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Aspergillus species from groundnuts (*Arachis hypogaea*) and mycotoxin production by toxigenic species

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ABSTRACT

Aims: Groundnut is an important food crop and is susceptible to contamination by *Aspergillus*. The present study was conducted to identify *Aspergillus* spp. from groundnuts as well as to detect mycotoxin production by toxigenic species.
Methodology and results: Molecular identification using ITS region, β-tubulin and calmodulin genes identified six species, *A. niger*, *A. tubingensis*, *A. flavus*, *A. aculeatus, A. sydowii* and *A. fumigatus.* Phylogenetic tree of combined sequences showed the isolates from the same species were grouped with reference strains in the same clade, thus the species identity was confirmed. Detection of mycotoxin biosynthesis genes can give an indication of mycotoxin production. Two ochratoxin A genes, PKS15KS and PKS15C-MeT were detected in seven *A. niger* isolates but none of the isolates produced ochratoxin A when quantification was conducted using Ultra-High Performance Liquid Chromatography. Two aflatoxin B1 biosynthesis genes, Nor-1 (norsolorinic acid) and Ver-1 (Versicolorin) genes were detected in *A. flavus* but only KDH7 and KL27b isolates produced aflatoxin B1 with concentrations of 1.0 µg/g and 1.1 µg/g, respectively.
Conclusion, significance and impact of the study: Various species of *Aspergillus* found on groundnuts may lead to potential mycotoxin contamination as toxigenic species were also recovered. The occurrence of *Aspergillus* spp. can reduce the quality of the legumes as well as reducing their shelf life.

Keywords: *Aspergillus*, groundnuts, molecular identification, ochratoxin A, aflatoxin B1

INTRODUCTION

Groundnut (*Arachis hypogaea*) or peanut is a legume belonging to the family Fabaceae. In Malaysia, groundnut is often used as an ingredient in cooking. Groundnuts are commercialised as raw or roasted, salted and consumed as a snack. Raw shelled groundnuts are available in almost all supermarkets and sundry shops in Malaysia.

Groundnuts cultivation and production are not extensive in Malaysia. The crop is more suitable to be used as crop rotation which is locally grown in rotation with other crops as well as grown as intercrop in small-holders farm. Groundnuts are grown in the riverine and in rainfed rice areas in Kelantan, Terengganu, Kedah and Pahang (Halim and Ramli, 1980).

Groundnuts are mainly imported from Vietnam, USA, China, Thailand and Hongkong. A total of 44,871 tonnes of groundnuts were imported, mainly in the form of shelled nuts as a response to high demands (Halimah and Lum, 1992).

Groundnuts are imported across the world and thus contamination can easily occur. Under favourable conditions during storage either in the shops and markets or shipments in long journey, groundnuts are prone to spoilage and contamination by diverse groups of microorganisms particularly storage fungi. One of the storage fungi which are widely distributed is *Aspergillus* spp. which can cause contamination in storage products including groundnuts.

Groundnut contamination by *Aspergillus* might cause health risk to human and livestock as groundnuts are commonly consumed directly. Moreover, mycotoxins that may be present in groundnuts are also toxic and have harmful effects on animals and humans. Identification of *Aspergillus* spp. and mycotoxin detection are important for implementing suitable control strategies for groundnuts storage and this will lead to improving quality control of groundnuts for consumer safety. Thus, the present study was conducted to molecularly identify *Aspergillus* species contaminating groundnuts and to determine the ability of toxigenic species, *A. niger* and *A. flavus* to produce ochratoxin A (OTA) and aflatoxin B1 (AFB1).

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MATERIALS AND METHODS

Fungal isolates

Aspergillus isolates were isolated from groundnuts using surface sterilization and direct plating methods (Samson *et al*., 2010). The groundnuts were purchased from sundry shops and supermarkets in Kuala Lumpur, Pulau Pinang, Sarawak, Kedah, Johor, Perak and Terengganu. The groundnuts in sundry shops and supermarkets were stored in gunny sacks or storage bins and were kept at room temperature. A total of 100 *g* groundnuts was randomly scooped from the gunny sacks or storage bins and purchased from each shop. The weight of the groundnuts is not vital for isolation of fungi from food and feed, but a sample size of 100 food particles must be used for isolation purposes (Samson *et al*., 2010).

