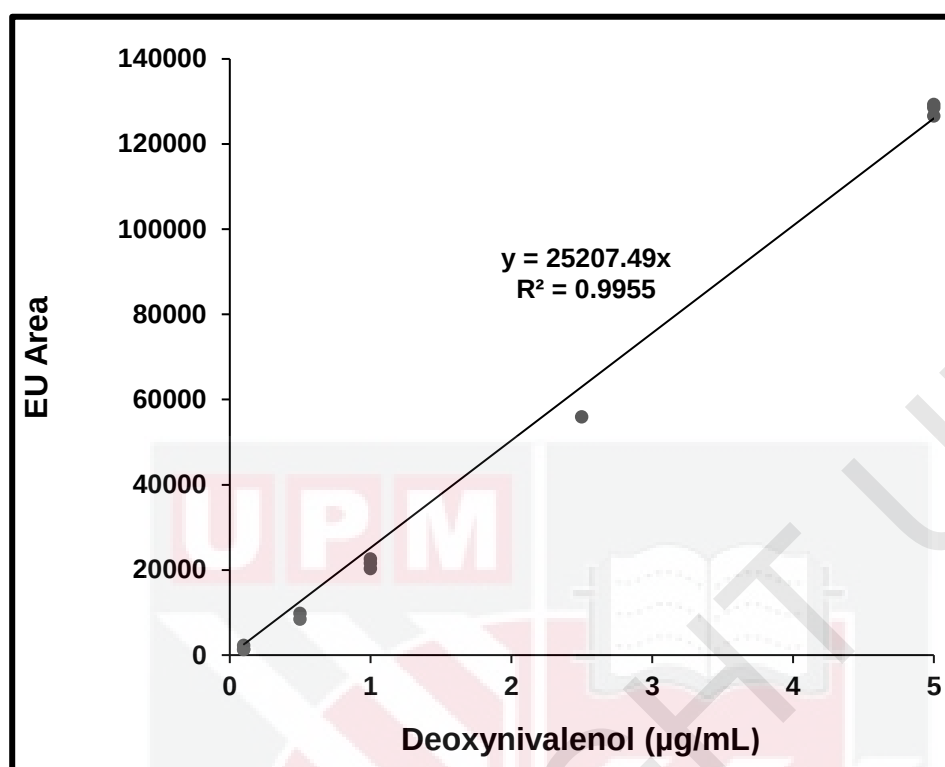
Appendix K: Standard curve of aflatoxins B₁ and G₁



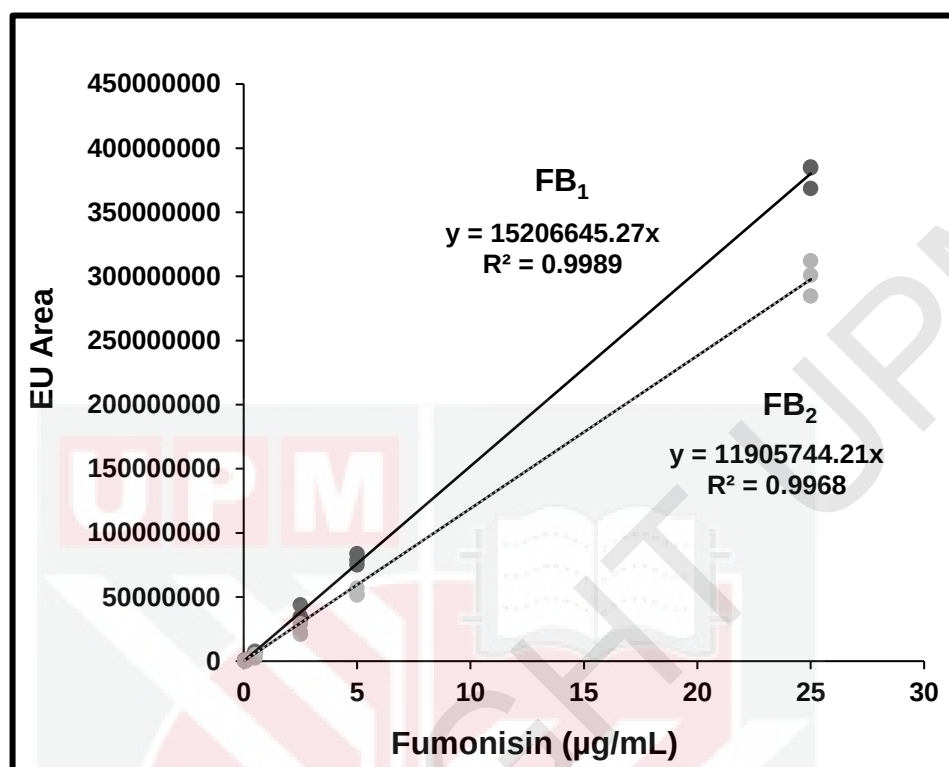
Appendix L: Standard curve of aflatoxins B₂ and G₂



