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**Discrimination between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* group contaminating Egyptian peanuts using molecular and analytical techniques**

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## Abstract

A wide range of *Aspergillus* section *Flavi* strains were isolated from Egyptian peanut samples. 18 of these strains were compared with 2 type strains (*A. flavus* SRRC G1907, *A. parasiticus* 2747) for aflatoxin production based on (a) qualitative fluorescence using a coconut cream agar medium (CAM), and (b) aflatoxin production on a conductive Yeast Extract-Sucrose (YES) medium using HPLC. These results were validated by using molecular approaches (the structural genes, *aflD* (nor-1), *aflM* (ver-1) and *aflP* (omt A) and the regulatory gene *aflR*) to discriminate between aflatoxigenic and non-aflatoxigenic strains of the *Aspergillus* section *Flavi* group in vitro and on peanut seeds. Overall, 13/18 strains producing aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and aflatoxin B<sub>2</sub> (AFB<sub>2</sub>) in the range 1.27- 213.35 µg/g medium were identified. In addition, 5 non-aflatoxin producing strains were found. The expression of these four genes was assessed using PCR (polymerase chain reaction) and RT-PCR (Reverse transcription polymerase chain reaction). PCR showed that all strains contained the four aflatoxin genes examined, regardless of expression profiles. Our results also showed that *aflD* expression is a reliable marker to discriminate between aflatoxin and non-aflatoxin producers. Interestingly, when an aflatoxin producing strain and three non-aflatoxigenic strains were subsequently grown on peanuts at 0.95 water activity, two of the non-producers were able to initiate aflatoxin biosynthesis. This suggests that growth of strains on the natural food matrix is important for confirming aflatoxigenic production potential.

## 1. Introduction

Peanuts are an important agricultural crop in Egypt. The country has a large number of peanut producers, with many farming small fields of 2.5 ha (WTO 1998). Egypt is a major peanut exporting country and the European market accounts for 68% of its exports. The US is also a major consumer of Egyptian nuts, importing over \$11 million worth of nuts annually (FAO 2006).

The major mycotoxins found in Egyptian peanuts are aflatoxins (El-Maghraby and El-Maraghy 1987). Contamination of peanuts with aflatoxins is one of the main factors that compromises their quality. Aflatoxins are carcinogenic fungal secondary metabolites produced by *Aspergillus flavus* and other closely related species. Aflatoxin B<sub>1</sub> has been demonstrated in animal species to be the most potent liver carcinogen known. A high incidence of Human liver cancer in central Africa and parts of Southeast Asia has been linked with aflatoxins (Groopman, and Kensler, 1996; Montesano, *et al.*, 1997).

In the last few years, an increase in the number of human liver diseases in Egypt has been reported. This initiated a multidisciplinary study to investigate the role of aflatoxins in high incidence of hepatotoxicity in Egypt (Badria 1996). Recently, several multiplex polymerase chain reaction (PCR) systems for aflatoxin-producing fungi were developed, capable of detecting several genes coding for enzymes at different stages of the aflatoxin biosynthesis pathway (Geisen 1996; Shapira *et al.*, 1996; Criseo *et al.*, 2001 ; Chen, *et al.*, 2002).

The use of reverse transcription polymerase chain reaction (RT-PCR) to assess aflatoxin production potential is attractive because it is based on the activity of genes rather than their presence (Sweeney *et al.*, 2000). Several studies have measured the expression of genes involved in the aflatoxin biosynthesis pathway to distinguish between aflatoxin producers and non-producers (Scherer *et al.*, 2005; Degola *et al.*, 2007; Rodrigues *et al.*, 2009). However, there is currently no agreement on single genes whose expression can be used to fully discriminate between aflatoxin and non-aflatoxin producers.

The objectives of this study were to isolate a range of *Aspergillus* section *Flavi* strains from peanuts from different regions of Egypt and to examine the ability of 18 such strains to produce aflatoxins based on (a) a qualitative medium-based assay, (b) HPLC, and (c) use of different biosynthetic and regulatory genes as indicators of aflatoxin production, and (d) to confirm the ability of aflatoxin producing and non-toxin producing strains after colonisation of Egyptian peanuts under specific temperature/water activity regimes.

## **2. Material and methods**

Strains, media and growth conditions

In this study, we isolated 18 strains of *Aspergillus* section *Flavi* group from Egyptian peanuts on (Malt Extract Agar (MEA) , [20.0 g/l malt extract (Difco), 2.0 g/l Peptone (Difco), 15.0 g/l Agar (Sigma)]), and they were identified according to morphological and microscopic characteristics. In addition 2 aflatoxigenic type strains, *Aspergillus flavus* SRRC G1907 (supplied by Dr. D. Bhatnagar, USDA), and *A. parasiticus* PRR-2747 were used for comparison. Each strain was assigned a name in the format EGPn, where EG means Egypt, P represents peanuts, and n is the isolate number. Fungal strains were sub-cultured before examination on Malt Extract Agar and grown for 7 days at 25 °C in the dark.