Molecular identification

From isolates of *Aspergillus* isolated from groundnuts, 61 isolates were chosen as representative isolates from 98 morphologically identified *Aspergillus* species. The isolates were chosen based on similarity of colony colours, shape of conidia, conidiophores and shape of vesicles. The isolates were molecularly identified using ITS region, β-tubulin and calmodulin genes.

For DNA extraction, mycelia were grown in malt extract broth in Universal bottles with three replicates for each isolate and incubated at 27 °C. Mycelia were harvested after 48 h as the level of sporulation was low and give a better yield of DNA after extraction.

The mycelia were dried using Whatman No. 1 filter paper, freeze-dried for 48 h and were ground into fine powder using liquid nitrogen. The fine powder of the mycelia was transferred into a sterile 2.0 mL microcentrifuge tube and approximately 60 mg of the mycelial powder was weighed for DNA extraction. The DNA was extracted using Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the protocols by the manufacturer.

For amplification of ITS region, ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) primers were used as described by White *et al*. (1990). β-tubulin gene was amplified using Bt2a (GGT AAC CAA ATC GGT GCT GCT TTC) and Bt2b (ACC CTC AGT GTA GTG ACC CTT GGC) primers (Glass and Donaldson, 1995) and calmodulin was amplified using CMD5 (CCG AGT ACA AGG ARG CCT TC) and CMD6 (CCG ATR GAG GTC ATR ACG TGG) primers (Hong *et al*., 2005).

PCR amplification of the ITS region, β-tubulin and calmodulin genes was performed in a total volume of 25 µL containing 0.5 µL of genomic DNA, 4.0 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 0.15 µL of 5U *Taq* polymerase (Promega, Madison, WI, USA) and 4.0 µL of 5 mM primers.

PCR was performed using a thermocycler (Bio-Rad MyCycler, Hercules, CA, USA) with the following cycles: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for ITS region and 56 °C for both β-tubulin and calmodulin genes, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. After PCR, the PCR products were sent to a service provider for DNA sequencing.

Phylogenetic analysis

MEGA5 software (Tamura *et al*., 2011) was used to perform multiple sequence alignment of the sequences and to generate phylogenetic tree. Phylogenetic tree of combined sequences of ITS region, β-tubulin and calmodulin were generated as combined sequences can give more accurate species phylogeny (Wiens, 1998). Sequences of ex-type strains of each *Aspergillus* species were also included in the phylogenetic analysis as reference isolates (Table 1). *Aspergillus ustus* was also included in the phylogenetic analysis as an outgroup.

Table 1: Ex-type strains of *Aspergillus* spp. included in phylogenetic analysis

Species	ITS region	β-tubulin	Calmodulin
A. niger	FJ629337	GU296687	FN594540
A. tubingensis	FJ629354	FJ629305	FN594448
A. flavus	KU296260	EF203146	EF202057
A. aculeatus	AY585558	HE577806	EU330198
A. sydowii	NR131259	EF652297	EU443971
A. fumigatus	KU296266	AY685169	AY689353
A. ustus	NR131284	EF591727	EF591719

Maximum-likelihood (ML) method was used to generate the phylogenetic tree. This method provided the most possible outcome and examines all possible topologies and to choose the one that shows the smallest amount of total evolutionary changes (Huelsenbeck, 1995). Nearest-Neighbour Interchange algorithm was used in ML method to search for topologies that fit the data better. Bootstrap values of 1000 replications were used to generate the tree.

Mycotoxin detection

Eleven isolates of *A. niger* were chosen for OTA analysis and nine isolates of *A. flavus* for AFB1 analysis (Table 2).