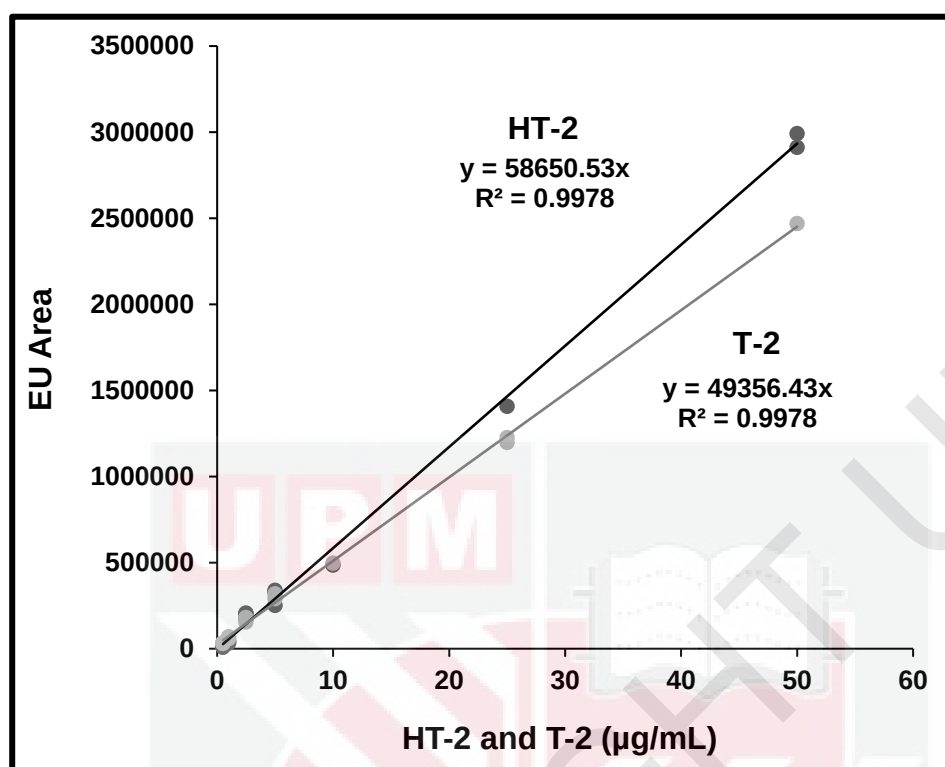
Appendix M: Standard curve of deoxynivalenol



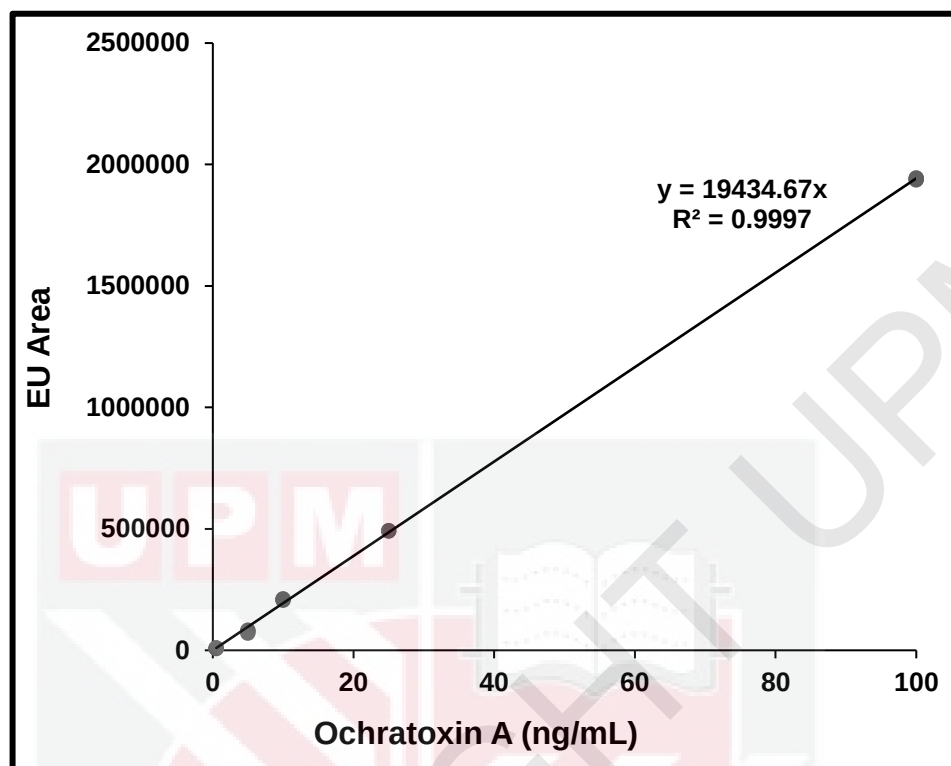
Appendix N: Standard curve of fumonisins B₁ and B₂



