UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF FUSARIUM AND ASPERGILLUS SPECIES FROM CORNMEAL IN MALAYSIA AND TOXIGENICITY OF MYCOTOXINS PRODUCED

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By

NITHIYAA A/P PERUMAL

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

IDENTIFICATION OF *FUSARIUM* AND *ASPERGILLUS* SPECIES FROM CORNMEAL IN MALAYSIA AND TOXIGENICITY OF MYCOTOXINS PRODUCED

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Chairman: Nur Ain Izzati Mohd Zainudin, PhD

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Corn is a vital food source for human consumption, animal feed as well as industrial processing. However, corn faces repeated spoilage and contamination by a huge range of fungi especially by *Fusarium* and *Aspergillus* species. These fungi are known of producing mycotoxins such as fumonisins (FBs), moniliformin (MON), zearalenone (ZEA), beauvericin (BEA) and aflatoxins (AFs). Therefore the main objectives of this study were to identify and determine the diversity of *Fusarium* and *Aspergillus* species as well as to quantify the mycotoxins produced by both fungi associated with cornmeal in Malaysia. In this study, cornmeal samples were obtained from 9 states throughout Malaysia and the cornmeal samples were surface sterilized and cultured onto Peptone Pentachloronitrobenzene Agar (PPA) to isolate the fungi. Single spore isolation was carried out onto Potato Dextrose Agar (PDA) to obtain pure culture. *Fusarium* and *Aspergillus* isolates were selected and preceded with morphological identification. The diversity of *Fusarium* and *Aspergillus* species were determined using Shannon-Weiner index and this is followed by the extraction of mycotoxins. The extracted mycotoxins were analyzed qualitatively using Thin Layer Chromatography (TLC) and *A. salina* bioassay, and quantitatively using Ultra-fast Performance Liquid Chromatography (UFLC). A total of 314 isolates of microfungi were obtained, 90.5% isolates belonged to *Aspergillus* species, namely *A. flavus* (76.8%), *A. niger* (7.6%), *A. nidulans* (4.5%) and *A. fumigatus* (1.6%). Another 9.5% isolates were *Fusarium* species, identified as *F. verticillioides* (4.5%), *F. semitectum* (3.2%) and *F. proliferatum* (1.9%). As for the mycotoxin analysis, 15 out of 16 *Fusarium* isolates produced MON, 12 isolates produced BEA and all 16 isolates produced FB_1. However none produced ZEA. In addition, 29 out of 40 *Aspergillus* isolates produced AFB_1 and only two isolates produced AFB_2. The analysis of *A. salina* revealed that all the five mycotoxins extracts were toxic to the brine shrimp despite the concentration of the mycotoxins. As a conclusion, a proper storage system for the corns should be implemented to avoid fungal contamination hence reducing the accumulation of mycotoxins.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN SPESIS *FUSARIUM* DAN *ASPERGILLUS* DARI JAGUNG BERPROSES DI MALAYSIA DAN UJIAN KETOKSIGENAN MIKOTOKSIN YANG DIHASILKAN

Oleh

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Jagung merupakan salah satu sumber makanan utama bagi manusia, haiwan serta untuk pemprosesan industri. Namun demikian, sumber jagung berulang kali menghadapi kerosakan dan kontaminasi oleh pelbagai jenis kulat terutamanya spesis *Fusarium* dan *Aspergillus*. Kulat-kulat ini boleh menghasilkan mikotoksin seperti fumonisins (FBs), moniliformin (MON), zearalenone (ZEA), beauvericin (BEA) dan aflatoxins (AFs). Oleh itu, objektif utama kajian ini adalah untuk mengenalpasti dan menentukan kepelbagaian spesis *Fusarium* dan *Aspergillus* serta mengkuantifikasi mikotoksin yang dihasilkan oleh kedua-dua kulat yang berassosiasi dengan jagung berproses di Malaysia. Dalam kajian ini, sampel jagung berproses diperolehi daripada 9 negeri di sekitar Malaysia and sampel jagung tersebut disterilasi sebelum dikultur ke atas agar Peptone Pentachloronitrobenzene (PPA) untuk pengasingan kulat. Teknik pengasingan spora tunggal dilakukan di atas Potato dextrose agar (PDA) untuk mendapatkan kultur kulat

yang tulen. Spesis *Fusarium* dan *Aspergillus* dipilih and seterusnya diidentifikasi secara morfologi. Diversiti spesis *Fusarium* dan *Aspergillus* ditentukan menggunakan Shannon-Weiner indeks dan langkah ini disusuli oleh pengekstrakan mikotoksin. Mikotoksin yang diekstrak telah dianalisis secara kualitatif menggunakan *Thin Layer Chromatography* (TLC) dan *A. salina bioassay* dan secara kuantitatif menggunakan *Ultra-fast Performance Liquid Chromatography* (UFLC). Sejumlah 314 isolat kulat mikro diperolehi, di mana 90.5% isolat terdiri daripada spesis *Aspergillus*, iaitu *A. flavus* (76.8%), *A. niger* (7.6%), *A. nidulans* (4.5%) dan *A. fumigatus* (1.6%). Selebihnya, 9.5% isolate adalah spesis *Fusarium* yang dikenalpasti sebagai daripada *F. verticillioides* (4.5%), *F. semitectum* (3.2%) and *F. proliferatum* (1.9%). Dari segi analisis mikotoksin, 15 daripada 16 isolat *Fusarium* telah menghasilkan MON, 12 isolat menghasilkan BEA dan kesemua 16 isolat didapati menghasilkan FB_1. Walaubagaimanapun tiada isolat yang menghasilkan ZEA. Selain itu, 29 daripada 40 isolat *Aspergillus* didapati menghasilkan AFB_1, manakala hanya dua isolat menghasilkan AFB_2. Analisis *A. salina* menunjukkan bahawa kesemua lima ekstrak mikotoksin adalah toksik kepada udang air masin tanpa mengira kepekatan mikotoksin tersebut. Sebagai kesimpulan, sistem penyimpanan jagung yang baik perlu dilaksanakan untuk mengelakkan kontaminasi kulat, sekaligus mengurangkan pengumpulan mikotoksin.

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I thank you all once again.

I certify that a Thesis Examination Committee has met on 23 November 2012 to conduct the final examination of Nithiyaa a/p Perumal on her thesis entitled "Detection and Toxigenicity of Mycotoxin Produced by *Fusarium* And *Aspergillus* species Isolated from Cornmeal in Malaysia" in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

NITHIYAA A/P PERUMAL

Date: 23 November 2012

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LIST OF ABBREVIATIONS

R_f	Retention factor
FBs	Fumonisins
FB_1	Fumonisin B_1
DON	Deoxynivalenol
BEA	Beauvericin
MON	Moniliformin
ZEA	Zearalenone
AFs	Aflatoxins
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_1
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_1
FA	Fusaric acid
FUS	Fusaproliferin
OTA	Ochratoxin
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
TLC	Thin Layer Chromatography
ELISA	Enzyme-Linked Immunosorbent Assay
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry

CZE	Capillary Zone Electrophoresis
HPLC	High Performance Liquid Chromatography
UPLC–MS	Ultra-High Performance Liquid Chromatography–Mass Spectrometry
UFLC	Ultra-fast Performance Liquid Chromatography
LC	Liquid Chromatography
NaOCl	Sodium hypochlorite
PPA	Peptone Pentachloronitrobenzene Agar
PDA	Potato Dextrose Agar
CLA	Carnation Leaf Agar
SNA	Spezieller Nahrstoffarmer Agar
TFA	Triflouroacetic acid
UV	Ultra-violet
i.d	Internal diameter
M	Molar
OPA	*o*-phthaldialdehyde
ANOVA	Analysis of variance
SE	Standard error

CHAPTER 1

INTRODUCTION

Corn plantation was introduced during 15th century by the Indian community in the highlands of Mexico (Wertz, 2005). In 19th century, the hybrid corns were developed by several scientists hence produced more varieties of corn and better yields. Therefore, the farmers started planting the hybrid corn in a large scale. The number of farmers that used hybrid corn increased throughout the 20th century. According to National Corn Growers Association, the yields of corn increased every year, for example in 1991 the average yield was 108.6 bushel per acre and in 2008 increased to 153.9 bushel per acre (USDA, 2009).

Corn serves as a staple food and an important part of the diet for millions of people worldwide. It contains high starch content and also good for feeding hogs, horses and other livestock such as chicken, cattle and swine (Fitting, 2006). Despite its vast importance, the farmers are facing problem to maintain the quality of corn. The major crisis faced by many corn farmers is fungal contamination. Corn plantations are basically susceptible to fungal infection during both pre-harvest and post-harvest periods. While in field it is colonized by numerous fungi, some of the fungi basically influence the quality of the grain during the pre-harvest period, particularly affecting the quality of the corn and causes ear rot disease. Fungi also infect the root, stem and leaf and therefore contribute to low quality corn. Contaminations during pre-harvest may persist until post-harvest such as during storage and processing, transporting and marketing (Etcheverry *et al*., 1999).

The fungal infection on corn continues till storage and the diversity of fungi inhabiting the corn changes. This is due to the alteration of the storage conditions, which involves the humidity and temperature in the particular area. The corn surface will also damage, causes by insect or animal and may enhance the filamentous fungi to invade the damaged corn (Etcheverry *et al*., 1999).

Several microscopic fungi which are associated with corn and reduce the quality of the yield are such as species of *Aspergillus*, *Chaetomium*, *Clasdosporium*, *Eurotium*, *Fusarium*, *Penicillium* and *Pythium* (Chelkowski, 1991). However, in the present study, most attentions are paid to *Aspergillus* species and *Fusarium* species as these fungi may persist from the field to storage.

Fusarium is a filamentous fungi widely distributed in soil and often acted as pathogen of plant, human and animal. Most species are saprophytes and relatively abundant members of the soil microbial community. Several species of *Fusarium* are important pathogens of plants, including corn and other cereals, causing root, stem and ear rot (Uhlig *et al*., 2007). The fungi that affected corn are capable to produce harmful primary and secondary metabolites. Therefore secondary metabolites which are known as mycotoxin can cause hazardous illness and sometimes fatal to the consumers such as animals and humans (Uhlig *et al*., 2007).

Approximately, a total 20 *Fusarium* species have been regularly associated with diseases occurring in small-grain cereals. *F. culmorum*, *F. graminearum* Schwabe (teleomorph: *Gibberella zeae*) and *F. avenaceum*, (teleomorph: *G. avenacea Cook*) were most frequently isolated (Miedaner, 1997). *Fusarium* species have the ability to produce mycotoxins that causes health problem. The common mycotoxins produced by *Fusarium* species are beauvericin (BEA), deoxynivalenol (DON), fumonisins (FBs), fusaric acid (FA), fusaproliferin (FUS), fusarins and moniliformin (MON) (Fotso *et al*., 2002; Logrieco *et al*., 2002; Muthomi *et al*., 2008). *Aspergillus* species are also harmful to both humans and animals, causing severe diseases. More than 185 species have been identified and 20 species are recognized as the pathogenic species. *Aspergillus* species are diverse, where it can be found in soil, wood, stored grains, plant remains, and mostly in the air inhaled by all living creatures. The several *Aspergillus* species found in stored grains are *A. flavus, A. parasiticus* and *A. niger* (Giorni *et al*., 2007).

Mycotoxins are secondary metabolites produced by fungi which does not participate in the growth and reproduction of the fungi, conversely effects the biochemical, physiological or pathological changes in other living organisms, such as other microorganism, plants, animals and human (Haschek *et al*., 2002). These compounds are known to produce a large range of effects in organisms such as mammals (rats, guinea pigs and etc), birds (chicken embryos), amphibians, arthropods, crustaceans, unicellular organisms, microorganisms and plants. Zearalenones (ZEA) produced by *Fusarium* species, is a nonsteroidal estrogenic mycotoxin that causes estrogenic syndrome such as enlargement of mammary glands and genital organs, and atrophy of testes in the affected animals. FBs cause leukoencephalomalacia, a brain lesion that is fatal to horses and also carcinogenic. MON causes muscle weakness, respiratory distress and cyanosis in animals (Desjardins, 2006a). BEA is a cyclic lactone trimer, a specific cholesterol acyltransferase inhibitor besides and toxic to several human cell lines and is able to induce apoptosis and DNA fragmentation (Reynoso *et al.*, 2004).

Aspergillus species are also able to produce various types of mycotoxins such as, aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), ochratoxin (OTA) and patulin (Giorni *et al.*, 2007; Morgavi *et al.*, 2003; Shundo *et al.*, 2009). AFs are toxin metabolites that functions as a hepatotoxic, hepatocarcinogenic, and also has mutagenic effects on animal and human (Almeida *et al.*, 1996). OTA however is known to exhibit nephrotoxin as well as hepatoxic and carcinogenic to humans, similar to AFs (Shundo *et al.*, 2009). According to Morgavi (2003), patulin also displays toxic effects where it acts as teratogenic, carcinogenic and mutagenic to other organisms.

Hence, these negative effects have been made into use as bioassays to detect the presence of mycotoxins in vast commodities (Panigrahi, 1993). *A. salina* larvae was selected as a screening system owing to the privilege that their eggs are commercially available, besides being able to hatch within 24 hours of incubation with minimal maintenance of the cultures (Jimenez *et al.*, 1997). Previously, Matthews (1995) also stated the use of brine shrimp, stating that these organisms are convenient, simple and less expensive. Besides, the sensitivity of these organisms is utilized greatly to quick scan the occurrence of the desires metabolites. Moreover, the use of higher animals are greatly restricted since only a small number of these animals are allowed for toxicity test which in this study accounts for the mortality rate of the organisms tested. As a result, only a handful of isolates can be tested. Hence, once again the use of brine shrimp was widely considered.

On the other hand, cornmeal contaminated with fungal have invited the attention of many farm breeders and scientists' worldwide. Farm animals that feed on the infected cornmeal are severely affected and causing death, hence causing serious lost to the farm breeders (Hazel and Patel, 2004). This problem proceeds when human consumes the infected animal or food and therefore risking their health. As one problem leads to another, this may eventually end up affecting the whole food chain in an ecosystem.

The objectives of this study were:

i) To identify and determine the diversity of *Fusarium* and *Aspergillus* species associated with cornmeal in Malaysia,

iii) To identify and quantify the mycotoxins produced by *Fusarium* and *Aspergillus* species associated with cornmeal in Malaysia.

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