OTA gene detection

The primers used for amplification of OTA genes were PKS15KS primers, PKS15KS-f (5-CAATGCCGTCCAACCGTATG-3) and PKS15KS-r (5-CCTTCGCCTCGCCCGTAG-3), and PKS15C-MeT primers, PKS15CMeT-f (5-GCTTTCATGGACTGGATG-3) and PKS15C-MeT-r (5-CATTTCGTTGATCCCATCG-3) (Ferracin *et al*., 2012). These primers were used to amplify polyketide synthase genes which involved in OTA biosynthesis in *Aspergillus*. The PKS15KS primer was used to amplify DNA fragments corresponding to β-ketoacyl synthase (KS) domain while PKS15C-MeT amplify DNA fragments corresponding to C-

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methyltransferase (C-Met) domain which are part of putative polyketide synthase gene gene, An15g07920 (Ferracin *et al*., 2012). Both C-Met and KS domains are found in An15g07920 gene which has been annotated as putative ochratoxin clusters (Pel *et al*., 2007).

Table 2: *Aspergillus* isolates used in OTA and AFB1 analyses

Aspergillus spp.	Isolates (OTA)	Isolates (AFB1)
A. niger	KDH 4	
A. niger	KDH5	
A. niger	KL19	
A. niger	KL25	
A. niger	KL29b	
A. niger	PNGM7	
A. niger	PRK 9b	
A. niger	SRW11	
A. niger	PNGT2	
A. niger	PNGT3	
A. niger	TGN2	
A. flavus		JOH5
A. flavus		JOH6
A. flavus		KDH7
A. flavus		KL16b
A. flavus		KL27b
A. flavus		KL29a
A. flavus		PRK3
A. flavus		SRW6b
A. flavus		TGN1

PCR amplification was performed in a total volume of 25 µL consisting of 0.5 µL of genomic DNA, 5.0 µL of 5× green buffer, 4.0 µL of 25 mM $MgCl_2$, 2.5 µL of each forward and reverse primers, 0.125 µL of *Taq* DNA polymerase (Promega), 0.5 µL of 10 mM dNTP mix (Promega) and sterile distilled water made up to 25 µL. PCR was run in a thermal cycler (Bio-Rad MyCycler) with the following conditions; initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 1 min, annealing for PKS15KS at 58.8 °C and PKS15C-MeT at 51 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Agarose gel (1%) was used to detect the PCR products. Wealtec Elite 300 power supply and buffer tank GES with 1X Tris-Borate-EDTA (TBE) buffer were used to run the electrophoresis. FloroSafe DNA stain (1st Base, Malaysia) was used to stain the gel. Electrophoresis was run at 80 V, 400 mA for 60 min. The sizes of the amplified bands were estimated by comparison with 100 bp DNA marker (GeneRulers™ DNA markers, Fermentas). After electrophoresis, the gel was viewed and visualized using Bio-RAD Molecular Imager Series® Gel Doc™ XR System and the gel photo was taken using The Discovery Series™ Quantity One® 1-D Analysis software version 4.6.5.

Aflatoxin B1 gene detection

Gene detection for AFB1 was performed using Nor-1 (norsolorinic acid) primers, Nor-1-f (5-ACCGCTACGCCGGCACTCTCGGCAC-3) and Nor-1-r (5-GTTGGCCGCCAGCTTCGACACTCCG-3) and Ver-1 (Versicolorin) primers, Ver-1-f (GCCGCAGGCCGCGGAGAAAGTGGT) and Ver-1-r (5-CGAAAAGCGCCA CCATCCACCCCAATG-3) as described by Rashid *et al.* (2008). These two primers were used as both primers are highly specific for the genes to be essential for AF biosynthesis (Hussain *et al*., 2015). These two genes are used to detect the production of AF and able to identify and distinguish aflatoxin-producer with non-aflatoxin producer (Rashid *et al*., 2008; Hussain *et al*., 2015).

PCR amplification was performed in a total volume of 25 µL by adding 12.5 µL of Econo *Taq* Plus Green 2× Master mix (Lucigen, Middleton, WI, USA), 0.25 µL of each forward and reverse primers, 1.0 µL of DNA template and 11 µL of sterile distilled water.

PCR amplification was carried out using a thermal cycler (Bio-Rad MyCycler) as follows: an initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 58–62 °C for 1 min for both primers, extension at 72 °C for 30 sec, and a final extension at 72 °C for 10 min. Electrophoresis conditions was the same as to detect OTA genes.