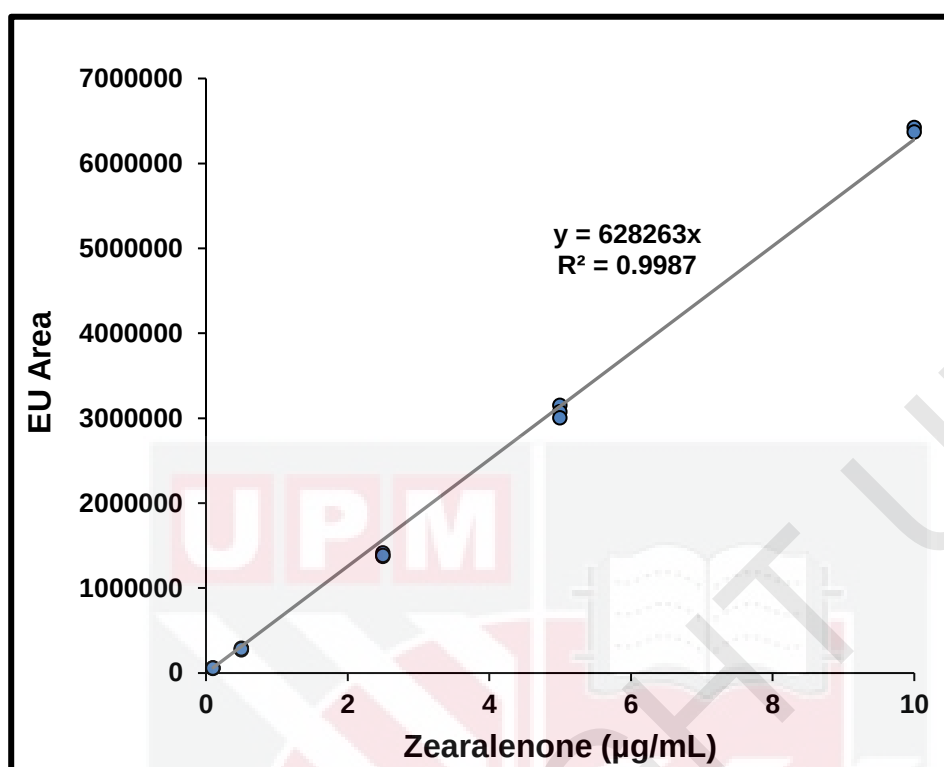
Appendix O: Standard curve of HT-2 and T-2



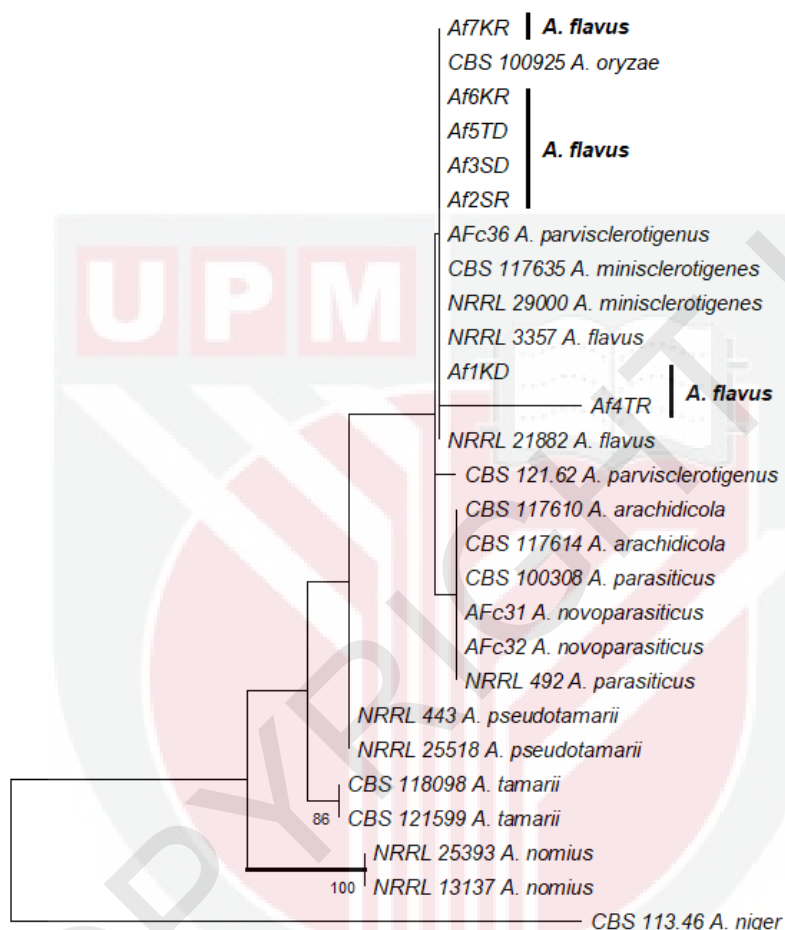
Appendix P: Standard curve of ochratoxin A