#### Screening for aflatoxin production using coconut cream agar medium

A preliminary screening for aflatoxin production by the strains was performed by growing them on coconut cream agar medium (CAM) based on fluorescence under long-wave UV light (365 nm). To prepare CAM, 50% coconut cream was homogenized for 5 min with 1000 ml of 100 °C distilled water. The homogenate was filtered through four layers of cheese cloth. Agar was added (20 g/l), and the mixture was heated to boiling and cooled to about 50°C. The mixture was then autoclaved, mixed and poured into sterile Petri dishes (Davis *et al.*, 1987). All strains were inoculated at 25°C for 8 days in the dark on a conducive Yeast Extract Sucrose (YES) medium (20 g/l Yeast extract, 150 g/l Sucrose, 15 g/l Agar).

#### Aflatoxin extraction and HPLC analysis

Three plugs (5 mm diameter) were cut out across the diameter of the colony using a cork borer. These were placed in 2 ml Eppendorf tubes and weighed. Aflatoxins were extracted by adding 1 ml of chloroform and shaking for 1 hour. The biomass was discarded after centrifugation and chloroform was evaporated to dryness. The residue was derivatized using TFA (Trifluoroacetic acid) as described in the AOAC (2000). Sample extracts were analyzed by HPLC using a Waters 600 E system controller, 470 fluorescence detector (Millipore Waters, Corporation Massachusetts USA) ( $\lambda_{exc}$  360 nm;  $\lambda_{em}$  440 nm) and a C<sub>18</sub> column (Phenomenex Luna ODS2 150 x 4.6 mm, 5  $\mu$ m). The analysis was performed at a flow rate of 1 ml min<sup>-1</sup> of the mobile phase (30% Methanol: 60 % Water: 10 % Acetonitrile) and the run time was 25 minutes. The limit of detection for AFB<sub>1</sub> was 1 ng /ml.

## **Molecular methods**

### **Isolation of RNA from the samples**

After 8 days incubation on cellophane-overlaid YES media, the entire mycelial colony was removed together with the cellophane layer. The mycelia were quickly frozen in liquid nitrogen and stored at -80 °C until extraction. A 0.5-1.0 g sub-sample of the mycelia was ground in a mortar with a pestle in liquid nitrogen. Approximately 250 mg of the mycelial powder was then used for isolation of total RNA. RNA extraction from the ground mycelia was accomplished with the RNeasy and Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. Then RNA was treated with DNase I (RNase free DNase I, Amplification Grade, Sigma) to digest residual DNA in the samples.

### **Aflatoxin gene expression**

The expression of three structural genes, *aflD* (nor-1), *aflM* (ver-1), and *aflP* (omt A), and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway was assayed for all the strains. The expression of the housekeeping gene ( $\beta$ -tubulin) was used as a control.

Reverse transcriptase PCR: RT was performed using Qiagen sensiscript<sup>®</sup> kit (Qiagen, UK) using oligo-dT primers to amplify mRNA. The reaction was assembled in a 20  $\mu$ l tube as follows: 1  $\mu$ M Oligo(dT) primer, 1 x reaction buffer, 4U sensiscript Reverse Transcriptase, 2  $\mu$ M dNTPs, 10 U RNase inhibitor, and 40 ng RNA sample in 12  $\mu$ L H<sub>2</sub>O (RNase free). The mixtures was incubated at 37 °C for 60 min followed by 93 °C for 5 min in a thermal cycler (Peltier Thermal cycler PTC-200 MJ Research), followed by rapid cooling on ice. Each 25  $\mu$ l PCR reaction contained 800  $\mu$ M dNTP, 1 x reaction buffer, 1.25 U Taq DNA polymerase I, 0.2  $\mu$ M of each primer, 1  $\mu$ l cDNA mixture, 12  $\mu$ L H<sub>2</sub>O (RNase and DNase free). PCR conditions were an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94°C, 60 s at 65°C and 90 s at 72°C, with a final extension at 72°C for 7 min (Schermer *et al.*, 2005). Primer sequences are detailed in Table 1. PCR products were visualized on a UV transilluminator (Gene Genius Bio Imaging system).

### **DNA isolation and amplification**

Total DNA was purified from all tested strains grown on cellophane-overlaid YES medium. 0.5 g of the mycelium was ground in a mortar with a pestle in the presence of liquid nitrogen. Approximately 100 mg of the mycelial powder was then used for isolation of total DNA. DNA extraction from the ground mycelia was accomplished with the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. PCR was performed as described previously, using the same set of primers designed for the RT-PCR.