Extraction of Ochratoxin A and Aflatoxin B1

OTA and AFB1 were extracted based on the method described by Bragulat *et al.* (2001). The isolates were cultured at three point's inoculation on Czapek Yeast Agar (25 ˚C) for 7 days. Agar plug (0.5 cm diameter) was removed from the centre of the growing colonies. After 7 days, three agar plugs were put in a Bijou bottle and mixed with 0.5 mL of methanol, shaken vigorously and left for 1 h at room temperature. After 1 h, the extracts were filtered through syringe filter (PTFE) with diameter of 0.2 µm × 13 mm. The extracts were then injected into a small vial with 3 mL syringe (NIPRO).

Ochratoxin A and Aflatoxin B1 Analysis Using UHPLC-FLD

Preparation of OTA Standard and OTA Analysis

OTA standard was purchased from Sigma Aldrich, USA. Stock solution was prepared by dissolving 1 mg of OTA in 1.0 mL of methanol (HPLC grade). Working standard solutions were prepared in five different concentrations, 2 µg/g, 4 µg/g, 6 µg/g, 8 µg/g, and 10 µg/g.

Analysis was performed using an Acquity UHPLC™ system (Waters) equipped with BEH C18 column (2.1 × 50 mm) connected to Fluorescence (FLR) detector (Waters). The mobile phase was acetonitrile (CH_3CN), deionized water and acetic acid (CH_3COOH) (57:41:2 v/v/v). The samples and standards (5 µL each) were injected into the UPLC system and run for 5 min.

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Excitation and emission wavelength were set at 330 nm and 440 nm, respectively. The flow rate was 0.2 mL/ min. The retention time and peak heights in the samples were compared with OTA standards using a calibration curve. The samples were quantified by comparing retention time and peak heights in the sample with OTA standards using a calibration curve.

Preparation of Aflatoxin B1 Standard and Aflatoxin Analysis

Aflatoxin B1 standard was purchased from Sigma Aldrich, USA. Stock solution was prepared by dissolving 1 mg of AFB1 in 1.0 mL of methanol (HPLC grade). Working standard solutions were prepared in five different concentrations, 2 µg/g, 4 µg/g, 6 µg/g, 8 µg/g, and 10 µg/g respectively. ACQUITY UHPLC™ system (Waters) equipped with BEH C18 column (2.1 × 50 mm) connected to FLD (Waters) was used for AFB1 analysis. Excitation and emission wavelength were set at 330 nm and 440 nm, respectively. The mobile phases were deionized water, acetonitrile (CH_3CN) and methanol (60:20:20 v/v/v). The samples and standards (5 µL each) were run for 4 min. The flow rate was 0.2 ml/ min. The samples were quantified by comparing retention time and peak heights in the samples with AFB1 standards using a calibration curve.

RESULTS AND DISCUSSION

Molecular identification

Internal transcribed spacer region, β-tubulin and calmodulin genes were successfully amplified and produced a single band of approximately 600 bp for all 81 isolates of *Aspergillus* spp. except *A. sydowii* that produced 500 bp of β-tubulin gene. All the isolates molecularly identified using ITS region, β-tubulin and calmodulin genes were deposited in the GenBank (Accession numbers - ITS : KY593515–KY593495; β-tubulin : KY587237 – KY587303; KY609932 – KY609941; calmodulin: KY593505–KY593514; KY609922–search of ITS region, β-tubulin and calmodulin genes, eight species were identified as *A. niger* (35 isolates), *A. tubingensis* (10 isolates), *A. aculeatus* (three isolates), *A. flavus* (nine isolates), *A. fumigatus* (two isolates), and *A. sydowii* (two isolates). The percentage of similarity produced by all isolates ranged from 99-100%.

