ISOLATION AND SELECTION OF AFLATOXINS PRODUCING *ASPERGILLUS FLAVUS* FROM PEANUT

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ABSTRACT

Aspergillus flavus is known as the main producer of carcinogenic aflatoxins. The presence of this fungus and aflatoxins is an important impactson food safety, human and animal health. Peanut is one of the most important crops in Vietnam and is suitable to substrate for aflatoxins producing fungus growth. The isolation and identification of *Aspergillus flavus* species from peanut is an initial step for further study in Aflatoxins contamination control in peanut. 28 peanut samples selected in Luc Nam district - Bac Giang province were used for fungus isolation. The isolated fungus were purified, conducted DNA extract, amplified PCR products then performed sequencing ITS gene and used BLAST software to check alignment to identification the strains. As the result, 4 strains alignedwith *Aspergillus flavus* strains from National Center for Biology Information - United Nation such as: TUHT115, KP214054.1, MTCC 8654, ZJ4-A. Those strains were checked and confirmed the aflatoxins production in peanut samples.

Keywords: *Aspergillus flavus*, Aflatoxins, peanut, Aflatoxin B1, Aflatoxin B2.

1. INTRODUCTION

The most significant mycotoxigenic *Aspergillus* species producing Aflatoxin are *A. flavus and A. parasiticus*. In the world, about 60 percent of approximately 1,400 isolates of the *A. flavus* group was aflatoxin producers. There are 26 isolated of the *A. flavus* groups producing aflatoxin on peanuts [1].

Studies about fungus prevalence and Aflatoxin contamination during harvest and storage has shown that up to 85 % of fungus naturally isolated in peanut are producing aflatoxins, in

which 65 % is *Aspergillus flavus*, 20.3 % is *Aspergillus parasiticus*, 7 % is *Aspergilus niger* and 1.2 % is *Aspergillus tereus* [2].

Aflatoxins including AFB1, B2, G1, G2 and M1 are classified into group 1 carcinogenesis substances by International Agency for Research on Cancer (IARC). In naturally contaminated foods, aflatoxins B1 and B2 or aflatoxins G1 and G2 usually occur together; however, B2 and G2 are less biologically active and there is limited or inadequate evidence of their carcinogenicity in experimental animals, respectively. Aflatoxin G1 is less mutagenic than aflatoxin B1. Aflatoxin M1 occurs almost exclusively in milk and milk products and is less carcinogenic than aflatoxin B1 [3].

Peanut is one of the most suitable substrate for *A. flavus* growth and to produce Aflatoxins [4]. The aim of this study was to find out the most common AF producing *A. flavus* strains in peanut of Viet Nam in order to contribute to the prevention of AF contamination in peanuts.

2. MATERIALS AND METHODS

2.1. Materials

Kernel peanut was collected in Luc Nam district, Bac Giang province.

Aspergillus flavus ATCC 204304 strain was provided by American Type Culture Collection.

Aflatoxin standards B1, B2, G1, G2 was purchased from Supelco (mix standards can be used).

2.2. Methods

2.2.1. Fungus isolation and purification

The fungus isolation was carried out following TCVN 8275-2: 2010 [9], based on morphology of isolated fungus colonies to select colonies which were expected to be *A. flavus*] [5, 6, 7].

The fungus was purified using spot inoculation, the most appropriate colonies were selected for further confirmation steps.

The seleted colonies were gathered from cultivated petri disks by using 0.05 % Tween 80, centrifuged at 1000 rpm / 5 min then the spores were decanted and preserved in 30 % glycerol.

2.2.2. Extraction and purification of DNA

- DNA was extracted by using TES (10 mM Tris-HCl, 1mM EDTA, pH 8.0, 1 % SDS), silica sand, vortexed then the supernatant was collected for purifying.
- DNA was purified by using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1), sodium acetate, ethanol; DNA was concentrated on a miVAC concentrator then added 100 µl of deionized water.
- DNA (Deoxyribonucleic acid) concentration was determinedby spectrophotometry at 260 nm and 280 nm.

