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

Amalina Kamaruddin and Latiffah Zakaria*

School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia.
Email: Lfah@usm.my

Received 18 February 2019; Received in revised form 14 June 2019; Accepted 20 June 2019

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 (Halm and Raml, 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).

*Corresponding author

DOI: http://dx.doi.org/10.21161/mjm.190375

ISSN (print): 1823-8262, ISSN (online): 2231-7538
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. The isolates 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 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 TGC ATR ACG TGG) primers (Glass 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 MgCl₂, 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 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

<table>
<thead>
<tr>
<th>Species</th>
<th>ITS region</th>
<th>β-tubulin</th>
<th>Calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger</td>
<td>FJ629337</td>
<td>OX296687</td>
<td>FN594540</td>
</tr>
<tr>
<td>A. tubingensis</td>
<td>FJ629354</td>
<td>FJ629305</td>
<td>FN594448</td>
</tr>
<tr>
<td>A. flavus</td>
<td>KU296260</td>
<td>EF203146</td>
<td>EF202057</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>AY585558</td>
<td>HE577806</td>
<td>EU330198</td>
</tr>
<tr>
<td>A. sydowii</td>
<td>NR131259</td>
<td>EF652297</td>
<td>EU443971</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>KU296266</td>
<td>AY685169</td>
<td>AY689353</td>
</tr>
<tr>
<td>A. ustus</td>
<td>NR131284</td>
<td>EF591727</td>
<td>EF591719</td>
</tr>
</tbody>
</table>

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'-CATGCGTCTCAGCCATGATAG-3') and PKS15KS-r (5'-CTTGGCCTGCAGGGATG-3') and PKS15C-MeT primers, PKS15CMeT-f (5'-GCTTTTCTGACTGAGATG-3') and PKS15C-MeT-r (5'-CATTTGCTTACCCATG-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-
methyltransferase (C-Met) domain which are part of putative polyketide synthase gene, An15g07920 (Ferrarci 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

<table>
<thead>
<tr>
<th>Aspergillus spp.</th>
<th>Isolates (OTA)</th>
<th>Isolates (AFB1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger</td>
<td>KDH 4</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>KDH5</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>KL19</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>KL25</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>KL29b</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>PNGM7</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>PRK 9b</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>SRW11</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>PNGT2</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>PNGT3</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td>TGN2</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>JOH5</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>JOH6</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>KDH7</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>KL16b</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>KL27b</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>KL29a</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>PRK3</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>SRW6b</td>
<td></td>
</tr>
<tr>
<td>A. flavus</td>
<td>TGN1</td>
<td></td>
</tr>
</tbody>
</table>

PCR amplification was performed in a total volume of 25 µL consisting of 0.5 µL of genomic DNA, 5.0 µL of 5x green buffer, 4.0 µL of 25 mM MgCl₂, 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 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.

Aflatoxin B1 gene detection

Gene detection for AFB1 was performed using Nor-1 (norsolorinic acid) primers, Nor-1-f (5'-ACCGCTACCCGGCCACTTGGGACGATGGCGGAC-3') and Nor-1-r (5'-GGTGCCGCTCAGTCTTGGAGACTGCC-3') and Ver-1 (Versicolorin) primers, Ver-1-f (GCGGCAGGCCGGAGAACCGTGT) and Ver-1-r (5'-CGAAAGCCGCA CCACTCACCACATG-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 (CH₃CN), deionized water and acetic acid (CH₃COOH) (57:41:2 v/v/v). The samples and standards (5 µL each) were injected into the UPLC system and run for 5 min.
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 (CH3CN) 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 (Embassy 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 (Adebaio and Dyaolu, 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 (Adebaio and Dyaolu, 2003) almonds, pistachios and walnuts (Molyneux et al., 2007).
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 fumagillin (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, KD7, 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 (KD7 and KL27b). The concentration levels of AFB1 produced by isolate KD7 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 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.
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 Aspergillus. 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 PKS15-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* (KD7 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. Guezlanne-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. Occurrence 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.
A. nigri and A. flavus are two well-known toxigenic species. Although polyketide synthase gene involved in OTA biosynthesis were detected in seven isolates of A. nigri, OTA was not produced by A. nigri which indicate that A. nigri 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.

ACKNOWLEDGEMENTS

This work was supported partially by a Research University Grant (1001/ PBIOLOGI / 811307) from the Universiti Sains Malaysia.

REFERENCES

Neighbour-joining. Molecular Biology and Evolution 12, 843-849.


Utrecht, The Netherlands: Centraalbureau voor Schimmecultures.