Phylogenetic relationship of the isolates is shown in Figure 1. From the tree, all the isolates from the same species including the reference isolates were grouped in the same clade. Isolates of *A. niger* (clade A) were separated from *A. tubingensis* (clade B) with 99% bootstrap value. Both *A. niger* and *A. tubingensis* isolates were grouped with the ex-type strains of *A. niger* and *A. tubingensis*. Clade C consisted of *A. aculeatus* isolates, Clade D, *A. fumigatus* isolates, Clade E, *A, flavus* isolates and Clade F, *A. sydowii* isolates. For molecular identification, sequence analyses of ITS region, β-tubulin and calmodulin genes were applied. These region and genes are recommended by Samson *et al.* (2011) for molecular identification of *Aspergillus* spp. ITS region is the most common region used to differentiate *Aspergillus* spp. as the region is also used to differentiate species within a section such as to distinguish between *A. flavus* and *A. tamari* (section *Flavi*) (Yazdani *et al.*, 2011), as well as between *A. niger* and *A. tubingensis* (section *Nigri*) (Varga *et al.*, 2007) of which these two species are closely related and their morphological characteristics are similar.

Phylogenetic analyses of combined sequences of ITS, β-tubulin and calmodulin showed there was very little variation or no variations observed among the isolates of the same species. Similar results were reported by Hong *et al.* (2005) of which the phylogenetic analysis of β-tubulin and calmodulin showed little variation among the isolates of *A. fumigatus* and *A. lentulus.* In a study by Krimitzas *et al.* (2013) using combined sequences of ITS, intergenic spacer region, β-tubulin and RNA polymerase II genes also did not show any variation among several species including between *A. niger* and *A. awamori,* and *A. amstelodami* and *A. rubrum.* Therefore, based on molecular identification and phylogenetic analysis using ITS, β-tubulin and calmodulin gene sequences, the identity of the *Aspergillus* isolates isolated from groundnuts was confirmed.

Aspergillus niger was the most prevalent species isolated from groundnuts, and the species has been reported as common species isolated from Southeast Asian food commodities (Pitt and Hocking, 2009). *Aspergillus niger* was also the most prevalent species isolated from groundnuts in Pakistan (Rasheed *et al.*, 2004), Eastern Ethiopia (Mohammed and Chala, 2014) and Egypt (Embaby and Abdel-Galel, 2014). In addition to groundnuts, *A. niger* has been isolated from other types of nuts including pecans (Pitt and Hocking, 2009), cashew nuts (Adebajo and Diyaolu, 2003), almonds, pistachios and walnuts (Molyneux *et al.*, 2007).

Aspergillus tubingensis has also been reported as contaminants of groundnut (Palencia *et al.*, 2014). Other than groundnuts, *A. tubingensis* has been found in maize (Palencia *et al.*, 2014) and grapes (Somma *et al.*, 2012). *Aspergillus aculeatus* has also been reported as contaminants of groundnut (Palencia *et al.*, 2014). Other than groundnuts, *A. aculeatus* is common contaminant of grapes (Somma *et al.*, 2012) and various post-harvest crops such as apples, pears, peaches, citrus, grapes, figs, strawberries, tomatoes, melons, dried fruit, beans, oil seed and nuts (JECFA, 2001).

Aspergillus flavus is one of the most common fungal contaminants of food and feed, as well as the main producer of aflatoxins. This species is also the most widely reported food borne fungus especially in the tropics (Pitt and Hocking, 2009). *Aspergillus flavus* is prevalent on different types of peanuts in Southeast Asia including pecans (Pitt and Hocking, 2009), cashew nuts (Adebajo and Diyaolu, 2003) almonds, pistachios and walnuts (Molyneux *et al.*, 2007).