Appendix Q: Standard curve of zearalenone



Appendix R: Maximum likelihood tree of *Aspergillus* section *Flavi* based on ITS dataset. Isolates with the initial code *Af* labelled as *A. flavus* in black are isolates tested in the present study. Branches in bold are highly supported with bootstrap values of > 90%. Bootstrap values of < 70% are not shown. *A. niger* strain CBS 113.46 is the outgroup taxon. Scale bars are the number of substitutions per site



Appendix S: Statistical analysis for data of multi-mycotoxin produced by mycotoxigenic strains co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia

Mycotoxin	Fungi	Normality test				Kruskal Wallis		
		Type	Test statistic D^a / W^b	N	p-value	Test statistic H	df	p-value
Aflatoxin B ₁	Af6KR	KS ^a	0.17	42	0.006	25.622	12	0.012
		SW ^b	0.92	42	0.005			
	Af7KR	KS	0.43	40	<0.001	35.031	12	<0.001
		SW	0.55	40	<0.001			
Ochratoxin A	An1KD	KS	0.21	37	<0.001	32.773	12	0.001
		SW	0.82	37	<0.001			
	An3KD	KS	0.18	41	0.002	34.400	12	0.001
		SW	0.83	41	<0.001			
Fumonisin B ₁	Fv21KD	KS	0.29	41	<0.001	36.692	12	0.001
		SW	0.81	41	<0.001			
	Fv92SR	KS	0.244	33	<0.001	25.947	12	0.011
		SW	0.792	33	<0.001			
	Fp9DD	KS	0.181	38	0.003	32.632	12	0.001
		SW	0.805	38	<0.001			
Fumonisin B ₂	Fv21KD	KS	0.264	44	<0.001	38.491	12	<0.001
		SW	0.791	44	<0.001			
	Fv92SR	KS	0.396	37	<0.001	29.044	12	0.004
		SW	0.503	37	<0.001			
	Fp9DD	KS	0.224	37	<0.001	32.577	12	0.001
		SW	0.771	37	<0.001			

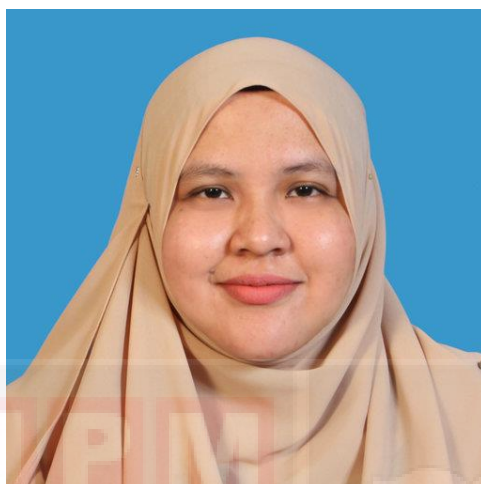
Mycotoxigenic fungal strains: Af6KR, Af7KR – *Aspergillus flavus* strains; An1KD, An3KD – *A. niger* strains; Fp9DD – *Fusarium proliferatum* strain; Fv21KD, Fv92SR – *F. verticillioides* strains. ^aKolmogorov-Smirnov test. ^bShapiro-Wilk test.

Appendix T: Mycotoxin inhibition/stimulation (%) of mycotoxigenic strains co-cultivated with antagonist candidates isolated from the grain maize farms in Terengganu, Malaysia.