### **Studies on peanuts**

Inoculation and culture conditions: An aflatoxin producing strain (*A. flavus* EGP3) and three non-aflatoxigenic strains (*A. flavus* EGP14, *A. flavus* EGP15 and *A. flavus* EGP16) were grown on peanuts modified with sterile water to 0.95  $a_w$ . A moisture adsorption curve was prepared for peanuts in order to accurately determine the amount of water required to add to obtain the target  $a_w$  level. This curve was obtained by adding different quantities of water to peanuts and calculating the moisture content as the difference in weight before and after drying at 130°C overnight and plotting against the  $a_w$  level measured with an AQUALAB® 3TE, USA.

20 g sub-samples of peanuts (three replicates per treatment) were put in glass jars covered with lids containing a microporous membrane and autoclaved. Peanut samples were inoculated with 0.2 ml of  $10^6$  spore suspension and vigorously shaken to cover the peanut with spores and incubated at 25°C for one week in polyethylene sandwich boxes containing glycerol/ water solutions to maintain the equilibrium relative humidity conditions.

### **Aflatoxin extraction and analysis**

10 g of each treatment and replicate was taken for clean up and aflatoxin extraction using an immunoaffinity column (Easi-Extract® Aflatoxin). Aflatoxin was analysed by HPLC as described previously.

### **RNA extraction and RT-PCR**

Total RNA was extracted from inoculated peanuts after the one week incubation using the RNeasy and Plant Mini Kit (Qiagen GmbH, Hilden, Germany) with minor modifications. An amount of 1 g of peanuts was ground in a mortar with a pestle in the presence of liquid

nitrogen, 500 µl of lysis buffer from RNasey kits and 0.5 g of polyvinylpolypyrrolidone (PVPP). Insoluble PVPP binds to both polysaccharide and phenolic compounds and prevents the undesirable binding between nucleic acids and these compounds (Chen *et al.*, 2000). RNA extraction was then performed according to the instructions provided by the manufacturer. RNA was treated with DNase I (RNase free DNase I, Amplification Grade, Sigma) to digest residual DNA in the samples. RT-PCR was performed as described above, using the same set of primers designed for RT-PCR.

### 3. Results

#### Screening the ability of the strains for aflatoxin production

A total of 18 strains of *Aspergillus* section *Flavi* isolated from Egyptian peanuts (EGP1-EGP18) were assessed for aflatoxin production during growth on YES medium using HPLC (Table 2). Thirteen of these strains, and two control strains tested (*A. flavus* SRRC G1907 and *A. parasiticus* 2747) produced aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and aflatoxin B<sub>2</sub> (AFB<sub>2</sub>), while *A. parasiticus* 2747 also produced AFG<sub>1</sub> and AFG<sub>2</sub>. The AFB<sub>1</sub> was in the range 1.27- 213.35 µg/g medium; one strain was a very high producer (213.35 µg/g ), nine strains were high producers (15.92-50.63 µg/g) and five strains were low producers (0.15-6.59 µg/g). Five of the strains showed no detectable levels of aflatoxin. Strains were grown on CAM and fluorescence was used to assess aflatoxin; our results showed a good correlation between fluorescence and levels of aflatoxin as measured by HPLC.

#### Analysis of aflatoxin gene transcription

Several genes code for proteins involved in the aflatoxin biosynthesis pathway. Measuring their transcriptional status should reveal if the strain is capable of producing aflatoxins. In this study we analysed the presence and transcription of four genes, *aflD* (nor-1), *aflM* (ver-1), *aflP* (omt A) and *aflR* in strains of *Aspergillus* section *Flavi*. The expression of the house keeping gene (β-tubulin) was used as a control.

PCR revealed that all strains tested contained all four genes (data not shown), therefore the ability to produce aflatoxin was not directly related to the presence or absence of these genes

(see Table 2). Transcription of the genes was subsequently assayed by RT-PCR. To ensure there was no DNA contamination in the RNA from each strain, PCR was performed following an RT reaction in the presence (+RT) or absence (-RT) of the reverse transcriptase enzyme. An additional no-template control PCR reaction for each strain confirmed that results were not due to contamination of PCR products (data not shown).