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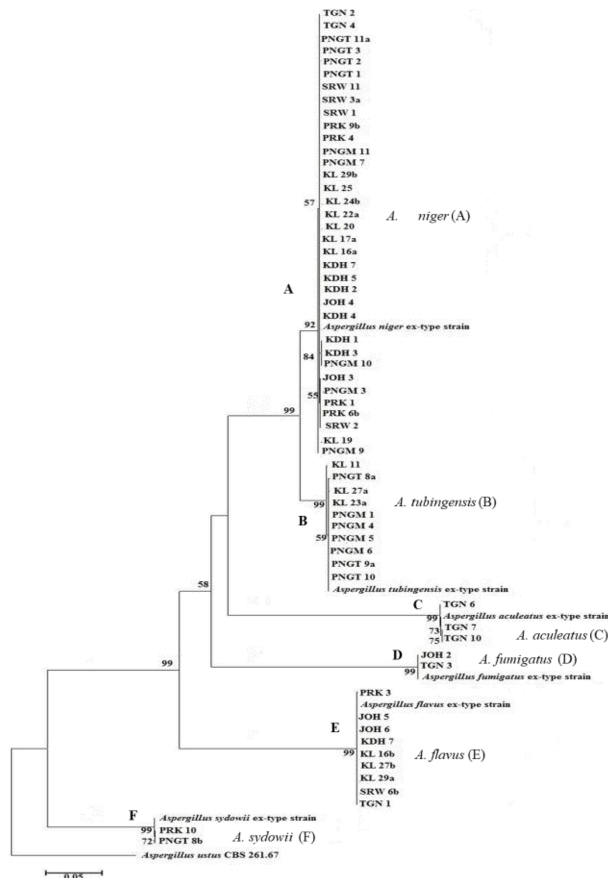

Figure 1: Maximum Likelihood tree generated based on combined sequences of ITS, β-tubulin and calmodulin of *Aspergillus* spp. from groundnuts. *Aspergillus ustus* is the outgroup.

Occurrence of *A. flavus* on groundnuts may lead to aflatoxin production although in the present study, aflatoxin was produced by only two isolates of *A. flavus*. Nevertheless, *A. flavus* is the main source of aflatoxins contamination in the world's food supplies (Pitt and Hocking, 2009). Studies by Rostami *et al*. (2009), Reddy *et al*. (2010), Rajarajan *et al.* (2013) and Guchi (2015) reported that aflatoxigenic *A. flavus* was the most predominant species contaminated groundnuts. Other than groundnuts, *A. flavus* is also prevalent on wheat, barley, oats, rye, maize and rice (Reddy *et al.,* 2010).

Aspergillus fumigatus has often been recovered from store commodities in the tropics and can grow at low water activity and high temperature (Pitt and Hocking, 2009). Besides groundnuts, *A. fumigatus* has been reported in other types of nuts such as hazelnuts, walnuts and peanuts (Pitt and Hocking, 2009) and cashew nuts (Adebajo and Diyaolu, 2003). The occurrence of *A. fumigatus* has also been reported on other types of food and feed such as dried fish, corn snacks, melon seeds, mango pickles, dried onion and different types of cheese (Pitt and Hocking, 2009); wheat grain (Misra *et al*., 2010) and maize (Makun *et al*., 2010). Contamination of *A. fumigatus* on food product may lead to production of metabolites particularly gliotoxin (Sugui *et al*., 2007) and fumagilin (Fallon *et al*., 2011).

Aspergillus sydowii is among the storage fungi found in Southeast Asian food and feed commodities. Pitt and Hocking (2009) reported that *A. sydowii* is commonly found on dried foods, including various types of nuts such as peanuts, pistachios, hazelnuts, walnuts and pecans. *Aspergillus sydowii* has been recovered from cereals such as barley, wheat, flour and pepper samples (Pitt and Hocking, 2009). Thus, it is not surprising that *A. sydowii* was recovered from groundnuts in the present study.

OTA gene detection and quantification

Seven isolates of *A. niger* produced the PKS15KS band which was approximately 776 bp and the PKS15C-MeT, 998 bp band. For analysis and quantification of OTA using UHPLC, the production of OTA by the *Aspergillus* isolates were detected by comparison of retention times with OTA standards at 2.9 min. None of the *A. niger* isolates produced OTA even though PKS15KS and PKS15C-MeT genes were detected in seven isolates of *A. niger* (KDH4, KL 19, KL 25, PNGM 7, PRK 9b, SRW11 and PNGT 3). From this analysis, OTA was not produced by *A. niger* isolates from groundnuts.

Aflatoxin B1 gene detection and quantification

Nor-1 and Ver-1 genes were detected in nine *A. flavus* isolates (JOH5, JOH 6, KDH7, KL16b, KL27b, KL29a, PRK3, SRW6b and TGN and KL6. A single band of approximately 400 bp for Nor-1 and 600 bp for Ver-1 gene were produced.

The production of AFB1 was detected at similar retention time with AFB1 standards at 1.9 min. Among the nine isolates of *A. flavus,* only two isolates produced AFB1 (KDH7 and KL27b). The concentration levels of AFB1 produced by isolate KDH7 was 1.0 µg/g and isolate KL27b, 1.1 µg/g.

OTA is a mycotoxin produced by several species of *Aspergillus* including *A. niger*, *A. ochraceus*, *A. carbonarius* and *A. melleus* which can contaminate various agricultural products. The first step to detect OTA production is to detect OTA biosynthesis genes. In this study, PKS15KS and PKS15C-MeT genes were detected in seven *A. niger* isolates. Both genes encode polyketide synthase genes in OTA biosynthesis (Ferracin *et al*., 2012; Kim *et al*., 2014). Similar to the present study, PKS15KS and PKS15C- MeT genes were used to detect the ability of *A. niger* isolates from Korean fermented food to produce OTA (Kim *et al*., 2014).

From 11 isolates of *A. niger*, PKS15KS and PKS15C-MeT genes were not detected in four isolates of *A. niger* (KDH 5, KL 29b, PNGT 2 and TGN 2) and may indicate that these isolates are non OTA producer. The results of this study were similar to a study by Kim *et al.* (2014) whereby PKS15KS and PKS15C-MeT genes were not

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detected in 16 isolates of *A. niger* from various Korean foods.

Based on UHPLC analysis, the seven isolates of *A. niger* (KDH4, KL 19, KL 25, PNGM 7, PRK 9b, SRW11 and PNGT 3) that were positive for OTA genes did not produce OTA. This might be due to the deletion or mutation of OTA gene clusters (Kim *et al*., 2014). The loss of the ability to produce OTA by *A. niger* might also be associated with deletion of nucleotides of the gene within the OTA gene clusters (Massi *et al*., 2016).

Besides mutation of OTA gene, environmental factors particularly water activity and temperature can also play a role on OTA production by *A. niger*. Milani (2013) reported that the production of OTA is at optimum temperature of 25 to 30 °C and 0.98 a_w. These conditions are common conditions where groundnuts are stored and the possibility of OTA production by ochratoxigenic fungi is higher. Production of OTA can occur in a few days if certain environmental conditions such as temperature, humidity and water activity are met.

OTA contamination in warm temperate areas and tropical region could be associated with *A. ochraceus* and black Aspergilli. According to Amezqueta *et al.* (2004), OTA can be produced by *A. niger* at 25-30 °C and 0.95-0.99 a_w. Therefore, fast drying and humidity control of food and feed in storage are necessary to avoid fungal invasion and toxin production.

Magnoli *et al.* (2007) reported that *A. niger* from stored peanuts produced OTA. In contrast, Sultan and Magan (2010) reported that none of *A. niger* isolates from groundnuts produced OTA which is similar with the present study. The results showed that the presence of PKS15KS and PKS15C-MeT genes were not necessarily an indication of OTA production. Although *A. niger* from groundnuts did not produce OTA, other species of black aspergilli have been reported to produce OTA, for example, *A. ochraceus* from peanut butter (Boli *et al*., 2013), *A. awamori*, *A. carbonarius* and *A. japonicus* from stored peanuts (Magnoli *et al*., 2006).

In the present study, two aflatoxin biosynthesis genes, Nor-1 and Ver-1 genes were detected. Both genes coded for key enzymes for aflatoxin production and are considered as an indicator of aflatoxin production by aflatoxigenic *Aspergillus* spp. (Rashid *et al*., 2008; Hussain *et al*., 2015). In several studies, Nor-1 and Ver-1 genes were initially detected to distinguish between aflatoxin and non-aflatoxin producers (Hussain *et al*., 2015; Davari *et al*., 2015).

Nor-1 and Ver-1 genes were detected in all nine *A. flavus* isolates tested in this study. However, based on UHPLC analysis, only two isolates of *A. flavus* (KDH 7 and KL 27b) produced AFB1 with concentrations of 1.0 µg/g and 1.1 µg/g, respectively. The inability to produce AFB1 by *A. flavus* might be due to deletion of the gene cluster (Yu *et al*., 2004). According to Criseo *et al.* (2001), although Nor-1 and Ver-1 genes are present in some non-aflatoxigenic isolates, occurrence of mutations such as substitution of some bases can cause formation of non-functional products.