2.2.3. Gel electrophoresis of PCR products

PCR amplifications were performed on 50 µL of a reaction mixture containing: 25 µL of Master mix 2X, 19 µL of H_20, 2 µL of each primer (ITS1 and ITS2), and 2 µL of DNA template.

PCR was carried out as follows: 1) one step at 96 ºC for 4 min; 2) 35 cycles of the following three steps: 45 sec. at 94 ºC, 1 min at annealing temp at 57 ºC), 1 min 20 sec at 72 ºC; and 3) one final 8 min step at 72 ºC. PCR products were separated by electrophoresis on a 1.5 % agarose gel with 0.5 % ethidium bromide in 1× TAE buffer (40 mM Tris base, 40 mM acetic acid, 1.0 mM EDTA, pH 8.0) and visualized under UV light. A DNA standard ladder was used to determine the relative length of PCR products.

2.2.4. ITS gene sequencing and comparison to strains in the gene bank

- ITS gene sequencing was done by 1st BASE Sequencing INT of Korea.
- The analysis data was then treated by FinchTV software to choose a suitable nucleotide chromatogram to compare with identified strains in NCBI (National Center for Biotechnology Information) of American by BLAST (Basic Local Alignment Search Tool), then choose the ITS gene sequencing with an alignment of more than 98 percent.

2.2.5. Confirmation of the aflatoxins production of chosen isolated strains

1 mL of spore solution collected in step 2.2.1 was added in 50 g of sterilized fresh peanut then cultivate in 10 days. Then analysis aflatoxin accumulation by LC-MS/MS, the used method is in-house method developed by National Institute for Food Control (NIFC) which accredited by Bureau of Accreditation of Vietnam.

3. RESULTS AND DISCUSSION

3.1. Fungus isolation and purification

28 peanut kernel samples which are determined to be contaminated with Aflatoxins (AF) in our study AF contamination in peanut in Luc Nam – Bac Giang in 2013 [8] are coded from A1 to A28. Peanut kernel samples was put on Sabouraud Dextrose Agar (SDA) at 25 ± 1 ^oC. The suspect *Aspergillus flavus* fungus are based on color change and spread boundary surrounded peanut kernel, the colony with pale green color in front and yellow color in the back (Figure 1).

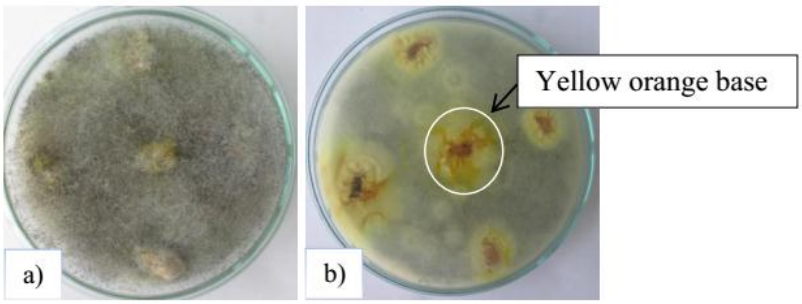

Figure 1. Front (a) and back (b) of peanut kernel in SDA after 3 days incubation.

After 5 days of cultivation on SDA at 25 ^{o}C, an intense yellow orange color was developed at the base of the colonies .The plating step would be repeated until obtaining a unique type of colonies.

The obtained colonies were cultivated, after 3 days the fungus color is yellow-green, after 5 days the color turned from yellow-green to green with the colonies diameter ranged from 3.0 - 5.5 cm (Figure 2). The morphology of isolated fungi are similar to the description of *Aspergillus* [5, 7] and *A. flavus* ATCC 204304 on SDA at 25 ^{o}C for morphological comparison (Figure 2).