Table 2 summarizes the results obtained by RT-PCR for all four genes examined. Electrophoresis patterns showed that of all the aflatoxin-producing strains (15 strains), five were negative for transcription of *aflR*, seven were negative for *aflP*, and one did not transcribe *aflM* (Figure 1A and 1B). Furthermore, of five non-aflatoxigenic strains, two transcribed *aflR* and *aflP* (Figure 2). Thus, transcription of these three genes does not fully discriminate between aflatoxin-producing and non-producing strains. However, transcription of the structural gene, *aflD*, gave positive results with all aflatoxin producing strains (see Figure 1A and 1B) and gave negative results with all non-aflatoxin strains (Figure 2). So the transcription of this gene was correlated with the ability to produce aflatoxins in all strains tested.

### **Studies on peanuts**

The production of aflatoxins was tested during colonisation by selected strains on peanuts (Table 3). The aflatoxin-producing strain EFGP3 also produced AFB<sub>1</sub> when grown on peanuts. Interestingly, the non-producing strains EGP14 and EGP15 synthesised AFB<sub>1</sub>; this was accompanied by the detection of *aflD* and *aflM* transcription (Figure 3). It is notable that EGP15 which produced no detectable levels when grown on YES media produced higher amounts than the producing strain (EGP3) when grown on peanuts. The strain EGP16 was confirmed to produce no aflatoxins on either substrate, which was consistent with a lack of *aflD* and *aflM* transcription.

## **4. Discussion**

In this study, we aimed to establish a molecular marker capable of detecting aflatoxin expression in *Aspergillus* section *Flavi* species contaminating Egyptian peanuts, confirming



the relationship between molecular tools and conventional analytical methods (fluorescence and HPLC) and to determine the behaviour of aflatoxigenic and non-aflatoxigenic strains when grown on peanuts. This study was carried out because very recently *A. flavus* has been found to be the most common species isolated from in shell peanuts and from seeds directly and was the most common mycotoxigenic component of the mycobiota in two seasons in Egypt (Sultan and Magan, 2010). Studies from the USA suggest a similar importance of *A. flavus* from other agricultural commodities (Cotty, 1997). Furthermore, potentially toxigenic species of *A. flavus* have been previously isolated from Egyptian peanuts (El-Maghraby and El-Maraghy 1987, 1988; Youssef *et al.*, 2008). However, no attempt was made in these previous studies to use molecular approaches to detect and differentiate between aflatoxin producing and non-producing species.

Our study revealed that both qualitative (fluorescence) and quantitative levels of aflatoxin measured by HPLC gave similar results. This is in contrast to previous studies which reported that fluorescence on CAM does not always correlated with aflatoxin analysis by HPLC (Scherin *et al.*, 2005; Giorni *et al.*, 2007). Indeed, Scherin *et al.* (2005) suggested that analysis of aflatoxin production by fluorescence developed by colonies grown on coconut agar (CAM) was not a reliable indicator of aflatoxin production. Using the conventional methods as a tool to differentiate between aflatoxin-producing and non-producing strains of the *A. flavus* group are based on culture on natural and/or artificial media which permits the release of aflatoxins have been studied previously by (Davis *et al.*, 1987; Abarca *et al.*, 1988; Bennett and Papa 1988). These methods have drawbacks: time-consuming, laborious and need the expertise of mycologists to avoid misidentification.

Aflatoxins are the end product of a biosynthetic pathway comprising many enzymatic steps. Measuring the level of expression of genes coding for these enzymes should provide a marker indicating whether a strain is capable of producing aflatoxin. However, despite previous work there is currently no agreement on a suitable marker for aflatoxin production, other than the measurement of aflatoxin production itself (Geisen 1996; Shapira *et al.*, 1996; Farber *et al.*, 1997; Criseo *et al.*, 2001; Scherin *et al.*, 2005; Degola *et al.*, 2007; Rodrigues *et al.*, 2009).

Using PCR we showed that all aflatoxin and non-aflatoxin producers harbour the four genes (*aflD*, *aflM*, *aflP* and *aflR*) examined. This discounts the possibility that a lack of aflatoxin production in certain strains is due to loss of the genes from the genome. This is supported by Chang *et al.* (2005) who reported that the loss of production of aflatoxins B<sub>1</sub> and B<sub>2</sub> in many

nonaflatoxigenic *A. flavus* isolates is not caused by large deletions or a complete loss of the aflatoxin gene cluster, but probably results from point mutations.

Based on our RT-PCR results we have shown that *aflD* transcription can be used as a marker to discriminate between aflatoxin and non-aflatoxin producers, while *aflM*, *aflP* and *aflR* failed to differentiate between aflatoxigenic and non-aflatoxigenic strains. Similarly Scherm *et al.* (2005) reported that *aflD* (nor-1), gave the best correlation of aflatoxin production and gene expression on inducing (YES) and non-inducing (YEP) media. The results also showed that the RT-PCR technique gave a positive correlation between aflatoxin production and aflatoxin gene transcription of the *A. parasiticus* strain examined. This indicates that the RT-PCR assay is valid to use for this species. Previously, Scherm *et al.* (2005) tested a RT-PCR assay with a set of aflatoxin gene primers against three aflatoxigenic strains of *A. parasiticus*. All gave positive results with the four genes we have used.

