EFFECT OF TEMPERATURE, CARBON DIOXIDE AND WATER ACTIVITY ON PRODUCTION OF PARENT AND MASKED MYCOTOXINS BY *Fusarium* spp. AT GERMINATION STAGE

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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December 2020

Chairman : Professor Jinap binti Selamat, PhD
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Maize is an important crop cultivated for food and feed in different parts of the world. Like any cereal, maize grain is susceptible to mycotoxin contamination on the farm, during storage and processing. Therefore, the aims of the present work were to (1) evaluate the biodiversity of *Fusarium* spp. in maize and soil samples collected from different farms, (2) access the effects of climate change factors (a_w, CO_2 and temperature) on the production of parent and masked mycotoxins in maize-based growth medium (*in vitro*), and, (3) determine the effect of climate change factors (a_w, CO_2 and temperature) on gene expression and production of parent and masked mycotoxins in germinating and autoclaved maize kernels by *F. verticillioides* and *F. graminearum*. A total of 68 *Fusarium* spp. were isolated from maize and soil samples. Molecular identification by DNA sequencing of TEF-1α gene regions confirmed the isolates. The fungal isolates found were *F. verticillioides, F. graminearum,* and *F. incarnatum*. *Fusarium* spp. contamination was found to be significantly higher in maize ($p < 0.05$) as compared to the soil samples regardless of the growth medium used. All representative isolates tested using LC-MS/MS were found to be mycotoxigenic. *F. verticillioides* tested showed Fumonisin production ability, FB_1 (12.0 ± 0.9 to 15,200 ± 432 µg/kg), FB_2 (3.39 ± 0.70 to 2510 ± 692 µg/kg) and FB_3 (12.0 ± 0.4 to 20.1 ± 2.1 µg/kg). While for *F. graminearum* DON (4,760 ± 580 µg/kg), 3-ADON (3,980 ± 62 µg/kg) and ZEN (50 ± 0.1 µg/kg) was detected and *F. incarnatum* produced FB_1 (14.3 ± 1.2 to 4,670 ± 835 µg/kg). *F. verticillioides* and *F. graminearum* were further selected because *F. incarnatum* could not withstand elevated temperature. *F. verticillioides* was acclimatised at 30 and 35°C while *F. graminearum* at 30 and 33°C. The effect of a_w, CO_2 and temperature were tested on the growth and mycotoxin production maize-based medium. *F. verticillioides* appears to be more tolerant to elevated temperature, a_w and CO_2 as compared to *F. graminearum*. However, *F. verticillioides* produced FB_1 (29.07 ± 6.70 to 140.49 ± 1.45 µg/kg) and FB_2 (21.46

± 14.10 to 32.17 ± 0.54 µg/kg). For the studies on gene expression, the total fungal biomass of the isolate was drastically reduced which was suspected to occur as a result of the effect of climate change factors. This negatively affected the extraction of RNA for further determination of the mycotoxigenic gene expression. However, the results obtained in the present study revealed that mycotoxins were detected in germinating and autoclaved maize kernel by those two acclimatised isolates. The optimum conditions for mycotoxin production were detected at 0.98 a_w, 400 ppm CO_2 and 30°C for both isolates in germinating and autoclaved maize kernel. However, masked mycotoxin was not detected in this study. At 30°C, the mycotoxins produced by *F. verticillioides* were FB_1 and FB_2 while, *F. graminearum* produced DON, ZEA and α-ZEA. Comparing *Fusarium* spp. and other species, *Aspergillus* spp. grow faster and have a high resistance to temperature than *Fusarium* spp. Therefore, more emphasis must be given to such species. However, it must be noted that the production of mycotoxins other than the targeted ones by these isolates may be possible under the effects of climate change factors. Also, further acclimatisation of the isolates to both temperature and CO_2 might affect the mycotoxigenic fungi due to their flexibility. Therefore, more research is also needed in this area to determine the general types of mycotoxins produced.