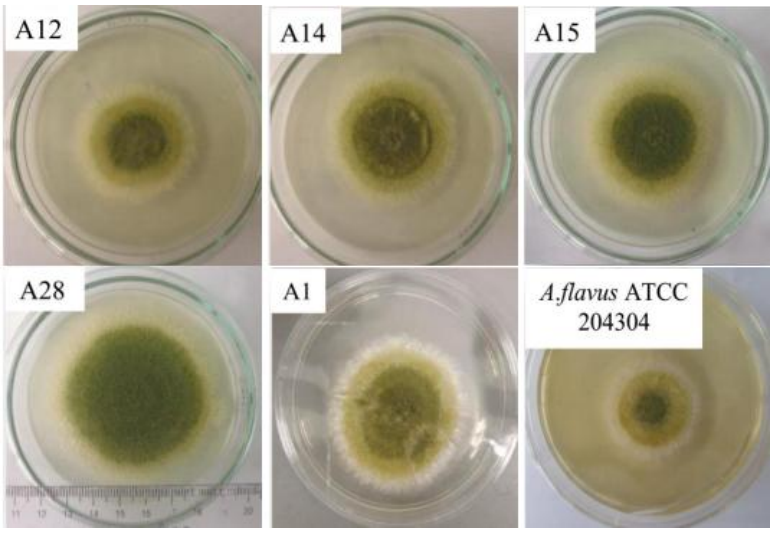

Figure 2. Colonies after 5 days cultivated.

With a spot inoculation of the isolated strains on SDA at 25 ± 1 ^{o}C, the best strains were identified based on their color: light yellow-green to dark green.

The isolated colonies were of white border, deep green center and spreading yellow-green (Figure 3).

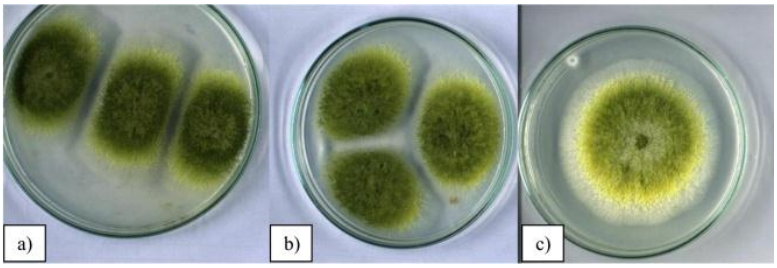

Figure 3. Spot inoculation of suspect colony in SDA media after 5 days (a, b) and ATTC 204304 strain (c).

The isolated fungi were stained using Lactophenol Amann dye; Key features were observed on an optical microscope at 40× for initial identification.

As the results, key features of the fungi were their conidia were pear-shaped or spherical, columnar with 1 or 2 layers and coarsely roughened; Apices were spherical to subspherical.

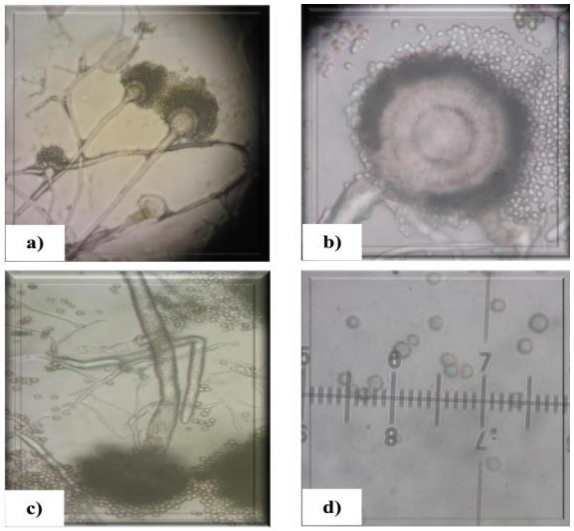

Figure 4. Conidia are pear-shaped to spherical, 1 layer columnar (a), 2 layers columnar (b); Apices spherical to subspherical, green color (c, d)

Note: Isolated strain (from A1 sample) are captured by CX31 microscope (made in Japan) with multiply 40× (Figure 4. a), multiply 90^{x} (Figure 4. b, c) and Axiovert 40 CFL inverted-microscope (made in Germany) with multiply 250× (Figure 4. d).

The picture of conidia spores taken with an electronic microscope scanner was given in Figure 5. The picture showed the coarsely roughened columnar, 12.9 µm in diameter. The picture was fitted with the research results given by Rodrigues P. et al. [10].

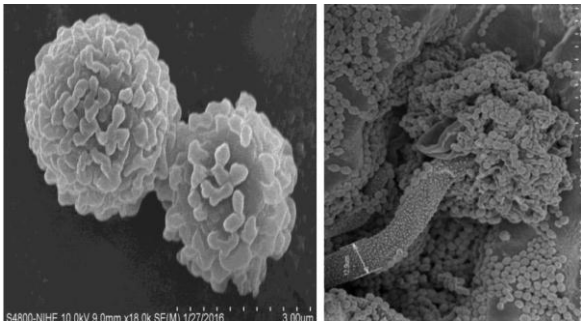

Figure 5. Electronic microscope isolated strain from A1 sample.

Based on the morphology and specific features of the colonies, we selected 11 strains for further confirmation including: A1, A3, A6, A12, A14, A15, A16, A18, A21, A25, A28.

3.2. Extraction and purification of DNA

11 selected strains and *A. flavus* ATCC 204304 were extracted, purified and DNA quality checked using spectrophotometry at 260 and 280 nm. DNA concentration and absorption ratio (A260/280) are presented in table 1.

Table 1. DNA absorption and A260/280.

Strain	Concentration (ng/ µl)	A260/A280
A1	20.39	1.87
A3	46.99	1.94
A6	62.62	1.83
A12	20.52	1.88
A14	38.88	2.03
A15	53.25	1.86
A16	42.86	1.91
A18	46.80	1.87
A21	69.21	2.05
A25	40.60	2.12
A28	35.63	2.01
A. flavus ATCC 204304	57.32	1.81

All DNA samples had an A260/280 ratio within 1.8 ÷ 2.2. It confirmed that DNA was purified and did not contain protein and RNA (Ribonucleic Acid).

3.3. Gel electrophoresis of PCR products

Total DNA samples are used as template for the specific PCR reaction with designed primers: ITS1 and ITS2, then gel electrophoresis is carried out for checking PCR reaction results as Figure 6.

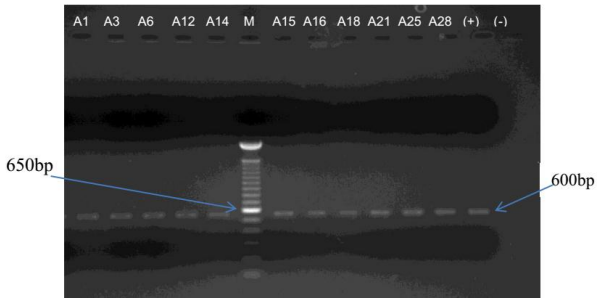

Figure 6. Gel electrophoresis of templates.

(+): Positive control (A. flavus ATCC 204304); (-): Negative control (blank); M: DNA standard ladder

The standard ladder (M) with 100 bp to 1,500 bp molecular size marker and light band length 650 bp. DNA samples have no subband, the major band with ITS 600 bp molecular size marker is appropriate with research results of P. Rodrigues et al. [10] and suitable for sequencing ITS gene.

3.4. ITS gene sequencing and comparison to strains in the gene bank

The amplified ITS by PCR reaction, sequencing transform and treatment by FinchTV software, identification by BLAST software in ncbi.nlm.nih.gov (The National Center for Biotechnology Information). The alignment between analyzed samples and NCBI supports for identifying the isolated strain. We found 4 alignment strains as in Table 2.

Table 2. Alignment between analyzed samples and NCBI.

Sample	NCBI strain	ADN compare	Species	Query cover (%)	Identity (%)
A1	TUHT115	*ITS1, ITS2*	*Aspergillus flavus*	100	100
A12	KP214054.1	*ITS1, ITS2*	*Aspergillus flavus*	100	100
A15	MTCC 8654	*ITS1, ITS2*	*Aspergillus flavus*	100	100
A16	ZJ4-A	*ITS1, ITS2*	*Aspergillus flavus*	99	100

The isolated fungus alignment with *Aspergillus flavus* species are kept for confirmation of aflatoxins production in next step.

3.5. Confirmation of the aflatoxin production of chosen isolated strains

The isolated *A. Flavus* are injected in peanut kernel with moisture of 16% in 10 days, then AFs presence is checked by being observed under UV lamp (λ = 365 nm), the blue fluorescent light covering the peanut kernel are observed as figure 8.

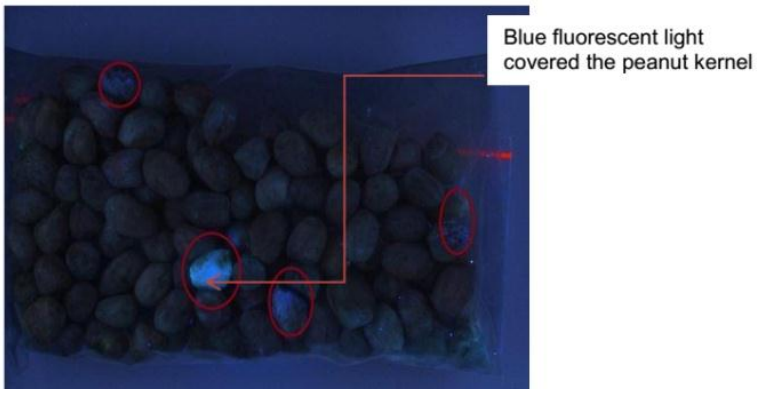

Figure 8. Peanut sample under UV lamp.

The sample are analyzed AFs content by LC-MS/MS. Results have shown that the samples are accumulated AFB1 and AFB2 accumulated 25.2 µg/kg. This result is suitable with research results of E.M. Embaby and Mona M. Abdel-Galel [11]. Chromatogram of AFs analysis is as Figure 9.

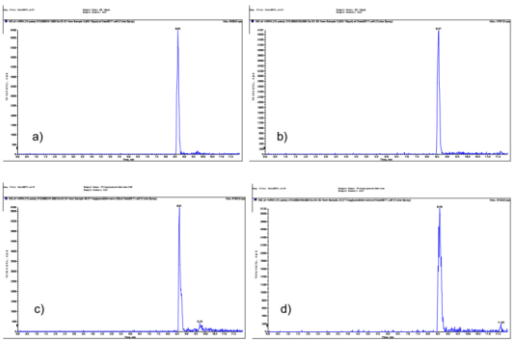

Figure 9. AFB1 chromatogram of standard (a) and sample (c); AFB2 chromatogram of standard (b) and sample (d).

4. CONCLUSIONS

Peanut collected from Bac Giang province were isolated and identified strains alignment with *A. Flavus* species in NCBI such as: TUHT115, KP214054.1, MTCC 8654, ZJ4-A. Those strains are clarified that they are aflatoxins production strains and they produce AFB1 and AFB2. These strains are stored for further research on inhibition of aflatoxins production.

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TÓM TẮT

PHÂN LẬP VÀ TUYỂN CHỌN *ASPERGILLUS FLAVUS* SINH AFLATOXINTỪ LẠC

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Aspergillus flavus được biết đến như nguyên nhân sinh chất gây ung thư aflatoxin. Sự có mặt của nấm này và aflatoxin có tác động quan trọng đến an toàn thực phẩm, sức khỏe con người và động vật. Lạc là một trong những cây công nghiệp quan trọng của Việt Nam và lạc là cơ chất thích hợp cho nấm sinh aflatoxin phát triển. Sự phân lập và định danh các loài *Aspergillus flavus* từ lạc là bước ban đầu cho những nghiên cứu tiếp theo về kiểm soát sự nhiễm aflatoxin trong lạc. 28 mẫu lạc lựa chọn từ huyện Lục Nam - tỉnh Bắc Giang được dùng để phân lập nấm. Nấm sau phân lập được làm thuần, tách chiết và làm sạch DNA, sản phẩm khuếch đại PCR được giải trình tự gen ITS, sử dụng phần mềm BLAST để kiểm tra sự tương đồng để định danh các chủng. Kết quả có 4 chủng tương đồng với loài *Aspergillus flavus* trên ngân hàng gen của Trung tâm thông tin sinh học quốc gia - Hoa Kì như: TUHT115, KP214054.1, MTCC 8654, ZJ4-A. Các chủng này được kiểm tra và xác nhận sự sinh aflatoxin trên các mẫu lạc.

Từ khóa: Aspergillus flavus, Aflatoxins, lạc, Aflatoxin B1, Aflatoxin B2.