Our results contrast with those of Rodrigues *et al.* (2009), who tested two genes, *aflD* and *aflQ*, in 31 isolates of *Aspergillus section Flavi* originating from Portuguese almonds and concluded that *aflD* expression was not a good marker for differentiating between aflatoxigenic and non-aflatoxigenic isolates based on testing 35 isolates (31 almonds isolates and 4 control strains). Only one almond isolate gave a false positive transcription.

Interestingly, *aflR* failed to discriminate between aflatoxin and non-aflatoxin producers, where 5/15 of aflatoxin producers were negative for transcription of *aflR*, and 1/5 of non-aflatoxin producer was positive for *aflR* transcription. This may be a result of sequence variability occurs within the entire *aflR* gene of *Aspergillus section Flavi*. It was found that a particular sequence variability differentiates some species in *Aspergillus section Flavi* and can be used to identify non-functionality of the AFLR protein (Lee *et al.*, 2006). Kale *et al.* (2007) reported that regulation of AF production in *A. parasiticus* sec- variants and loss of aflatoxigenicity is not caused by defects in *aflR* or *aflJ* or *laeA* (three known AF-regulatory genes). It is possible that the sec-strains lack additional positive regulators of secondary metabolic pathways that act independently of or in conjunction with *AflR* and *AflJ*.

The fact that *aflD* transcription became detectable when a non-producing strain (on YES media) when grown on peanuts and produce aflatoxins suggests that *aflD* transcription is a good marker for aflatoxin production, and suggests that *aflD* plays a functional role in the adaptive growth on different media. It is worthwhile to note that the conducive medium (YES

medium) is not definitive assay to confirm whether strains are aflatoxigenic or non-aflatoxigenic per se. It is used for carrying out ecophysiological studies with known aflatoxin producers (Schmidt-Heydt et al., 2009). In the present study *A. flavus* EGP3 did not produce any aflatoxin on YES, however, it produced higher aflatoxin than *A. flavus* EPG14 (an aflatoxin producer on YES) in peanuts. This suggests that this may be another factor that should be borne in mind as original nutritional substrate (in this case peanuts) may be important. Aflatoxin production by *A. flavus* has been shown to be influenced by nutritional conditions (Chang *et al.*, 2000; Yu *et al.*, 2003). The data suggests that a lipid-rich nutritional environment (such as peanuts) represent a type of stress which induces aflatoxin production. Previously, Yu *et al.* (2003) concluded that substrate-induced lipase gene expression might be indirectly related to aflatoxin formation by providing the basic building block (acetate) for aflatoxin synthesis. Also supplementation of lipid (0.5% soya bean or peanut oil) in the non-aflatoxin-conducive medium (PMS) promoted aflatoxin production of two non-aflatoxigenic *A. flavus* isolates (Yu *et al.*, 2003).

This work presented in this paper is the first detailed molecular analyses of strains of *Aspergillus* section *flavi* group from Egyptian peanuts. It also highlights the dynamic nature of aflatoxin production, and the potential use of *aflD* transcription as a marker for aflatoxin. This could be used to develop a more rapid molecular assay for the detection of aflatoxin-producing species in contaminated peanuts and other food matrices.

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Table 1: Details of primer sequences, target gene, annealing temperature and expected PCR/RT-PCR product length in base pairs (bp).

Primer pair	Gene	Primer sequence (5'- 3')	Optimal Annealing Temp. (°C)	PCR product size (bp)	RT-PCR product size(bp)
<b>Tub1-F</b>	<b>Tub 1</b>	GTCCGGTGCTGGTAACAAC	65	902	837
<b>Tub1-R</b>		GGAGGTGGAGTTTCCAATGA			
<b>NOR1-F</b>	<b>aflD</b>	ACCGCTACGCCGGCACTCTCGGCAC	65	400	400
<b>NOR1-R</b>		GTTGGCCGCCAGCTTCGACACTCCG			
<b>VER1-F</b>	<b>aflM</b>	GCCGCAGGCCGCGGAGAAAGTGGT	65	537	487
<b>VER1-R</b>		GGGGATATACTCCCGCGACACAGCC			
<b>OmtA-F</b>	<b>aflP</b>	GTGGACGGACCTAGTCCGACATCAC	65	797	624
<b>OmtA-R</b>		GTCGGCGCCACGCACTGGGTTGGGG			
<b>AflR-F</b>	<b>aflR</b>	CGAGTTGTGCCAGTTCAAAA	55	999	999
<b>AflR-R</b>		AATCCTCGCCCACCATACTA			

