INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
14–170/2015/17–3–440–448
DOI: 10.17957/IJAB/17.3.14.170
http://www.fspublishers.org

Full Length Article

Mycotoxins Production by *Fusarium* and *Aspergillus* Species Isolated from Cornmeal

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Abstract

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). The main objective of this study was to quantify the mycotoxins produced by both fungi associated with cornmeal in Malaysia. The extracted mycotoxins were qualitatively analyzed using *A. salina* bioassay, and quantitatively using Ultra-fast Performance Liquid Chromatography (UFLC). Three hundred and fourteen 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 9.6% isolates were *Fusarium* species, identified as *F. verticillioides* (4.5%), *F. semitectum* (3.2%) and *F. proliferatum* (1.9%). As for the mycotoxin analysis, out of 40 isolates of *Aspergillus*, 29 isolates produced AFB_1 and only two isolates produced AFB_2. Fifteen out of 16 *Fusarium* isolates produced MON, 12 isolates produced BEA and all isolates produced FB_1, but none of them produced ZEA. The analysis of *A. salina* revealed that all the five mycotoxins extracts were toxic to the brine shrimp despite the concentration of the mycotoxins.

Keywords: *Fusarium; Aspergillus*; Mycotoxins; Aflatoxins; Cornmeal

Introduction

The major crisis faced by many corn farmers is fungal contamination. Corn plantations are susceptible to fungal infection during both pre- and post-harvest periods. While in the field, numerous fungi colonize it. Some of the fungi 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, 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 until storage and the diversity of fungi inhabiting the corn changes. This is due to the alteration of the storage conditions, which involves humidity and temperature in the particular area. The surface of the corn faces damage caused by insect or animal and thus enhances the filamentous fungi to invade the damaged corn (Etcheverry *et al.*, 1999). 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).

Mycotoxins are known as harmful secondary metabolites produced by various filamentous fungi such as *Fusarium*, *Aspergillus* and *Penicillium* species, which usually colonize crops and grains. There are various types of mycotoxins discovered by the modern science nowadays, such as fusarin, fumonisins (FUM), moniliformin (MON), deoxynivalenol (DON), zealenone (ZEA) and aflatoxins (AFs). Mycotoxins are capable of causing several health effects in animals and human, and several deaths are also reported. Some mycotoxins are known to cause nephrotoxicity (Hartl and Stenzel, 2007). Other mycotoxins such as DON and ZEA inhibit the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and affect the immune system (Langseth *et al.*, 2000). Besides infecting animal and human, these mycotoxins are also harmful to microorganism such as fungi and even bacteria, where the growth of these microbes will be retarding and finally dies. The toxicity of mycotoxins can be determined using laboratory test organism, for instant chick embryo and rodents. Besides the above, *Artemia salina* (brine shrimp) bioassays are also used for the toxicity test. This is because the brine shrimp possess great sensitivity towards slight dosage of toxins. Thus, in this study we used brine shrimp as a test organism.

On the other hand, the aid of more accurate devices

To cite this paper: Zainudin, N.A.I.M. and N. Perumal, 2015. Mycotoxins production by *Fusarium* and *Aspergillus* species isolated from cornmeal. *Int. J. Agric. Biol.*, 17: 440–448

such as Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC), Ultra-fast Performance Liquid Chromatography (UFLC), Enzyme-linked Immunosorbent Assay (ELISA) and Mass spectrometry-based assays are used to detect and quantify the produced compounds (Akiyama *et al*., 1998; Younis and Malik, 2003; Maragos and Busman, 2010). In this study, the extracted mycotoxins were quantitatively analyzed using UFLC.

Hence, the objective of this study are to detect and quantify the mycotoxins produced by *Fusarium* and *Aspergillus* species using UFLC and to test the toxigenicity of the mycotoxins produced using *A. salina* bioassay.

Materials and Methods

Isolation of Fungi

The cornmeal samples were surface sterilized with 0.5% sodium hypochlorite (NaOCl) and rinsed twice with sterile distilled water. The sterile samples were blotted to dry on sterilized filter papers. The sterilized cornmeal samples were plated onto Peptone Pentachloronitrobenzene Agar (PPA) using a sterilized forceps, to isolate the fungi. The fungal cultures were single conidiated using the streak plate technique (Crawford *et al.,* 1993) and identified into species level (Leslie and Summerell, 2006).

Conidial Suspension

Only 16 *Fusarium* isolates - 3 isolates of *F. proliferatum*, 6 isolates *F. semitectum* and 7 isolates *F. verticillioides* and 40 *Aspergillus* isolates - 37 isolates of *A. flavus*, and a single isolate each from *A. fumigatus*, *A. niger* and *A. nidulans* were used for mycotoxin production study. All the isolates were cultured on PDA and incubated for 7 days. The spores were dislodged using 10 mL of sterile distilled water and filtered using gauze to remove the fragments of mycelia. A heamocytometer was used to adjust the concentration of the spore suspension to $1x10^5$ spores/mL according to Nur Ain Izzati *et al.* (2008) with slight modification.