AFB1 production was only detected in two isolates of *A. flavus* in which isolate KDH7 produced 1.0 µg/g of AFB1 and isolate KL27b produced 1.1 µg/g. Guezlane-Tebibel *et al.* (2013) classified aflatoxigenic *Aspergillus* section *Flavi* according to the concentrations of AFB1 produced on CYA. The four groups of concentration levels were classified as high (> 1.1 µg/g), moderate (0.11 to 1 µg/g), low (0.011 to 0.10 µg/g) and very low (0.005 to 0.01 µg/g). Based on this classification, isolates KDH7 and KL27b can be classified as moderate producers, suggesting that are risk of AFB1 contamination of groundnuts by *A. flavus*. AFB1 concentration from 24.0 to 87.5 µg/kg has been found in peanuts while in peanut products, from 22.0 to 84.6 µg/kg (Hoeltz *et al*., 2012). Amiri *et al.* (2013) reported that AFB1 was detected in several types of nuts including peanuts, almonds, walnuts and hazelnuts with high concentration levels (0.016 – 15.744 µg/kg). In addition to nuts, AFB1 has been reported as contaminants in other food products such as maize and brown rice with levels of AFB1 ranging from 0 to 149.32 µg/kg (Karthikeyan *et al*., 2013) and 1.07 to 24.65 µg/kg (Asghar *et al*., 2014), respectively.

Seven isolates of *A. flavus* did not produce AFB1 and these isolates are considered as non-aflatoxigenic isolates. Occurence of non-aflatoxigenic isolates of *A. flavus* are common in groundnuts (Yin *et al*., 2009; Okun *et al*., 2015). In addition to groundnuts, non-aflatoxigenic *A. flavus* was also found in maize (Probst *et al*., 2011; Okun *et al*., 2015) and cotton seed (Cotty, 1997).

Contamination of *Aspergillus* on groundnuts may occur during pre-harvest and post-harvest, influenced by several factors such as poor storage condition, mechanical damage when harvesting, inadequate drying and poor transportation condition. Improper handling during pre-harvest including crop rotation, tillage, planting date, irrigation and fertilization which may influence the incidence of *Aspergillus* infestation especially *A. flavus* on groundnuts (Torres *et al*., 2014). Contamination of *Aspergillus* on groundnuts during post-harvest could be attributed to cleaning, grading, transportation, storage, processing, packaging, and retailing (Kimatu *et al*., 2012). However, contamination during post-harvest can be prevented by quick drying of pods, controlling storage pests, storing the peanuts at low moisture content less than 10% and using mechanical threshers (Waliyar *et al*., 2013).

Occurrence of *Aspergillus* spp. on groundnuts may lead to contamination of mycotoxin and can be harmful to livestock as well as to human. Mycotoxin contamination can affect the quality of groundnut and may reduce the germination rate with loss of carbohydrate, protein and oil content (Begum *et al*., 2013). The occurrence of *Aspergillus* spp. on groundnuts can also reduce the quality of the legumes as well as reducing their shelf life.

CONCLUSION

Several species of *Aspergillus* were isolated and identified from groundnuts, namely *A. niger, A. tubingensis, A. flavus, A. aculeatus*, *A. sydowii* and *A.*

fumigatus. Among the species, *A. niger* and *A. flavus* are two well-known toxigenic species. Although polyketide synthase gene involved in OTA biosynthesis were detected in seven isolates of *A. niger*, OTA was not produced by *A. niger* which indicate that *A. niger* isolates from groundnuts are not OTA producers. Aflatoxin biosynthesis gene, Nor-1 and Ver-1 genes were detected in nine isolates of *A. flavus*. However, only two isolates of *A. flavus* produced AFB1, and is classified as a medium AFB1 producers. The present study showed that the presence of polyketide synthase genes (PKS15KS and PKS15C-MeT) and aflatoxin biosynthesis genes (Nor-1 and Ver-1) do not necessarily lead to OTA and AFB1 production.

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