Table 2: Comparison between the results obtained by PCR, RT-PCR and conventional methods. Key: CMA, coconut cream agar medium; HPLC, High Pressure Liquid Chromatography. Key: A., *Aspergillus*

Strains	PCR					RT-PCR					CAM fluorescence	HPLC Aflatoxin B <sub>1</sub> µg/g
	<i>β-tub</i>	<i>aflR</i>	<i>aflD</i>	<i>aflP</i>	<i>aflM</i>	<i>β-tub</i>	<i>aflR</i>	<i>aflD</i>	<i>aflP</i>	<i>aflM</i>		
<i>A. flavus</i> SRRC G1907	+	+	+	+	+	+	+	+	-	+	+	1.42
<i>A. parasiticus</i> 2747	+	+	+	+	+	+	+	+	+	+	+	0.15
<i>A. Flavi</i> EGP1	+	+	+	+	+	+	-	+	-	+	+	50.63
<i>A. Flavi</i> EGP2	+	+	+	+	+	+	+	+	-	+	+	34.28
<i>A. Flavi</i> EGP3	+	+	+	+	+	+	-	+	-	+	+	21.33
<i>A. Flavi</i> EGP4	+	+	+	+	+	+	-	+	+	+	+	6.59
<i>A. Flavi</i> EGP5	+	+	+	+	+	+	+	+	+	+	+	15.92
<i>A. Flavi</i> EGP6	+	+	+	+	+	+	+	+	+	+	+	1.48
<i>A. Flavi</i> EGP7	+	+	+	+	+	+	-	+	-	+	+	1.27
<i>A. Flavi</i> EGP8	+	+	+	+	+	+	+	+	+	+	+	44.83
<i>A. Flavi</i> EGP9	+	+	+	+	+	+	+	+	+	+	+	213.35
<i>A. Flavi</i> EGP10	+	+	+	+	+	+	-	+	-	-	+	19.74
<i>A. Flavi</i> EGP11	+	+	+	+	+	+	+	+	-	+	+	19.38
<i>A. Flavi</i> EGP12	+	+	+	+	+	+	+	+	+	+	+	33.65
<i>A. Flavi</i> EGP13	+	+	+	+	+	+	+	+	+	+	+	36.49
<i>A. Flavi</i> EGP14	+	+	+	+	+	+	-	-	-	-	-	0
<i>A. Flavi</i> EGP15	+	+	+	+	+	+	-	-	-	-	-	0
<i>A. Flavi</i> EGP16	+	+	+	+	+	+	+	-	-	-	-	0
<i>A. Flavi</i> EGP17	+	+	+	+	+	+	-	-	+	-	-	0
<i>A. Flavi</i> EGP18	+	+	+	+	+	+	-	-	-	-	-	0



Table 3: Comparison of the results obtained by RT-PCR and HPLC for 4 *Aspergillus* section *Flavi* strains grown on YES medium and peanuts. Key: A., *Aspergillus*

Sample	Aflatoxin B <sub>1</sub> on YES µg/g	Aflatoxin B <sub>1</sub> on Peanut µg/g	RT-PCR on peanut					RT-PCR on YES				
			β-tub	<i>aflR</i>	<i>aflD</i>	<i>aflP</i>	<i>aflM</i>	β-tub	<i>aflR</i>	<i>aflD</i>	<i>aflP</i>	<i>aflM</i>
<i>A. Flavi</i> EGP3	21.33	0.67	+	-	+	-	+	+	-	+	-	+
<i>A. Flavi</i> GP14	0	0.94	+	-	+	-	+	+	-	-	-	-
<i>A. Flavi</i> GP15	0	0.01	+	-	+	-	+	+	-	-	-	-
<i>A. Flavi</i> GP16	0	0	+	-	-	-	-	+	+	-	-	-