Antagonist candidate	Mycotoxin inhibition / stimulation (%)										
	Aflatoxin B ₁			Ochratoxin A		Fumonisin B ₁				Fumonisin B ₂	
	Af6KR	Af7KR	An1KD	An3KD	Fp9DD	Fv21KD	Fv92SR	Fp9DD	Fv21KD	Fv92SR	
Ba42DD	23.61	100	-17.74	-26.21	-8.30	73.38	80.33	-21.66	90.93	100	
Pj46SD	75.34	89.11	4.94	54.90	56.80	22.71	42.07	59.74	37.48	65.50	
Pj81SR	65.50	55.71	47.68	43.32	57.00	23.75	67.23	65.04	48.57	81.20	
Sc40DD	-39.85	-48.27	-28.31	64.62	53.40	62.49	71.59	50.92	72.90	100	
Tc28DD	50.76	100	-71.31	75.41	70.71	33.91	83.09	79.74	37.16	100	
Ta39KR	-36.65	100	97.68	94.95	94.64	94.87	78.14	98.24	99.13	96.10	
Ta31KR	12.23	100	97.22	98.27	60.96	94.58	86.61	92.53	98.77	100	
Th20SR	-33.97	100	67.57	89.47	94.50	98.93	66.59	100	99.62	100	
Th24KD	-26.26	100	98.52	100	92.41	98.98	65.33	99.82	99.25	100	
Th33SR	12.43	100	100	95.87	83.53	96.12	68.72	92.78	99.62	100	
Th36SR	-87.49	100	82.23	60.33	92.71	96.89	81.70	99.67	99.20	100	
Ty34KD	-178.86	100	80.69	63.12	79.63	94.72	-22.87	99.13	98.45	96.20	

Data are means of four replicates ($n = 4$). Mycotoxigenic fungal strains: Af6KR, Af7KR – *Aspergillus flavus* strains; An1KD, An3KD – *A. niger* strains; Fp9DD – *Fusarium proliferatum* strain; Fv21KD, Fv92SR – *F. verticillioides* strains. Antagonist candidates: Ba42DD – *Bjerkandera adusta*; Pj46SD, Pj81SR – *Penicillium janthinellum*; Sc40DD – *Schizophyllum commune*; Tc28DD – *Trametes cubensis*; Ta39KR – *Trichoderma asperelloides*; Ta31KR – *T. asperellum*; Th20SR, Th24KD, Th33SR, Th36SR – *T. harzianum*; Ty34KD – *T. yunnanense*. Negative values indicate stimulation of mycotoxins.

BIODATA OF AUTHOR



Siti Nur Ezzati Yazid was born on June 3rd, 1991 at Hospital Segamat, Segamat, Johor darul Ta'azim. She received her primary education at Sekolah Rendah Jalan Gurney (2) (1998-2003), Kuala Lumpur, before continuing with her secondary education at Sekolah Menengah Agama Kuala Lumpur (SMAKL) (2004-2008), Kuala Lumpur. She completed her pre-university education at Universiti Sains Islam Malaysia (USIM), Negeri Sembilan darul Khusus, and continued her tertiary education in Bachelor of Science (Food Biotechnology) at the same university (2010-2014).

She then enrolled for Master of Food Safety and Quality Assurance (MSc) at the Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM), Selangor darul Ehsan (2015-2017) under the supervision of Assoc. Prof. Dr. Nik Iskandar Putra Samsudin. Her MSc project title was "*Formulation of Maize-Based Semi-Synthetic Growth Media for the Ecophysiological Studies of Aflatoxigenic Aspergillus flavus in Maize Agro-Ecosystem*" from which she managed to publish one ISI journal article (Q1), and win the Syarikat Bumi

Sains Award for Poster Presentation under the Food Microbiology category during the International Congress of the Malaysian Society for Microbiology.

She then pursued her Doctor of Philosophy (PhD) at the Institute of Tropical Agriculture and Food Security also in UPM under the same supervisor (2017-2024). Her PhD project title was "*Biocontrol Potential of Indigenous Fungi against Mycotoxigenic Fungi and Mycotoxin Contamination in Grain Maize Agro-Ecosystems in Terengganu, Malaysia*" from which she managed to publish five ISI journal articles (2 Q1, 3 Q2). While completing her PhD, she was awarded with the Graduate Research Fund (GRF) scheme, thus involved in assisting her supervisor in the teaching and learning program. She aspires to become a lecturer / researcher upon the completion of her PhD, and has participated in several national and international research activities throughout her PhD candidature at UPM.

As of March 1st, 2025 her *h*-Index for Scopus was 4 (50 citations), her *h*-Index for ResearchGate was 5 (59 citations), and her *h*-Index for Google Scholar was also 4 (66 citations).

LIST OF PUBLICATIONS

This paper partially forms the Literature Review of this thesis (ISI Q1)

Yazid, S. N. E., Jinap, S., Ismail, S. I., Magan, N., & Samsudin, N. I. P. (2020). Phytopathogenic organisms and mycotoxigenic fungi: Why do we control one and neglect the other? A biological control perspective in Malaysia. *Comprehensive Reviews in Food Science and Food Safety*, 19(2), 643-669. <https://doi.org/10.1111/1541-4337.12541>.

This paper partially forms the Objective 1 of this thesis (ISI Q1)

Yazid, S. N. E., Ng, W. J., Selamat, J., Ismail, S. I., & Samsudin, N. I. P. (2021). Diversity and toxigenicity of mycobiota in grain corn: A case study at pioneer grain corn plantations in Terengganu, Malaysia. *Agriculture*, 11(3), 237. ISI Q1 <https://doi.org/10.3390/agriculture11030237>

This paper partially forms the Objective 3 of this thesis (ISI Q2)

Yazid, S. N. E., Tajudin, N. I., Razman, N. A. A., Selamat, J., Ismail, S. I., Sanny, M., & Samsudin, N. I. P. (2023). Mycotoxigenic fungal growth inhibition and multi-mycotoxin reduction of potential biological control agents indigenous to grain maize. *Mycotoxin Research*, 29, 177-192. ISI-Q2 <https://doi.org/10.1007/s12550-023-00484-4>

This paper partially forms the Objective 2 of this thesis (ISI Q2)

Yazid, S. N. E., Selamat, J., Ismail, S. I., Sanny, M., & Samsudin, N. I. P. (2024). Molecular and aflatoxigenicity analyses of *Aspergillus flavus* isolates indigenous to grain corn in Malaysia; potentials for biological control. *Journal of Applied Microbiology*, 135(6), 145.

This paper partially forms the Objective 4 of this thesis (ISI Q2)

Yazid, S. N. E., Selamat, J., Ismail, S. I., Sanny, M., & Samsudin, N. I. P. (2025). Efficacy of fungal antagonists against aflatoxins, ochratoxin A, and fumonisins at different pathogen:antagonist inoculum ratios on grain corn agar and grain corn kernel. *Journal of Applied Microbiology*. In press.

LIST OF CONFERENCES

POSTER PRESENTATION

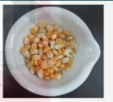
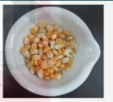
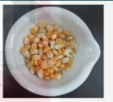
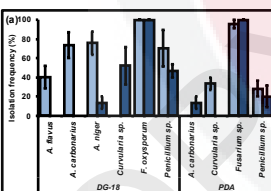
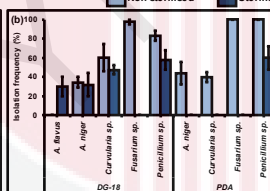
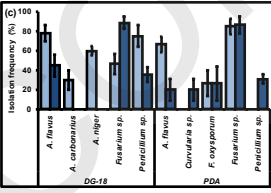
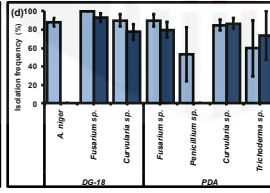
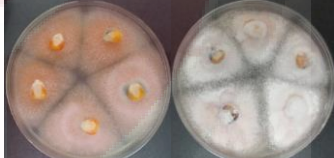
Poster presented at the Food Safety Postgraduate Mobility Programme (2018), Bogor, Indonesia.

Evaluation of Mycological Contamination in Grain Maize: A New Crop Source for Wealth Generation in Malaysia

Siti Nur Ezzati YAZID^a, *Nik Iskandar Putra SAMSUDIN^{a,b}, Jinap SELAMAT^{a,b}, Siti Idera ISMAIL^c

^aLaboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, UPM
^bDepartment of Food Science, Faculty of Food Science and Technology, UPM
^cDepartment of Plant Protection, Faculty of Agriculture, UPM




INTRODUCTION	OBJECTIVE															
- Grain maize (<i>Zea mays</i> L.) is an important cereal grain widely used in poultry and livestock diets as a major source of energy, and it has been cultivated in many parts of the world. - Malaysia recently embarked in the grain maize commercial planting in an effort to reduce the import dependency for feed materials. - However, Malaysian tropical climate is highly conducive for the proliferation of microfungi including the mycotoxigenic ones, and is therefore at risk of the subsequent mycotoxin contamination, including in grain maize. - The mycotoxigenic fungal colonisation of maize crops on the field may cause damages (e.g., ear rot, stalk rot, seedling blight) which result in severe quality, safety and yield reduction. - Furthermore, mycotoxins may persist even after grain processing, thereby contaminating the resulting animal feed. - Ingestion of mycotoxin-contaminated feed exposes poultry and livestock to several diseases such as liver and gastrointestinal damages, or to a lesser extent, cause a feed refusal. - Common mycotoxins that have been found in maize are T-2 toxin, HT-2 toxin, fumonisins, zearalenone, nivalenol, and deoxynivalenol.	To evaluate the level and biodiversity of mycological contaminants in selected pioneer grain maize farms, in Terengganu, Malaysia.															
MATERIALS AND METHODS																
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr style="background-color: #2c3e50; color: white;"> <th></th> <th>Debris</th> <th>Kernel</th> <th>Soil</th> <th>Tassel</th> </tr> </thead> <tbody> <tr> <td style="text-align: center;">Kg. Dadong</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td style="text-align: center;">Rhu Tapai</td> <td></td> <td></td> <td></td> <td></td> </tr> </tbody> </table>			Debris	Kernel	Soil	Tassel	Kg. Dadong					Rhu Tapai				
	Debris	Kernel	Soil	Tassel												
Kg. Dadong																
Rhu Tapai																
   Common diseases of grain maize that are caused by fungal pathogens: (a–b) ear rot / cob rot, and (c) stalk rot.	Sampling was done at two pioneer grain maize farms in Terengganu, Malaysia. Samples were subjected to surface and non-surface sterilisation to allow for slow- and fast-growing fungal isolates, respectively. Both treatments were directly cultivated onto PDA (total fungi) and DG-18 (xerophilic fungi) growth media, and incubated at 30°C for seven days.															
RESULTS AND DISCUSSION																
 (a) Kernels from Kg. Dadong  (b) Tassels from Kg. Dadong  (c) Kernels from Rhu Tapai  (d) Tassels from Rhu Tapai Isolation frequency (%) of (a) kernels, (b) tassels from Kg. Dadong and (c) kernels, (d) tassels from Rhu Tapai following direct plating on DG-18 and PDA, and incubation at 30°C for seven days. Data are means of triplicate (n = 3) with bars indicating standard error (± SE).																
 The air- and soilborne <i>Fusarium</i> spp. were found to be the predominant isolates obtained from the maize farm. Other mycotoxigenic isolates such as <i>Aspergillus</i> spp. were also found but at lower frequency. - The kernel and tassel samples were predominated by <i>Fusarium</i> spp. regardless the origin of samples, the growth media used or the surface sterilisation treatment. - Considering that <i>Fusarium</i> spp. are slow-growing fungi and often outgrown by other fast-growing pre-harvest fungi (e.g., <i>Aspergillus</i> spp.), the high <i>Fusarium</i> spp. isolation frequency obtained indicates that grain maize is a suitable breeding ground for its proliferation. - This in turn indicates the potential for various <i>Fusarium</i>-related maize diseases (seedling blight, stalk rot, cob rot) and also the contamination of <i>Fusarium</i>-related mycotoxins, which consequently will reduce the yield, safety and quality of the grain maize harvest, and incur economic losses. - This is in agreement with a previous study which indicated the contamination of <i>Fusarium</i> spp. in maize samples collected from maize farms in different states in Malaysia (e.g., Johor, Pahang, Sabah) (Zainudin <i>et al.</i>, 2011). <i>Fusarium</i> spp. have also been predominantly found in pre-harvest maize samples in Italy (Covarelli <i>et al.</i>, 2011).																
CONCLUSIONS	REFERENCES															
- The kernels and tassels of grain maize samples collected from Terengganu, Malaysia were contaminated with various fungal genera with <i>Fusarium</i> spp. being the most predominant genus. - Besides <i>Fusarium</i> spp., other mycotoxigenic fungal species (<i>Aspergillus</i> spp.) were also isolated which in turn pose potentials for the production of aflatoxins and ochratoxin A in the maize kernels. - Future works as listed below are warranted to further validate the findings.	- Covarelli, L., Beccari, G., & Salvi, S. (2011). Infection by mycotoxigenic fungal species and mycotoxin contamination of maize grain in Umbria, central Italy. <i>Food and Chemical Toxicology</i> 49(9), 2365-2369. - Zainudin, N. A. I. M., Sidique, S. N. M., Johari, N. A., Razak, A. A., & Salleh, B. (2011). Isolation and identification of <i>Fusarium</i> species associated with <i>Fusarium</i> ear rot disease of corn. <i>Pertanika Journal of Tropical Agricultural Science</i> 34(2), 325-330.															
CORRESPONDING AUTHOR	FUTURE WORK	ACKNOWLEDGEMENT														
 E-mail : nikiskandar@upm.edu.my Telephone : +603 – 8946 8259 Facsimile : +603 – 8942 3552	- To molecularly identify the fungal isolates - To identify the types and quantity of mycotoxins produced using HPLC-FLD - To search for indigenous biological control agents to inhibit the fungal proliferation	The authors would like to acknowledge the financial support received from the Malaysian Ministry of Education (MOE) under the High Impact Centre of Excellence (HiCoE) grant scheme (vot no.: 6369114).														

ORAL PRESENTATION

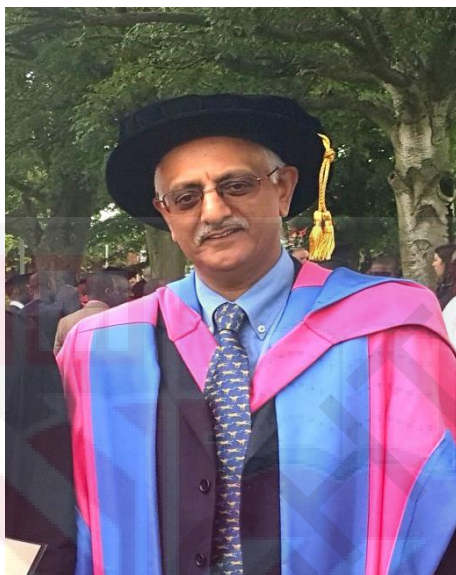
Siti Nur Ezzati Yazid, Jinap Selamat, Siti Izera Ismail, Maimunah Sanny, and Nik Iskandar Putra Samsudin (2023). Effect of different pathogen:antagonist inoculum ratios on aflatoxin and fumonisin production by *Aspergillus flavus* and *Fusarium verticillioides* indigenous to grain maize of Malaysia. The 3rd International Joint Symposium on Agriculture and Food Security, Chiang Rai, Thailand.

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Siti Nur Ezzati Yazid, Jinap Selamat, Siti Izera Ismail, and Nik Iskandar Putra Samsudin (2019). Biodiversity and mycotoxigenic potentials of mycobiota from pioneer grain maize plantations in Malaysia. Universiti Putra Malaysia – Kasetsart University Postgraduate Colloquium and Research Forum, Selangor, Malaysia.

IN LOVING MEMORY

PROFESSOR NARESH MAGAN, BSc, MSc, PhD, DSc
July 1953 – April 2023



The earlier part of this PhD work is dedicated to the late Prof. Naresh MAGAN, who through my main supervisor (Assoc. Prof. Dr. Nik Iskandar Putra SAMSUDIN), who was in turn Prof. MAGAN's former PhD student (2012 – 2015; Cranfield University, United Kingdom), taught me a lot on biological control of mycotoxigenic fungi. In 2020, we published a review paper together on the control of phytopathogenic organisms and mycotoxigenic fungi in the *Comprehensive Reviews in Food Science and Food Safety* journal. That review paper forms a part of the Literature Review section of my PhD thesis.

Prof. MAGAN obtained a BSc (honours) in Botany (1976) and MSc in Plant Pathology (1977) from the University of Exeter, UK, and PhD in Agricultural Botany from the University of Reading, UK (1982). He then worked as a Postdoctoral Scientist at Rothamsted Research, Harpenden (1982 – 1984), and

as a Senior Science Officer at Glasshouse Crops Research Institute, Littlehampton (1984 – 1986). He joined Cranfield University in 1986 as a Lecturer, promoted to a Senior Lecturer in 1993, Reader (equivalent to Associate Professor) in 1997, and Personal Chair (equivalent to full Professor) in Applied Mycology in 1999. In 2013, he was awarded with the Doctor of Science (DSc) degree by Cranfield University for his research on "Ecophysiology of Spoilage and Mycotoxigenic Fungi".

In a research and academic career spanning nearly 50 years, Prof. MAGAN has produced nearly 80 PhD students and 100 MSc students, and published nearly 400 scientific papers including articles, books, book chapters, and reviews (Scopus *h*-Index 66; Google Scholar *h*-Index 87). He was an active member of various scientific societies including *Association of Applied Biologists*, *British Mycological Society* (President, 2011 – 2012), *British Society of Plant Pathology*, *Society for Applied Microbiology*, and *Society of General Microbiology*, and scientific journals including *Fungal Biology* (formerly known as *Transactions of the British Mycological Society*; 1896 –1989, and later *Mycological Research*; 1989 – 2010), *International Journal of Food Microbiology*, *Journal of Applied Microbiology*, *Letters in Applied Microbiology*, and *World Mycotoxin Journal*. His passing after a short illness is certainly a great loss to his family, friends, and the scientific community as a whole, particularly those who are working in the areas of mycology and mycotoxins. Nevertheless, his contributions will live on through the hearts and minds of his many students all over the world.

"...to the world, you were just a teacher, but to your students, you were the world..."