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

KESAN SUHU, KARBON DIOKSIDA DAN AKTIVITI AIR TERHADAP PENGHASILAN MIKOTOKSIN UTAMA DAN TERSEMBUNYI OLEH *Fusarium* spp. PADA PERINGKAT PECAMBAHAN

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Jagung adalah tumbuhan yang sangat penting di beberapa kawasan di dunia sebagai makanan bagi manusia dan haiwan. Seperti bijirin yang lain, bijirin jagung juga mudah terdedah kepada pencemaran mikotoksin di ladang, semasa penyimpanan dan pemprosesan. Oleh itu, objektif kajian ini adalah untuk; (1) menilai biodiversiti *Fusarium* spp. di dalam sampel jagung dan tanah yang diambil dari beberapa ladang jagung, (2) mengakses kesan faktor-faktor perubahan iklim (suhu, karbon dioksida dan aktiviti air) terhadap pengeluaran mikotoksin utama dan mikotoksin tersembunyi di dalam medium pertumbuhan berasaskan jagung *(in vitro*) dan, (3) mengenalpasti kesan faktor-faktor perubahan iklim (suhu, karbon dioksida dan aktiviti air) terhadap ekspresi gen dan penghasilan mikotoksin utama dan mikotoksin tersembunyi. Sejumlah 68 *Fusarium* spp. telah diasingkan daripada sampel jagung dan tanah. Pengenalan molekul menggunakan penjujukan DNA TEF-1α kawasan gen telah memastikan isolat tersebut. Tanpa mengambilkira media yang digunakan, jumlah bilangan kulat iaitu *F. verticillioides, F. graminearum,* and *F. incarnatum*. *Fusarium* spp. yang dihasilkan daripada sampel jagung adalah lebih tinggi ($p < 0.05$) berbanding sampel tanah. Semua isolat diuji menggunakan LC-MS/MS didapati adalah mikotoksigenik. *F. verticillioides* menunjukkan kebolehan untuk menghasilkan Fumonisin, FB_1 (12.0 ± 0.9 hingga 15,200 ± 432 µg/kg), FB_2 (3.39 ± 0.70 hingga 2510 ± 692 µg/kg) dan FB_3 (12.0 ± 0.4 hingga 20.1 ± 2.1 µg/kg). Manakala, untuk *F. graminearum*, DON (4,760 ± 580 µg/kg), 3-ADON (3,980 ± 62 µg/kg) dan ZEN (50 ± 0.1 µg/kg) telah dikesan dan *F. incarnatum* menghasilkan FB_1 (14.3 ± 1.2 hingga 4,670 ± 835 µg/kg). Hanya *F. verticillioides* dan *F. graminearum* telah dipilih selanjutnya kerana *F. incarnatum* tidak boleh menahan suhu yang tinggi. *F. verticillioides* telah disesuaikan pada suhu 30 dan 35°C, manakala, *F. graminearum* pada suhu 30 dan 33°C. Kesan aktiviti air, karbon dioksida dan suhu telah diuji pada pertumbuhan dan pengeluaran mikotoksin pada medium berasaskan jagung. *F. verticillioides* lebih

bertoleransi terhadap suhu yang tinggi, aktiviti air dan karbon dioksida, jika dibandingkan dengan *F. graminearum*. Namun begitu, *F. verticillioides* menghasilkan FB_1 (29.07 ± 6.70 hingga 140.49 ± 1.45 µg/kg) dan FB_2 (21.46 ± 14.10 hingga 32.17 ± 0.54 µg/kg). Untuk kajian mengenai gen ekspresi, jumlah biomass kulat yang telah diisolat berkurang secara drastik di mana disyaki berlaku akibat faktor perubahan iklim. Ini memberi kesan negatif kepada pengekstrakan RNA untuk penentuan lanjut tentang mikotoksigenik gen ekspresi. Walau bagaimanapun, hasil yang diperoleh dalam kajian ini menunjukkan bahawa mikotoksin berjaya dikesan di dalam biji jagung bercambah dan diautoklaf oleh kedua-dua isolat tersebut. Keadaan optima untuk penghasilan mikotoksin dikesan pada 0.98 aw, 400 ppm CO_2 dan 30°C bagi kedua-dua isolat dalam biji jagung bercambah dan diautoklaf. Walau bagaimanapun, mikotoksin tersembunyi tidak dikesan dalam kajian ini. Pada suhu 30°C, mikotoksin yang dihasilkan oleh *F. verticillioides* adalah FB_1 dan FB_2, manakala, *F. graminearum* menghasilkan DON, ZEA dan α-ZEA. Jika dibandingkan *Fusarium* spp. dengan spesis yang lain, *Aspergillus* spp. tumbuh lebih cepat dan mempunyai rintangan tinggi terhadap suhu berbanding *Fusarium* spp. Oleh itu, penekanan lebih tinggi perlu diberikan kepada spesis ini. Walau bagaimanapun, perlu diingatkan bahawa penghasilan mikotoksin selain daripada yang ditarget oleh isolat tersebut adalah berkemungkinan di bawah faktor-faktor perubahan iklim. Selain itu, aklimatisasi selanjutnya bagi isolat terhadap suhu dan karbon dioksida mungkin mempengaruhi kulat mikotoksigenik disebabkan kelenturannya. Oleh itu, penyelidikan yang lebih banyak perlu dijalankan bagi bidang ini bagi menentukan jenis-jenis mikotoksin umum yang dapat dihasilkan.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

a_w	Water activity
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
CO_2	Carbon dioxide
DG18	Dichloran 18% glycerol agar
DON	deoxynivalenol
3-ADON	3-acetyl-Deoxynivalenol
DRBC	Dichloran Rose Bengal Chlorophenerin
EU	European Union
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FAO	Food and Agriculture Organization of the United Union
FAOSTAT	FAO Statistical Databases (United Nations)
F. graminearum	*Fusarium graminearum*
F. verticillioides	*Fusarium verticillioides*
GMP	Good Manufacturing Practice
HRMS	High-resolution mass spectrometry
IARC	International Agency for Research on Cancer
IPCC	International Panel on Climate Changes
IF	Isolation frequency
Kg	Kilogram
LC-MS/MS	Liquid chromatography-tandem mass spectrophotometry
LOD	Limit of detection

LOQ	Limit of quantification
MEA	Malt Extract Agar
MeOH	Methanol
ML	Maximum Likelihood
mL	Millilitre
MMA	Milled maize agar
TEF	Translation elongated factor
ULPC	Ultra Performance Liquid Chromatography
WHO	World Health Organization
α-ZEA	Alpha - Zearalenol

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Climate change is described as a global change of weather that may occur as a result of human activities such as clearing of the forest, burning of fossil fuel and others (Paterson and Lima, 2011), causing a drastic increase in carbon dioxide (CO_2) and temperature in the atmosphere resulting to drought, precipitation and change in rainfall (Chakraborty *et al*., 1998). The climate change scenario has become the center of attention to the scientific community due to the impact it may have on plants, mycotoxigenic fungi, and mycotoxin contamination. Several researchers have predicted that about 1.5 µmol of CO_2 and also 0.03% of temperature is expected to increase yearly (Medina *et al*., 2017; Magan *et al*., 2011). However, based on the presently available data, in the next 20-50 years, the concentration of atmospheric CO_2 is expected to double or tripled (from 350 - 400 to 800 - 1200 ppm) (Medina *et al*., 2015).

Vaughan *et al*., (2014) reported that in the future, climate change would increase the susceptibility of crops such as maize, rice, and wheat to mycotoxigenic fungal pathogens. Based on a global basis, about 1/3 variations in the yield of crops may be as a result effect of climate change (Ray *et al*., 2015). In Europe, the European Food Safety (EFSA) has examined the potential impact of climate change and suggested that this could depend on geographical region, which could be detrimental or advantageous (Medina *et al*., 2015). Similarly, climate change is also expected to have a negative impact in some parts of Asia, Central and South America, they are important producers of various crops such as wheat, maize and soya beans.

Mycotoxigenic fungi can be affected by the impact of climate change factors. Mycotoxins produced by these fungi are toxic chemical substances of secondary metabolites (Andrade *et al*., 2017; Rahmani *et al*., 2009). *Aspergillus, Fusarium and Penicillium* are the most important fungal genera that can produce mycotoxins under suitable conditions (Lee and Ryu, 2017; Roseanu *et al*., 2010). Currently, more than 400 fungal metabolites have been discovered however, not all are toxigenic (Paterson and Lima, 2011). The major fungi associated with maize is *Fusarium* spp. especially *F. verticillioides* and *F. proliferatum* (Cendoya *et al*., 2014).

Plants are capable of protecting themselves against xenobiotic compounds such as mycotoxins by transforming them into conjugated forms (Berthiller *et al*., 2005). These conjugated compounds are referred to as masked mycotoxins due to their ability to escape the normal routine analytical methods. Their mechanism of production could be natural or could be formed as a defense against infection by

xenobiotics (Rychlik *et al*., 2014). Furthermore, these metabolites can be formed as a result of metabolism by plant, animal or alongside during processing of foods from contaminated raw materials.

Several researchers have reported that *Fusarium* spp. are the major species affecting cereal grains during pre and post-harvest under favorable environmental conditions such as temperature, water activity and CO_2 (Smith *et al*.,2016; Medina *et al.*, 2015). The species of *Fusarium* is a natural producer of both parent and masked mycotoxins which are mainly isolated from various food and feed products. Most of the frequently occurring *Fusarium* mycotoxins are DON, 3-ADON, 15-ADON, ZEA, NIV, DAS, HT-2 toxins, T-2 toxins, NEO (Mankevičienč *et al*., 2011). In cereals, some of the mycotoxins produced accumulated in the kernel as a result of fungal contamination which may lead to their carryover in processed food and feed in toxicologically relevant concentrations (Duan *et al*., 2016; Rodrigues and Naehrer, 2012). Hence, this has become a global issue.

In Malaysia, maize has been recently gazetted as one of several wealth-creating crops. The interest in maize plantation stemmed from the need to reduce the country's dependence on maize import to feed its livestock. Basically, nearly 100% maize for feed is imported from other countries which caused a heavy economic burden. Therefore, the Malaysian government under the Department of Agriculture with co-operation from Green World Genetics (GWG), has taken an initiative to cultivate Malaysian first commercialized maize. The pilot project of maize cultivation was carried out using GWG 888 (maize seed).

Therefore, there is a need to carry out extensive research on the prevalence of *Fusarium* spp., and the effect of climate change on the prevalent species at different stages in maize and their mycotoxigenic potentials.

1.2 Problem statement

Mycotoxins are toxic chemical substances contaminating a large variety of food and feed in a relevant toxicological quantity. This occurs due to suitable environmental conditions such as temperature, humidity, water activity and CO_2. Indeed, many researchers in Malaysia have reported contamination of animal feed. Malaysia is a tropical country with high humidity, temperature and rainfall therefore a high risk of mycotoxin contamination is expected to occur (Bhat *et al*., 2010). However, *Fusarium* spp. is among the mycotoxigenic fungi that invade many varieties of agricultural products and can produce both parent and masked mycotoxins that are harmful to both human and animals.

Masked mycotoxin is of great threat to both human and animal health due to its ability to hydrolysed into their parent compound in the animal and human body, it is reabsorbed into the blood thereby increasing its exposure and toxicological properties (Berthiller *et al*., 2009). Although, various research has been conducted on the co-occurrence of parent and masked mycotoxins in different varieties of food and feed, unfortunately, classical risk analysis does not include the presence of masked mycotoxin into account as the total mycotoxins and current regulatory limits for food and feed are based on parent mycotoxins only.

Besides, contamination of maize in the field cannot be totally overcome due to the ubiquitous nature of fungi. Several issues of *Fusarium* contamination in different product has been reported in Malaysia. Therefore, due to these reasons, extensive research focusing on the diversity, identification, toxigenic potentials and the influence of climate change on *Fusarium* spp. is needed in order to ensure the safety of both humans and animal and also to recommend a relevant way to tackle the issue of contamination by *Fusarium* spp. as a result of future climate change.

Furthermore, the gene expression by the *Fusarium* spp. must also be investigated based on the effect of climate change to understand the impact on the clustered gene of *Fusarium* spp. in maize since there are no reported kinds of literature on such study. This information is important in order to understand the behavior of *Fusarium* spp. in maize under certain climatic conditions, which in turn could be used as an actual dataset based on the previous prediction about the effect of climate change factors on mycotoxigenic fungi.

1.3 Significance of the study

The data obtained from this study will contribute to better awareness of the prevalent *Fusarium* spp. in maize. The data and information will also be useful to the Policy Makers (National Policy on Climate Change) about the current occurrence and the feature perception of *Fusarium* based on the influence of climate change, the target for intervention due to the present occurrence of *Fusarium* spp. in maize.

1.4 Objective

1.4.1 Objective of the study

Generally, this study aimed to evaluate the prevalent *Fusarium* spp. in maize and determine the effect of climate change on the production parent and masked mycotoxins by the *Fusarium* spp. in maize at different developmental stages.

1.4.2 Specific objectives

1. To evaluate the biodiversity of *Fusarium* spp. in maize and soil samples collected from different maize farms.
2. To access the effects of climate change factors (CO_2, temperature and a_w) on the production of parent mycotoxins in maize-based growth medium (*in vitro*).
3. To determine the effect of climate change factors (CO_2, temperature and a_w) on gene expression and production of parent and masked mycotoxins in germinating and autoclaved maize kernels by *F. verticillioides* and *F. graminearum*

1.5 Research approach

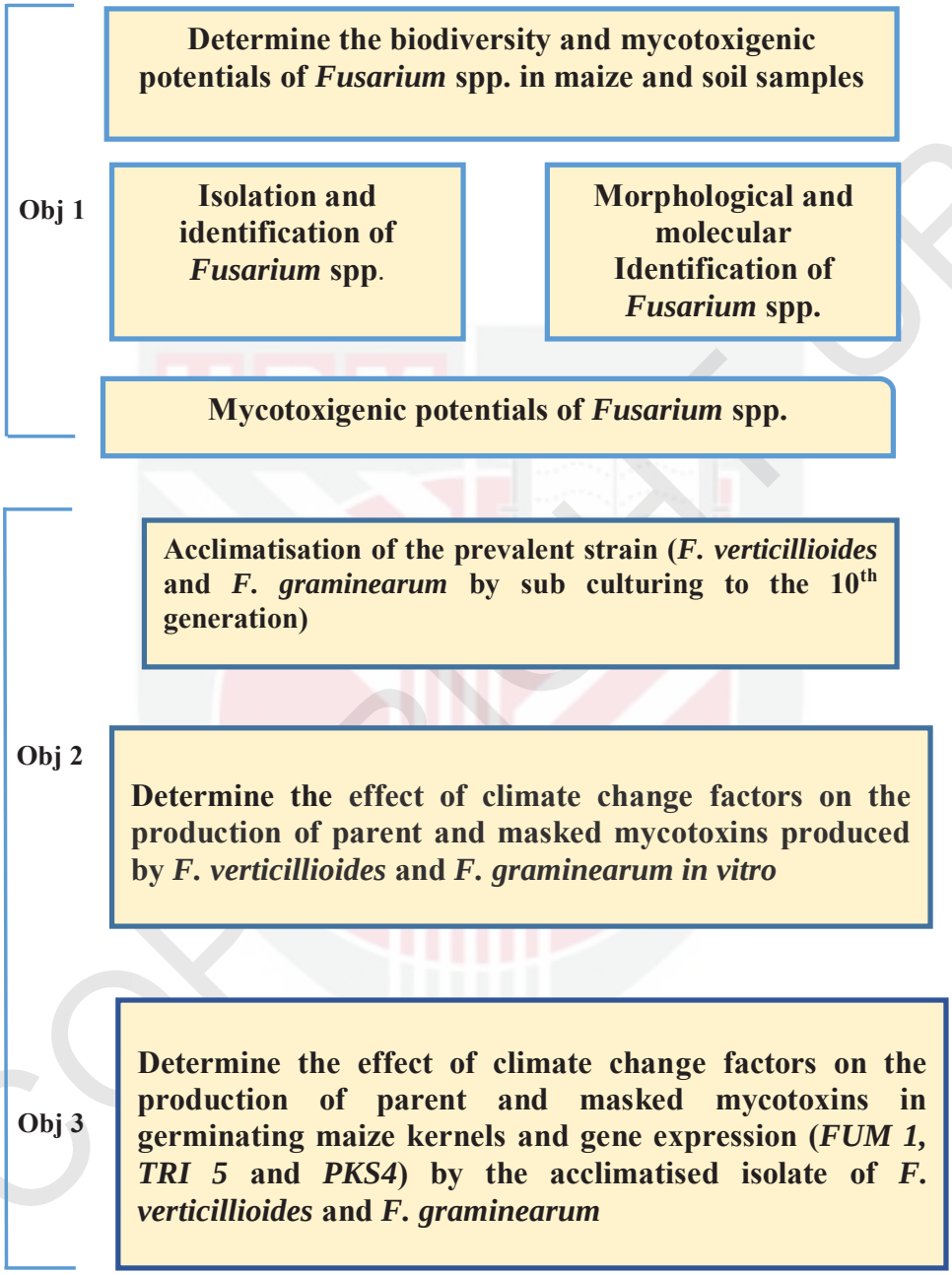

Figure 1.1 : Flow chart of the study

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