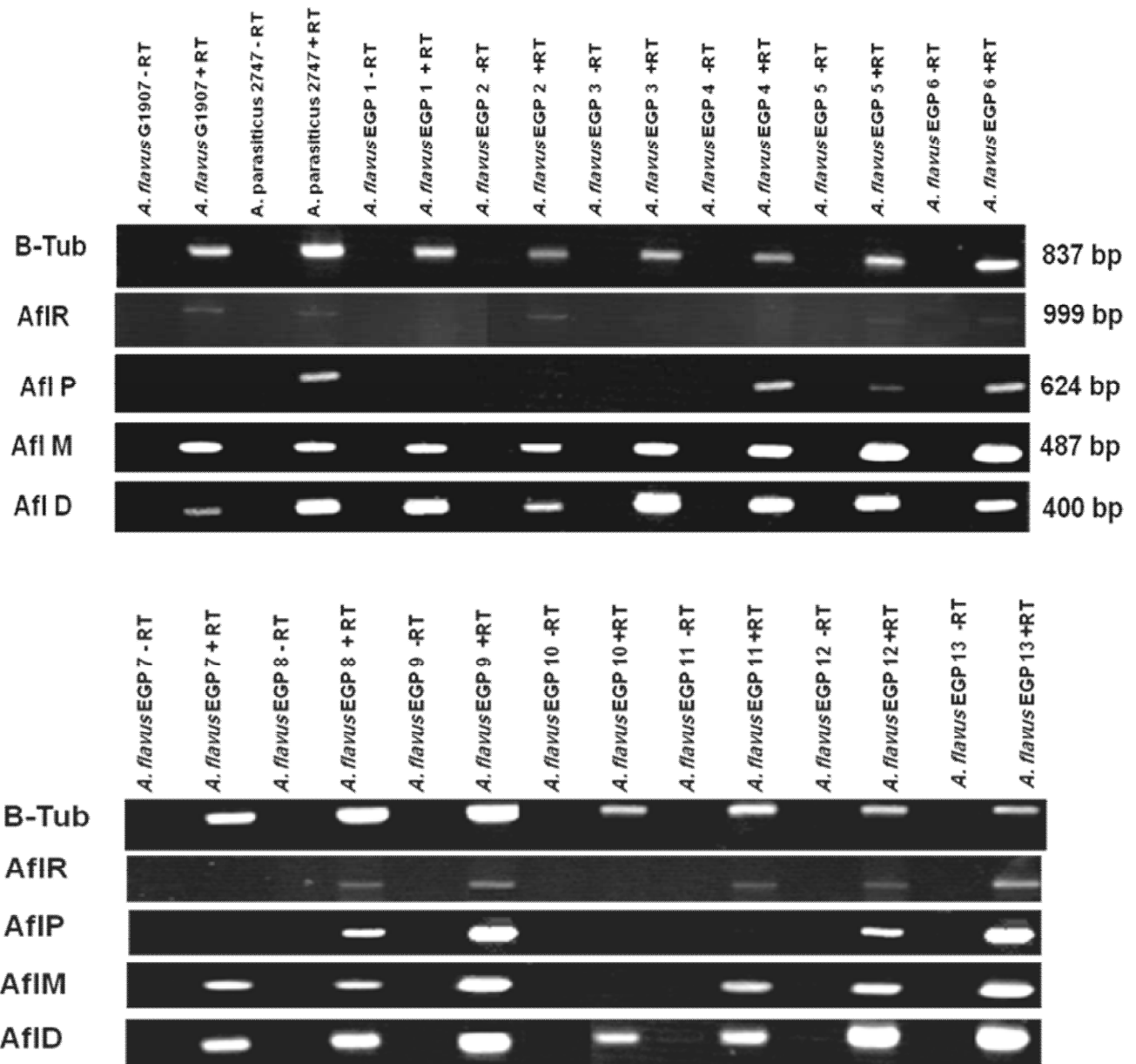


Figure 1A and 1B. Transcription of primer set ( $\beta$ - tubulin, *aflD*, *aflM*, *aflP* and *aflR*) detected by RT-PCR in 15 aflatoxigenic strains. RNA from each strain was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2 % agarose gel, stained with ethidium bromide and visualized under UV.