Inoculation of Spores and Sample Extraction

With minor modification on the method described by Munkvold *et al.* (1998), approximately 85 g of cornmeal was washed and added with 10 mL distilled water in a conical flask. The flask was covered with cotton and autoclaved for 15 min at 121ºC. Then 5 mL of sterile distilled water was added to the cornmeal and re-autoclaved for 30 min. About 1 mL of spore suspension was added to the sterile cornmeal and shaken once a day for three continuous days to allow even distribution of the spores. The cultures were then incubated in the dark at room temperature (25±2ºC) for 28 days. All treatments were done in triplicates. Controls were set up by inoculating sterile distilled water instead of fungal spore.

For the extraction of MON, 20 g of colonized cornmeal sample was added with 100 mL acetonitrile: H_2O (95: 5; v/v) and was grinded using a waring blender. The extracts were filtered using Whatman No. 4 filter paper. The extracts were then partitioned with 150 mL hexane and the bottom layer liquid was collected and evaporated to dryness using a rotary evaporator (Heidolph 3 Model, Laborato 4010) at 60°C and re-dissolve in methanol (Sewram *et al*., 1999).

As for the extraction of FB_1, 25 g of the colonized cornmeal was grind in 100 mL of methanol: H_2O (3:1; v/v). The extract was filtered through Whatman no. 4 filter paper. The pH of the sample extract was adjusted to pH 6 using 1M sodium hydroxide. The extract was then dried using a rotary evaporator at 60°C and re-dissolved in methanol (Shephard, 1998).

For the extraction of ZEA, 20 g of colonized cornmeal sample was extracted with methanol: 1% aqueous NaCl (55:45; v/v) by grinding in a waring blender. The extract was filtered in Whatman No. 4 filter paper and treated with 150 mL *n*-hexane to remove fat, and fractionated with dichloromethane. The extract was further evaporated to dryness using a rotary evaporator at 60°C. The residue obtained after solvent evaporation was redissolved in 2 mL of methanol: H_2O (40:60; v/v) solution and then filtered through a 0.2 µm membrane filter and eluted, first with 2 mL of methanol: H_2O (40:60; v/v) (fraction 1) and then twice with 2 mL of methanol (fraction 2). Fraction 2 will be evaporated again to near dryness and redissolved in 0.5 mL of methanol (Bottalico *et al*., 1985).

For the extraction of BEA, 15 g of colonized cornmeal sample was grind with 75 mL of solvent (acetonitrile: methanol: H_2O, [16:3:1; v/v/v]) in a waring blender, and filtered using Whatman No. 4 filter paper. The filtrate was defatted twice with 25 mL of hexane and the bottom layer was collected and evaporated to dryness using a rotary evaporator. The residue was dissolved in 50 mL of solvent methanol: H_2O (1:1; v/v) and extracted twice with 25 mL of dichloromethane. The dichloromethane phase that contains BEA was collected and evaporated to dryness with a rotary evaporator at 60°C (Munkvold *et al*., 1998).

Extraction of AFs was done according to Khayoon *et al.* (2010), where 20 g of colonized cornmeal sample was extracted with 80 mL acetonitrile: H_2O (9:1; v/v) mixture for 30 min by shaking under high speed, and then filtering using a No. 4 Whatman filter paper. The filtrate was dried using rotary evaporator at 40°C and re-dissolved in methanol.

Analysis of Mycotoxins using Ultra-Fast Liquid Chromatography (UFLC)

UFLC analysis was done using a Shimadzu (Kyoto, Japan) equipped with two LC-20AD pumps, a SIL-20A

autosampler, a Shimadzu RF-10A XL fluorescence detector and a Shimadzu SPD-20AV UV/VIS detector. A reversed phase LC column, Thermo Scientific, Hypersil GOLD, C18, 250 mm x 4.6 mm i.d with a particle size of 5 µm was used.

Moniliformin (MON): The extracted compounds were first diluted with methanol using tenfold serial dilution and filtered through 0.20 µm membrane syringe filter (Disposable Syringe Filter, PTFE Hydrophilic Membrane, Advantec) into 2 mL clear vials prior to 10 µL injection into the column. The mobile phase used was 0.1 M ammonium acetate: methanol: triethylamine (90:10:0.1 v/v/v) at pH 8.24, with a flow rate of 1 mL/min (with slight modification to Sewram *et al*., 1999). The peaks were observed using Ultra-Violet (UV) detector with a wavelength of 254 nm. Ten different concentrations of the standard were prepared and run to obtain a standard curve. The standard and the samples were analyzed simultaneously, and the results obtained were compared with reference to the standard curve obtained to determine the concentration of MON produced.

Fumonisin B_1 (FB_1): The samples were derivatized by adding 50 µL of sample extracts with 200 µL of *o*-phthaldialdehyde (OPA) reagent. 10 µL of derivatized samples and standard were injected into the column, using 80% methanol and 0.1 M aqueous sodium dihydrogen phosphate as the mobile phase with a flow rate of 1 mL/min. The peaks were observed using fluorescence detector. The sample peaks were compared with the standard curve to determine the concentration of FB_1 produced (Shephard, 1998).

Zearalenone (ZEA): Ten microliters of the extracted compounds together with the standard were injected into the UFLC system. Methanol: H_2O (65:35 v/v) was used as the mobile phase with a flow rate of 1 mL/min. The peaks were observed using an UV detector with a wavelength of 236 nm. The sample peaks were compared to the standard curve to determine the concentration of ZEN produced (Jimenez *et al*., 1997).

Beauvericin (BEA): Ten microliters of extracted compounds and standard were injected into the UFLC system with acetonitrile: H_2O (85:15 v/v) as the mobile phase with a flow rate of 1 mL/min. The peaks were observed using an UV detector with a wavelength of 225 nm. The sample peaks were compared to the standard curve to determine the concentration of BEN produced (Munkvold *el at*., 1998).

Aflatoxin B_1 (AFB_1) and B_2 (AFB_2): For derivatization, 100 µL of triflouroacetic acid solution and 300 µL of n-hexane were added to the residue from the sample extracts and to the AF standards. The mixture was vortexed for 30s, and kept in the dark for 15 min at room temperature. Then 900 µL of acetonitrile: H_2O (1:9 v/v) was added to the vial and vortexed for another 30s. The mixture was left to stand to allow the two layers to be separated. Ten microliters of the derivatised product (bottom layer) was injected into the UFLC column, with a mobile phases consisting of acetonitrile: methanol: H_2O (8:20:72 v/v/v) and a flow rate of 1 mL/min. The peaks were observed using an UV detector with a wavelength of 360 nm. The sample peaks were compared with the standard curve to determine the concentration of AFB_1 and AFB_2 produced (Khayoon *et al*., 2010).

***Artemia salina* Bioassay**

Dried eggs of *A. salina* were hatched in 3.8% sterile artificial seawater for 48 h at room temperature (25±2ºC) in a tank. The bioassay was conducted according to Logrieco *et al*. (1996). Thirty matured larvae were transferred into 2 x 2 mm glass petri dishes, loaded with 5 µL of mycotoxin extracts, and brought up to 3 mL using sterile artificial seawater. As for the control, 5 µL of artificial seawater, methanol and acetonitrile each were loaded in separate petri dishes. Three replicates were done for each mycotoxin extracts and the controls. After 24 h of incubation, the dead *A. salina* in each plate were calculated and recorded. The data obtained were analyzed using one-way ANOVA (Logrieco *et al*., 1996).

Results

***Aspergillus* and *Fusarium* Species Isolated from Cornmeal**

Three hundred and fourteen isolates of microscopic fungi were successfully isolated from corn samples obtained from nine states throughout Malaysia (Table 1). Out of this, 284 isolates belong to the genus *Aspergillus*, which comprises of *A. flavus* (241 isolates), *A. niger* (24 isolates), *A. nidulans* (14 isolates) and *A. fumigatus* (5 isolates), whereas the remaining 30 isolates belong to the genus *Fusarium*, which includes *F. verticillioides* (14 isolates), *F. semitectum* (10 isolates) and *F. proliferatum* (6 isolates). *A. flavus* dominated all other microscopic fungi with 76.8%, and was most frequently isolated from corn samples from Sandakan, Sabah. Out of 314 microfungi isolates were obtained, only 56 isolates (16 isolates of *Fusarium* species and 40 isolates of *Aspergillus* species) were used in mycotoxin production study.

***F. proliferatum*:** The macroconidia were thin-walled, slender and straight with 3-5 septa. The microconidia were abovoid with flattened base, 0-1 septate and formed in chains of moderate length and also in false heads from monophialides and polyphialides (Fig. 1A, 1I).

F. verticillioides: Macroconidia produced were long, slender, straight and thin walled. The apical cell of macroconidia was curved and tapered, and the basal cell was notched. Microconidia were oval or abovoid, formed long chains or false head (Fig. 1B, 1C and 1J) attached at branched monophialides.

***F. semitectum*:** Macroconidia had a curved surface on

Table 1: List of *Fusarium* and *Aspergillus* isolates and their respective locations

Species	Isolate Code	Location (City, State)
F. proliferatum	B29C, B30C, B32C, B33C, B35C	Serdang, Selangor
	P301C	Kepala Batas, Pulau Pinang
F. semitectum	SS302C, SS307C, SS308C, SS318C, SS323C	Sandakan, Sabah
	C325C	Kuantan, Pahang
	P326C, P328C	Kepala Batas, Pulau Pinang
	C329C	Mentakab, Pahang
	C333C	Lanchang, Pahang
F. verticilloides	N334C	Bukit Pelanduk, Seremban
	B335C, B336C, B338C, B343C, B362C, B363C, B367C, B369C, B387C, B393C, B394C	Serdang, Selangor
	J405C	Labis, Johor
	P410C	Sungai Ara, Pulau Pinang
A. flavus	P1C, P2C, P3C, P4C, P5C, P6C, P7C, P8C, P9C, P10C	Kepala Batas, Pulau Pinang
	P11C, P12C, P13C, P14C, P15C, P16C, P17C, P18C, P19C, P20C, P21C, P22C, P23C, P24C, P25C P26C	Sungai Ara, Pulau Pinang
	C27C, C28C, C29C, C30C, C31C, C32C, C33C, C34C, C35C, C36C, C37C, C38C, C39C, C40C, C41C, C42C, C43C, C44C, C45C, C46C, C47C	Mentakab, Pahang
	C48C, C49C, C50C, C51C, C52C, C53C, C54C, C55C, C56C, C57C, C58C, C59C, C60C, C61C, C62C, C63C, C64C, C65C, C66C, C67C, C68C, C69C	Bentong, Pahang
	C70C, C71C, C72C, C73C, C74C, C75C	Kuantan, Pahang
	C76C, C77C, C78C, C79C, C80C, C81C	Lanchang, Pahang
	C82C, C83C, C84C, C85C, C86C, C87C	Temerloh, Pahang
	C88C, C89C, C90C, C91C, C92C, C93C, C94C, C95C, C96C, C97C	Karak, Pahang
	C98C, C99C, C100C, C101C, C102C, C103C, C104C, C105C, C106C, C107C	Maran, Pahang
	M108C, M109C, M110C, M111C, M112C, M113C, M114C, M115C, M116C, M117C, M118C	Melaka
	B119C, B120C, B121C, B122C, B123C	Bertam, Selangor
	B124C, B125C	Semenyih, Selangor
	B126C, B127C, B128C, B129C, B130C, B131C, B132C, B133C, B134C	Kelang, Selangor
	B135C, B136C, B137C, B138C, B139C, B140C	Serdang, Selangor
	B141C, B142C, B143C	Tanjung Karang, Selangor
	T144C, T145C, T146C, T147C, T148C, T149C, T150C, T151C, T152C, T153C, T154C, T155C, T156C, T157C, T158C, T159C	Marang, Terengganu
	T160C, T161C	Kuala Terengganu, Terengganu
	SS162C, SS163C, SS164C, SS165C, SS166C, SS167C, SS168C, SS169C, SS170C, SS171C, SS172C, SS173C, SS174C, SS175C, SS176C, SS177C, SS178C, SS179C, SS180C, SS181C, SS182C, SS183C, SS184C, SS185C, SS186C, SS187C, SS188C, SS189C, SS190C, SS191C, SS192C, SS193C, SS194C, SS195C, SS196C	Sandakan, Sabah
	Q197C, Q198C, Q199C, Q200C, Q201C, Q202C, Q203C, Q204C, Q205C, Q206C, Q207C, Q208C, Q209C, Q210C, Q211C	Bintulu, Sarawak
	Q212C, Q213C, Q214C, Q215C, Q216C, Q217C, Q218C, Q219C, Q220C, Q221C	Miri, Sarawak
	J222C, J223C, J224C, J225C, J226C, J227C, J228C, J229C, J230C, J231C, J232C, J233C, J234C, J235C, J236C, J237C, J238C	Labis, Johor
	N239C, N240C, N241C	Bukit Pelanduk, Seremban
A. fumigatus	C242C	Kuantan, Pahang
	C243C, C244C, C245C	Temerloh, Pahang
	Q246C	Bintulu, Sarawak
A. niger	P247C, P248C, P249C, P250C	Kepala Batas, Pulau Pinang
	C251C, C252C, C253C	Mentakab, Pahang
	C254C, C255C, C256C, C257C	Bentong, Pahang
	C258C	Lanchang, Pahang
	B259C, B260C	Bertam, Selangor
	T261C, T262C, T263C	Marang, Terengganu
	T264C, T265C	Kuala Terengganu, Terengganu
	Q266C, Q267C, Q268C	Bintulu, Sarawak
	J269C, J270C	Labis, Johor
A. nidulans	C271C	Lanchang, Pahang
	B272C, B273C	Kelang, Selangor
	B274C	Serdang, Selangor
	Q275C	Bintulu, Sarawak
	M276C, M277C, M278C, M279C, M280C, M281C, M282C, M283C	Ayer Keroh, Melaka
	J284C	Labis, Johor

vertical and a straight surface at the dorsal. Microconidia were oval-shaped with 1-septate, mesoconidia with 3 septa were abundance in aerial mycelia (Fig. 1D, 1K). Conidiophores were monophialides with/without polyphialides.

***A. flavus*:** Some isolates have black sclerotia produced upon maturation. Conidiophores were smooth and moderate in length measuring 800.0 µm. The vesicles were globose upon maturity with heads, 20.0-45.0 µm in diameter, and have both uniseriate and biseriate

Table 2: Concentrations of mycotoxins produced by each isolate of *Fusarium* species

Fusarium sp.	Isolate no.	Concentration of MON (ppm)	Concentration of ZEA (ppm)	Concentration of BEA (ppm)	Concentration of FB_1(ppm)
F. proliferatum	B30C	173.72	ND	32.20	554.35
	B32C	176.53	ND	90.20	252.13
	P301C	66.0	ND	20.30	298.25
F. semitectum	SS302C	2926.20	ND	ND	795.0
	SS318C	1910.30	ND	ND	416.90
	C325C	72.15	ND	57.0	2580.30
	P326C	1262.60	ND	56.80	787.13
	SS329C	164.58	ND	50.30	423.89
	C333C	2426.80	ND	36.20	683.07
F. verticillioides	N334C	1779.80	ND	106.0	571.95
	B335C	ND	ND	25.80	558.59
	B336C	122.80	ND	ND	1050.07
	B363C	1204.0	ND	ND	439.68
	B393	849.0	ND	83.80	222.56
	J405C	2164.60	ND	63.0	461.01
	P410C	2058.30	ND	66.90	380.33

ND= not detected

sterigmata (Fig. 1E). Conidia are 3.0-6.0 µm in size with smooth cell wall (Fig. 1L).

***A. fumigatus*:** Conidial heads measuring 70.0-80.0 µm with short conidiophores rising vertically from the hyphae (Fig. 1F). The conidiophores measure 300.0 µm in length. The conidial head comprises a vesicle like structure, 20.0-30.0 µm in size that holds the uniseriate sterigmata. Conidia were green in color and subglobose with a diameter between 2.4-3.7 µm (Fig. 1M).

***A. niger*:** The conidial head was brownish black and splits into several irregular and regular columns of conidial chain (Fig. 1G). The diameter of the conidial heads ranges from 70-120 µm. The conidiophores were hyaline, brown in color, measuring more than 500 µm. Vesicles were hyaline, globose and brown in color, 30-75 µm in diameter with uniseriate sterigmata. The conidia were brown to black, globose, rough, diameter ranging between 4.0-5.0 µm (Fig. 1N).

***A. nidulans*:** Conidial heads have short columnar with a diameter of 40.0-80.0 µm (Fig. 1H). Conidiophores were moderate in length (less than 150 µm) and brown in color. The vesicles were hemispherical in shape, small, globose and flattened at the apical part, and diameters ranging from 8.0-12.0 µm, with uniseriate and biseriate sterigmata. Conidia were light brown in color, globose and rough walled and measuring 3.0-4.0 µm in diameter (Fig. 1O). The ascospore have rough wall, reddish brown in color with a diameter of 4.0-5.0 µm.

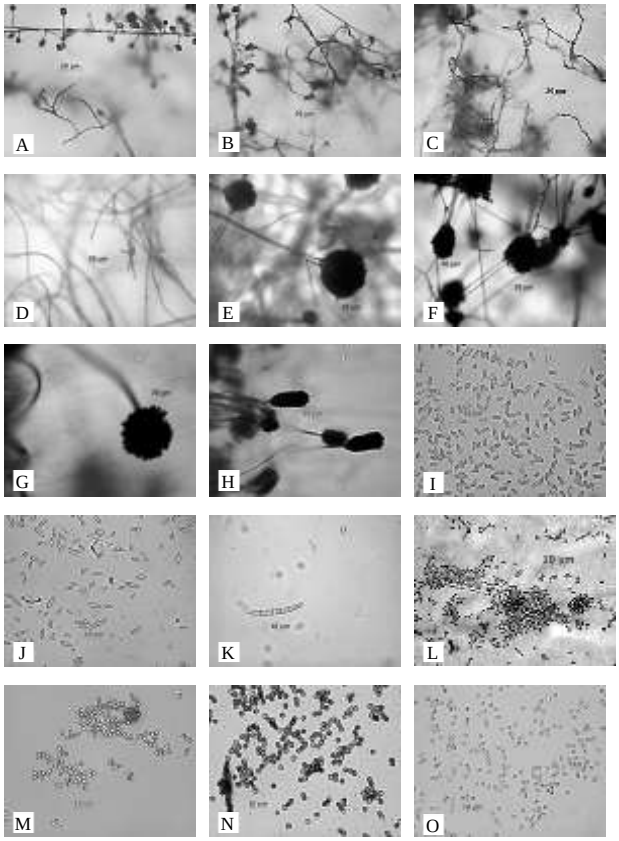

Fig. 1: Conidia observed *in situ*. A: *F. proliferatum* formed microconidia in false heads and chains, B-C: *F. verticillioides* formed microconidia in false heads and chains (usually produced long chain as in C compared to *F. proliferatum*), D: *F. semitectum* formed conidia in singly, pair "rabbit ear", E-H: Conidia in conidiophore of *A. flavus* (E), *A. fumigatus* (F), *F. niger* (G), *F. nidulans* (H). I-O: Conidia of *F. proliferatum* (I), *F. verticillioides* (J), *F. semitectum* (K), *A. flavus* (L), *A. fumigatus* (M), *A. niger* (N), *A. nidulans* (O)

Production of Mycotoxins by *Fusarium* Species

According to the results attained in Table 2, isolate SS302C (*F. semitectum*) produced significantly high level of MON, which is 2926.20 ppm of MON, subsequently 66 ppm by isolate P301C and not detected in isolate B335C. No production of ZEA detected in all of the isolates. Particularly, 106 ppm of BEA was produced by *F. verticillioides* isolate N334C and 20.30 ppm by isolate P301C. Isolates SS302C, SS318C, B336C and B363C did not produce BEA. All isolates of *Fusarium* species

Table 3: Concentrations of AFs produced by the respective isolates of *Aspergillus* species

Aspergillus sp.	Isolate no.	Concentration (ppm)	
		AFB_1	AFB_2
A. flavus	P1C	226.55	4.48
	P2C	221.52	ND
	P13C	156.90	ND
	P14C	ND	ND
	C27C	133.44	ND
	C28C	138.85	ND
	C53C	580.26	ND
	C54C	138.03	ND
	C58C	171.70	ND
	C61C	91.60	ND
	C70C	100.41	ND
	C76C	147.60	ND
	C82C	180.73	ND
	C88C	99.16	ND
	C98C	ND	ND
	M108C	ND	ND
	M109C	ND	ND
	B119C	ND	ND
	B124C	ND	ND
	B126C	13.52	ND
	B135C	29.88	ND
	T144C	34.22	ND
	T145C	ND	ND
	T160C	4.08	ND
	SS162C	35.15	ND
	SS163C	27.57	ND
	SS164C	413.74	ND
	SS165C	40.78	ND
	SS193C	39.43	ND
	Q197C	33.30	ND
	Q198C	ND	ND
	Q212C	11.95	ND
	Q213C	26.37	ND
	J222C	17.07	ND
	J238C	23.80	ND
	N239C	59.51	ND
	N240C	11.43	21.87
A. nidulans	C242C	ND	ND
A. niger	P256C	ND	ND
A. fumigatus	Q284C	ND	ND

produced FB_1, and *F. semitectum* isolate C325C produced significantly FB_1 higher than other isolates, which is 2580.30 ppm. However, isolate B393C seemed to produce the slightest quantity of FB_1 that was 222.56 ppm. The analysis of MON (Table 2) revealed that *F. semitectum* produced the most, as the average production of MON was 1460.44 ppm, followed by *F. verticillioides* (1168.36 ppm) and lastly *F. proliferatum* (138.75 ppm). The average amount of FB_1 (Table 2) produced by both *F. verticillioides* and *F. proliferatum* are comparably low, which was 526.31 ppm and 368.24 ppm, respectively compared to 947.72 ppm by *F. semitectum*. For BEA analysis, *F. verticillioides* produced the highest BEA with an average concentration of 49.23 ppm, followed by *F. proliferatum* with an average concentration of 47.57 ppm and *F. semitectum* with an average concentration of 33.38 ppm (Table 2).

Production of Mycotoxins by *Aspergillus* Species

Table 3 showed that eight isolates, out of 37 isolates of *A. flavus* did not produce AFB_1, and 35 isolates did not produce AFB_2. Isolate C53C produced the highest amount of AFB_1, which is 580.26 ppm, and isolate T160C produced the least amount of AFB_1, which is 4.08 ppm. As for the production of AFB_2, only isolates P1C and N240C were detected producing 4.48 ppm and 21.87 ppm, respectively. Isolates of *A. nidulans*, *A. niger* and *A. fumigatus* did not produce either AFB_1 or AFB_2.

The analysis of AFB_1 and AFB_2 using UFLC analysis, eight isolates (P14C, C98C, M108C, M109C, B119C, B124C, T145C and Q198C) were negative for the production of AFB_1 and AFB_2. The analysis of AFB_1 and AFB_2 using UFLC showed that the level of AFB_1 produced were higher that the level of AFB_2.

Toxigenicity Test using *Artemia salina* Bioassay

The analysis of MON showed that isolate J405C has the highest mean of dead brine shrimps which was 22.7 shrimps (75.6%) (Table 4). The lowest was isolate B335C with a mean death of 8.3 shrimps (27.8%). All 16 isolates tested were significantly different from the three controls (blank, acetonitrile and methanol). This means, the extracts of all isolates tested, regardless of the presence of MON proved to be toxic to the brine shrimps.

The analysis of BEA showed that isolate J410C caused the highest mortality rate with a mean of 28.7 dead shrimps (95.6%). The lowest was isolate SS302C with a mean of 1.7 dead shrimps (5.6%). All isolates differed from the control indicating their toxicity, except isolate SS302C that laid in the same subset as the controls. This shows that the extract of SS302C was not lethal to the brine shrimps.

The analysis of ZEA revealed that isolate B393C has the highest mean of dead shrimp with 26.0 shrimps (86.7%), which was the most toxic. The least toxic isolate was P326C with a mean of 17.0 dead shrimps (56.7%). All isolates were considerably harmful compared to the controls.

The analysis of FB1 showed that all isolates except SS302C and SS318C have a mean of 30.0 dead shrimps, which was the highest. The lowest mean was shown by isolate SS318C that was 12.0 dead shrimps (40%). This indicated that all isolates with mean 30.0 individual dead shrimp were highly toxic to the brine shrimps.

The results in Table 5 also showed the analysis of AFs which proved that isolate P13C recorded the highest mean of dead brine shrimp, which was 22.7 shrimps (75.7%). The lowest mean was recorded by isolate B119C with a mean of 3.0 dead shrimps (10%).

Table 4: The mortality rate of *A. salina* tested with the mycotoxins produced by *Fusarium* isolates

Fusarium sp.	Isolate code	Mortality rate of *A. salina* treated with							
		MON		FB_1		ZEA		BEA	
		AVG	%	AVG	%	AVG	%	AVG	%
F. proliferatum	B30C	12.7	42.3	30.0	100.0	21.3	71.1	19.0	63.0
	B32C	12.7	42.3	30.0	100.0	19.7	65.6	27.3	91.1
	P301C	11.3	37.7	30.0	100.0	18.0	60.0	15.3	51.1
F. semitectum	SS302C	18.7	62.3	16.0	53.3	23.0	76.7	1.7	5.6
	SS318C	16.3	54.3	12.0	40.0	20.7	68.9	6.7	22.2
	C325C	18.3	61.0	30.0	100.0	23.0	76.7	20.3	67.8
	P326C	14.3	47.7	30.0	100.0	17.0	56.7	21.3	71.1
	SS329C	9.3	31.1	30.0	100.0	18.3	61.1	23.3	77.8
	C333C	16.0	53.3	30.0	100.0	19.7	65.6	17.3	57.8
F. verticillioides	N334C	13.7	45.6	30.0	100.0	18.7	62.2	26.7	88.9
	B335C	8.3	27.8	30.0	100.0	20.7	68.9	11.7	38.9
	B336C	17.7	58.9	30.0	100.0	20.7	68.9	6.3	21.1
	B363C	20.7	68.9	30.0	100.0	25.3	84.4	9.0	30.0
	B393C	13.7	45.6	30.0	100.0	26.0	86.7	28.7	95.6
	J405C	22.7	75.6	30.0	100.0	23.3	77.8	27.3	91.1
	P410C	22.3	74.4	30.0	100.0	19.3	64.4	28.0	93.3

*AVG = Average value

Discussion

Fusarium toxins are produced in the field throughout the growth of the maize plant. However, unsuitable storage conditions such as high moisture content can induce the fungi to produce mycotoxins (Rychlik and Asam, 2008). This statement is supported by the findings of this study, where both *Fusarium* and *Aspergillus* isolates were cultured onto sterilized cornmeal with sufficient moisture content. This condition has in fact favoured the production of mycotoxins by the respective fungal isolates.

According to the literatures on the production of MON, it is stated that the main producers of MON are *Fusarium* species in the Section Liseola, *F. subglutinans* and *F. semitectum* (Leslie and Summerell, 2006). Contrary to the literature, *F. proliferatum* seemed to be the least producer of MON, although *F. proliferatum* is categorized under Section Liseola. In this case, it could be accepted that the production of MON may vary among the *Fusarium* species (Desjardins *et al*., 2006), even different by the isolates in the same species as the production of MON could be affected by the surrounding factors such as temperature and humidity.

The analysis of ZEA showed that none of the three *Fusarium* species produced ZEA as the analysis gave negative results. These findings correspond to the prainy done by Nuryono *et al.* (2005) and Glenn (2007). They stated that the main producers of ZEA were *F. graminearum, F. pseudograminearum, F. crookwellense, F. equiseti* and *F. culmorum*. *F. proliferatum* and *F. verticillioides* are non-producers of ZEA (Leslie and Summerell, 2006). Regardless of the fact above, some researchers have found that certain culture of *F. verticillioides* produce ZEA, despite the fact that this species is a non-ZEA producer (Haschek *et al*., 2002). *F. semitectum* otherwise is known to produce ZEA (Leslie and Summerell, 2006; Ezekiel *et al*., 2008), but the results obtained in this study contrasted with the results obtained by Ezekiel *et al*. (2008). This phenomenon is justified based on the findings by Ezekiel *et al*. (2008) who suggested that ZEA production is highly dependent on the strain of *Fusarium* species. Although the fungi may belong to the same species, they are physiologically different from each other based on their individual strain. Hence, different strains of fungi in the same species may not be able to produce mycotoxins.

The analysis of FB_1 in this study indicated that all three species, *F. proliferatum*, *F. semitectum* and *F. verticillioides* are capable of producing FB_1. These results are in agreement with the findings of Kurtzman (2003) who had stated that *F. verticillioides* and its related species, *F. proliferatum*, are frequent contaminants of corn-based feed and are capable of producing high levels of the FB_1 mycotoxin (Kurtzman, 2003). It has to be taken into consideration that although *F. verticillioides* and *F. proliferatum* are known FB_1 producers, the strains used in this study may have altered ability to produce FB_1. This abnormality can be validated in line with the fact that the level of FB_1 produced by both *F. verticillioides* and *F. proliferatum* are greatly dependent on the genetic of the strains involved (Tseng *et al*., 1995; Reynoso *et al*., 2004). There are reports, which stated that the production of FB_1 by different strains of *F. proliferatum* differs greatly. Other reports have also showed that only low or even no FB_1 production by *F. proliferatum* (Ghiasian *et al*., 2005). *F. semitectum* although known to be a poor producer of FB_1, seemed to produce the highest FB_1, which may be due to the condition of the environment in which the fungal isolates were cultured and incubated.

As for the production of BEA, *F. proliferatum* and *F. semitectum* are the well-known producers (Josephs *et al*., 1999). *F. verticillioides* on the contrary in found to produce BEA only in trace amount (Leslie and Summerell, 2006). In

Table 5: The mortality rate of *A. salina* tested with the AFs produced by *Aspergillus* isolates

Aspergillus sp.	Isolate code	Mortality rate of *A. salina* treated with AFs	
		AVG	%
A. flavus	P1C	22.3	74.3
	P2C	7.3	24.4
	P13C	22.7	75.7
	P14C	14.7	48.9
	C27C	16.7	55.7
	C28C	12.7	42.2
	C53C	18.3	61.0
	C54C	8.7	28.9
	C58C	14.7	48.9
	C61C	16.0	53.3
	C70C	9.3	31.1
	C76C	8.3	27.8
	C82C	11.3	37.8
	C88C	13.3	44.4
	C98C	15.7	52.2
	M108C	6.7	22.3
	M109C	11.3	37.8
	B119C	3.0	10.0
	B124C	5.3	17.7
	B126C	4.7	15.7
	B135C	5.3	17.7
	T144C	5.3	17.7
	T145C	11.7	38.9
	T160C	9.6	32.0
	SS162C	11.7	38.9
	SS163C	8.3	27.8
	SS164C	13.0	43.3
	SS165C	14.3	47.8
	SS193C	9.7	32.3
	Q197C	4.0	13.3
	Q198C	10.0	33.3
	Q212C	3.3	11.0
	Q213C	5.3	17.8
	J222C	7.0	23.3
	J238C	11.7	39.0
	N239C	12.0	40.0
	N240C	6.0	20.0
A. nidulans	C242C	15.3	51.0
A. niger	P256C	14.3	47.7
A. fumigatus	Q284C	12.7	42.3

*AVG = Average value

this study, *F. verticillioides* produced the highest BEA with an average concentration of 49.23 ppm, followed by *F. proliferatum* with an average concentration of 47.57 ppm and *F. semitectum* with an average concentration of 33.38 ppm. As stated by Desjardins *et al.* (2006), the production of mycotoxins may differ among species and that the production of mycotoxins by certain *Fusarium* isolates may or may not occur.

The ability of the isolates of *A. flavus* to produce AFB_1 and AFB_2 supports the finding that *A. flavus* is indeed one of the main producers of AFs besides *A. fumigatus* (Munkvold *et al*., 1998). According to Leong *et al*. (2010), the highest amount of AFs detected in shelled raw groundnuts was 711 µg/kg, which is 0.71 ppm. This incident can be used as a benchmark to evaluate the fact that the amount of AFs quantified occurs in large concentrations. The occurrence of this is considered a norm in tropical and subtropical regions such as in Malaysia, where the hot and humid weather provides optimal conditions for *A. flavus* molds to grow (Leong *et al*., 2010). Not all the isolates of *A. flavus* in this study produced AFs, this is because, the production of AFs are isolate-specific.

The extraction of MON, FB_1, BEA, ZEA and AFs involved both acetonitrile and methanol. Hence, positive controls consisting of both these chemicals were set up separately to investigate the tolerance level of the brine shrimp. It turned out that the acetonitrile and methanol treatment did not differ from the blank control, which contained only artificial seawater. This proves that the content of methanol and acetonitrile in the mycotoxin extracts does not interfere with the mortality rate of the brine shrimp. In all the mycotoxins analysed using this bioassay, there was a similarity. It was observed that, although some of the isolates were mycotoxin-free their extracts were noted to be toxic to the brine shrimps. The means of the dead brine shrimps were significantly different from the means of the controls used, which indicates that the extracts of the mycotoxin-free isolates are toxic to the test organism. This incident may be due to the presence of certain organic compounds such as fatty acids that might have been extracted from the cornmeal. These compounds could have been toxic to the brine shrimps (Hlywka *et al*., 1997).

The bioassay results for FB_1 showed that despite the concentrations of the toxin, whether high or low, the mortality of the brine shrimps was 100%. Only isolates SS302C and SS318C from *F. semitectum* caused the least death among other isolates. This finding is in accordance with Jimenez *et al*. (1997) who stated that extracts containing FB_1 are highly toxic to brine shrimp larvae.

The isolates that were all ZEA-free also caused death to the brine shrimps. This may be due to the presence of additional compounds produced by the fungal isolates (Jimenez *et al*., 1997). Besides, not all extracts of mycotoxins were purified, hence there are residues of fatty acid compound and other organic matters extracted from the cornmeal. This could contribute to the mortality of the brine shrimp larvae.

Mycotoxin production ability by *Fusarium* and *Aspergillus* species are monitored by their specific isolates. Although belonging to the same species, they still differ in terms of strain specificity. The loss of toxin production by a *Fusarium* strain is possibly due to mutation, where the strains were cultured for long periods as well as being repeatedly cultured. In addition, diverse choice of substrate such as cornmeal, wheat, rice barley and so on including different environmental conditions for growing the fungal cultures could determine the status of mycotoxin production.

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(Received 27 February 2014; Accepted 23 July 2014)