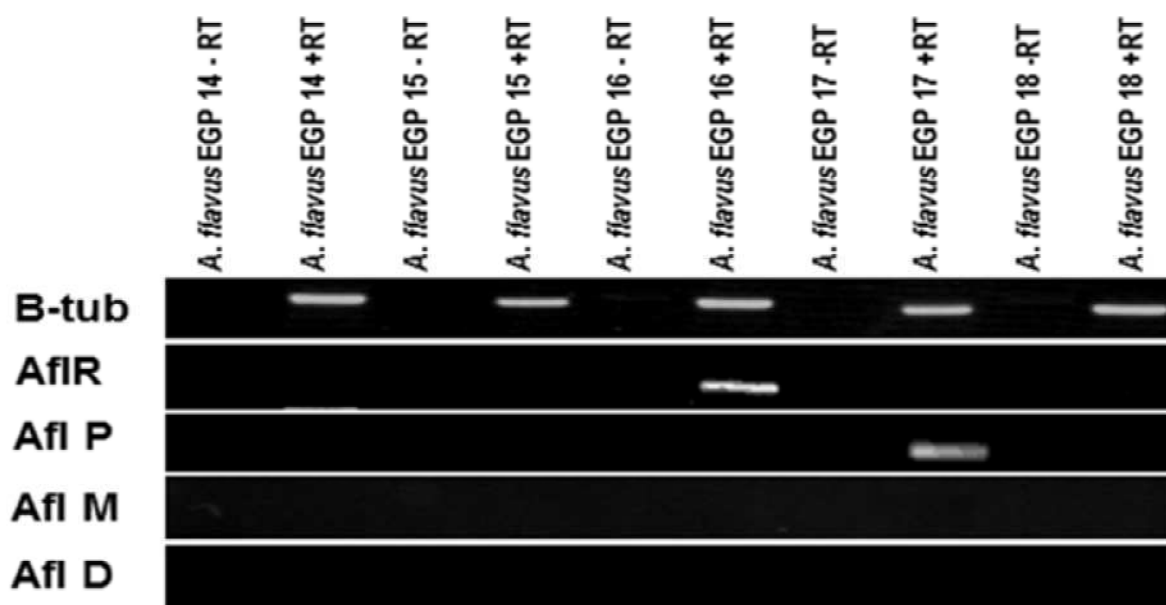


Figure 2: Transcription of primer set ( $\beta$ - tubulin, *aflD*, *aflM*, *aflP* and *aflR*) detected by RT-PCR in 5 non-aflatoxigenic strains. RNA from each strain was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2 % agarose gel, stained with ethidium bromide and visualized under UV.

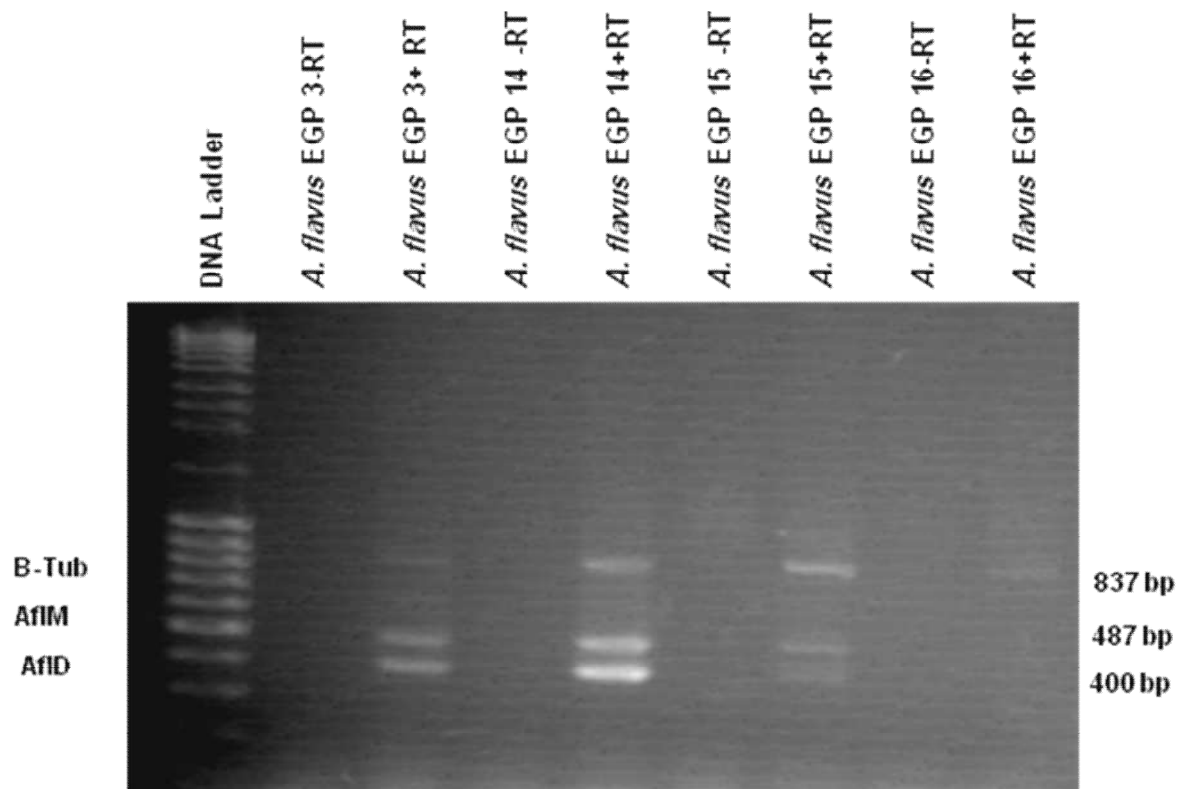


Figure 3: Transcription of  $\beta$ - tubulin, *aflD*, *aflM*, *aflP* and *aflR* detected by RT-PCR in 4 strains grown on peanuts. RNA from each strain was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV.