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Molecular Characterization of Aflatoxin Biosynthesis Genes of *Aspergillus flavus* from Peanuts Production Area

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ABSTRACT

In this study, molecular analysis of (100%) all fungal isolates, which were sampled from soil and air besides from infected peanut plants in the peanut planting area, were identified in β-tubulin gene by Polymerase Chain Reaction (PCR). PCR products of fungal isolates were restricted by *Bgl*II enzyme within Restriction Fragment Length Polymorphism (RFLP). The intergenic spacer (IGS) region for aflatoxin biosynthesis genes (*aflJ-aflR*) were determined in 254 (78.2%) *A. flavus* isolates using PCR-RFLP. Selected 100 isolates were detected as *A. flavus* by β-tubulin sequence gene fragments and comparisons of sequence showed 96–100% similarity. 254 out of 325 isolates contained aflatoxin biosynthesis genes (*aflJ-aflR*), whereas 213 out of 254 isolates produced aflatoxin. The results acquired in study remarked that *A. flavus* was the species responsible for aflatoxin contamination. Aflatoxin gene cluster in populations can be advantage for comprehension of the toxicological risk as well as the election of biocontrol isolates.

Key words: Air, *Aspergillus flavus*, β-tubulin, IGS, Infected peanut plant, PCR-RFLP, Soil.

INTRODUCTION

Aflatoxins are provided from toxic metabolites synthesized by *Aspergillus* spp., *Aspergillus flavus* and *Aspergillus parasiticus* in particular. They are produced as aflatoxin B_1, B_2, G_1, G_2 that are also accepted to be mutagenic, carcinogenic and teratogenic (Lereau *et al.* 2012; Kumar *et al*. 2019).

The identification of fungi by morphological characters is based on culture and microscopic techniques (Mangal *et al.* 2014). In addition, morphological characters should be verified by the aflatoxigenic profile of the fungi (Samson *et al.* 2007). However, morphological methods do not differentiate between toxigenic or non-aflatoxigenic fungi. Improving of fast and susceptible methods for the detection of aflatoxigenic fungi is necessary for assessment of the potential health risk on soil, air and plant. The different molecular techniques are used to identify *Aspergillus* species that have aflatoxin biosynthesis genes (Chang *et al.*, 2005; Singh *et al*. 2017).

Aflatoxigenic fungi may also be identified by PCR-based methodology. Firstly, the standard PCR is used to detect aflatoxigenic fungi. In this technique, the contiguous the β-tubulin gene regions in fungal DNA are amplified, sequenced, and compared with sequences in GenBank (Gonzalez-Salgado *et al.* 2008). Secondly, isolated fungi are screened for aflatoxin biosynthesis genes by PCR as regulatory genes (*aflR, aflS*), sterigmatocystin-o-methyltransferase (*omt-A*), versicolorin A dehydrogenase (*ver-1*), norsolorinic acid reductase (*nor-1=aflD*), polyketide synthase (*pksA*) and apa-2 (Somashekar *et al.* 2004). Thirdly, the identification is performed by PCR-RFLP targeting the *aflJ-aflR* intergenic spacer (IGS) of the aflatoxin biosynthesis genes (Khoury *et al.* 2011).

Somashekar *et al.* (2004) reported that PCR-RFLP was indicated to cut *aflR* amplicons with *Pvu*II restriction enzyme to distinguish between *A. flavus* and *A. parasiticus*. This result showed that PCR-RFLP was identified as *A. flavus, A. nomius* and *A. tamarii* having aflatoxin biosynthesis genes (Midorikawa *et al.* 2014).

This study was carried out to determine the existence of the genes (*aflJ-aflR*) contained in the pathway of aflatoxin biosynthesis in *A. flavus* isolates from soil, air and infected plant at planting and harvesting of experimental area in Adana. Then, positive PCR product of *aflJ-aflR* intergenic region was performed to identify by PCR-RFLP. It was also used to detect β-tubulin sequences in 100 out of 325 *A. flavus* isolates characterized by RFLP associated with specific morphological characters from peanut cultivation areas. Moreover, this is the first study of molecular diagnosis of *A. flavus* isolates by PCR-RFLP method of *aflJ-aflR* Intergenic Spacer from a Turkish peanut planting area.

MATERIALS AND METHODS

Research Areas: This study has been performed in 2015 and 2016 at the fields of Cukurova University located in Adana, Turkey as a second crop peanut. Halisbey variety

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belonging to Virginia market type was used as a plant material in this experiment.

Fungal isolates: Soil and air samples were isolated during planting and harvesting periods in experimental area. Also, samples were isolated from infected peanut plants.

Morphological identification: Isolates were identified as *A. flavus* using Czapek agar (CZA) and according to the method of Samson *et al.* (2007).

Molecular identification: Identification of *A. flavus* was performed by using Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) (Khoury *et al.* 2011).

DNA extraction: DNA was isolated from *A. flavus* isolates Czapek Yeast Agar (CYA). Petri dishes were incubated at 25ºC for 2 days. After, the young mycelium was added to 1.5 ml microcentrifuge tubes. Extraction of DNA was done according to Lui *et al.* (2000). Mycelium was taken into 1.5 ml centrifuge and mixed in 500 µl lysis buffer for 10 min. incubated at room temperature. 150 µl of potassium acetate was added to the mixture. An equal volume of isopropyl alcohol was mixed into the microcentrifuge tube. These tubes were spun at 10,000 rpm for 2 min and the supernatant was discarded. Pellet was washed with 300 µl of 70% ethanol, and spun at 10,000 rpm for 1 min. Then supernatant was discarded. Pellet was air dried for 1-2 hours, then suspended in 50 µl of pure water. DNA was stocked at -20ºC.

Polymerase Chain Reaction Analysis: According to Khoury *et al.* (2011), primer pair IGS-F/R was designed to target the intergenic spacer (IGS) for aflatoxin biosynthesis genes, *aflR* and *aflJ* (Ehrlich *et al*., 2003; 2007) that corresponded to PCR product of 674 bp. The primer sequences were as follows: IGS-F, 5′ -AAGGAATTCAGG AATTCTCAATTG-3′; IGS-R, 5′ -TCCACCGGCAAATCG CCGTGCG-3′. The β-tubulin gene was amplified to a 340 bp fragment on genomic DNA with primers Tub-F (5′ -CTCG AGCGTATGAACGTCTAC-3′) and Tub-R (5′ -AAACCC TGGAGGCAGTCGC-3′).

The amplification was performed in 50 µL reaction volume, containing 5 µl 10 x PCR buffer, 0.3 units Taq 5 U/ µl, 1 µL dNTP, 1.5 µL $MgCl_2$, about 50 ng genomic DNA, 1 µl of each primer. The reaction cycling parameters were: 94ºC for 4 min, 35 cycles of 94ºC for 40 s, 58ºC for 40 s, 72ºC for 1 min, and extension of 10 min at 72ºC. The PCR amplified products were divided by electrophoresis on 0.8% w/v agarose gel.

Digestion of PCR products: After amplification, the restriction enzyme *Bgl*II was used to digest on PCR products. 1.5 µl of enzyme and 4 µl of buffer, 15µl of PCR product and 19.5 µl of purified water were added into a tube and total volume of 40 µl was incubated for 3 hours at 37ºC (Khoury *et al.* 2011). The digested fragments were separated on 2% agarose gel.

DNA sequencing: Medsantek sequenced the PCR products. Alignment of the partial β-tubulin gene sequence was conducted using the software package MEGA sequence analysis software version 6.0 (Kumar *et al.* 2012).

Comparing the partial DNA sequences of different species were detected with Basic Local Alignment Search Tool (BLAST). DNA sequences of isolates were carried out using Clustal X (Chenna *et al*. 2003) alignment program. Phylogenetic tree was arranged using MEGA version 6.0 (Kumar *et al.* 2012). Using the neighbour-joining method was constructed phylogenetic tree (Saitou and Nei, 1987). The sequence similarities were carried out with BLAST searches programs in the National Center for Biotechnology Information (NCBI).

Aflatoxin production by *A. flavus*: Total aflatoxin production potential in suspension were analyzed using the isolates. The cultures were grown in potato dextrose agar at 28°C for 7 days. The fungal spore suspension was collected using filter paper into an erlenmeyer. Spore concentration in the inoculum was supplied of $5x10^6$ spores/ml. (Abbas *et al*. 2011). The mobile phase was a mixture of water: acetonitrile: methanol (600:200:300, V/V/V) with addition of 132 mg KBr and 385 ml HNO_3. The flow rate was 1 ml/ min and the injection volume was 100 ml. The Agilent 1100 HPLC system was used. The detection was performed at ex = 360 nm and em = 440 nm. All the data were shown as a µg/kg (AOAC, 2002).

RESULTS AND DISCUSSION

Totally 325 *A. flavus* were isolated from soil and air samples during planting and harvesting periods, and also isolated from infected peanut plants on secondary peanut growing areas in trials (Table 1). Firstly, these isolates were identified as growth, colony color and microscopic property. *A. flavus* colonies with hyphae showed color green turning to yellow-green on Czapek agar (CZA). The reverse color was cream. *A. flavus* isolates had with rough conidia with thin walls. The average size of conidia and colony diameter of *A. flavus* were 3 µm and 7 cm, respectively.

The PCR was conducted using the primer pairs for *aflJ* and *aflR* genes targeted the *Aspergillus* DNA. As seen in Fig 1A, in all PCR products with β-tubulin primers were observed at the size of 340 bp. Moreover, IGS-F/R the primer pairs were amplified at a size of 674 bp. Positive bands obtained with IGS primers show that PCR products have *aflJ* and *aflR* biosynthetic genes (Fig 1B). It has been determined that *A. flavus* isolates were obtained from infected peanut plants and soil taken during the planting and harvesting periods which has aflatoxin biosynthesis genes, *aflJ-aflR*. In the PCR product performed with IGS primer pairs weren't observed at the band size of 674 bp from airborne *A. flavus* isolates and it was also determined that *aflJ-aflR* did not have aflatoxin biosynthesis genes.

Table 1: Morphological defination and isolation of *A. flavus* from infected peanut plants, soil and air samples during planting and harvesting periods.

Isolated Fungi	Planting period		Infected peanut plants	Harvesting period		
	Soil	Air		Soil	Air	Total
			2015			
A. flavus	41	11	60	32	8	152
			2016			
A. flavus	17	32	74	30	20	173
Total	58	43	134	62	28	325

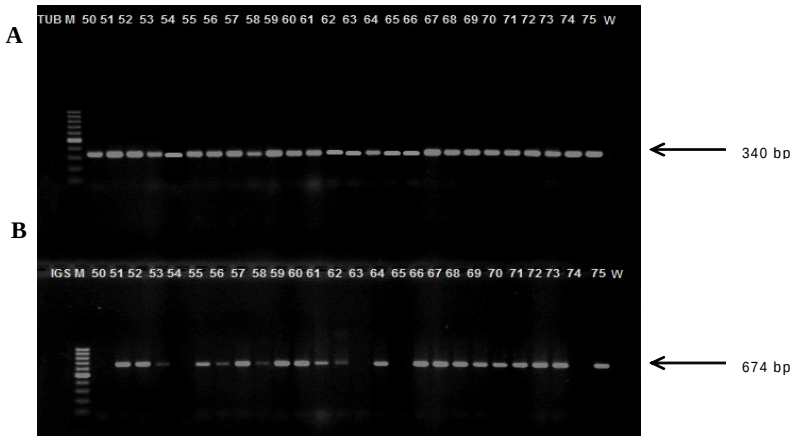

Fig 1: (A) Examination of β-tubulin primer pairs of agarose gel electrophoresis (Amplified 340 bp), Lane M: 100 bp DNA marker, Lane 50 to 75: bands were observed at the size of 340 bp in all these fungal DNA, Lane W: negative control;
(B) Agarose gel electrophoresis of PCR products using IGS-F/R primer pairs (Amplified 674 bp), Lane M: 100 bp DNA marker, Lane 50, 54, 63, 65, 74: no DNA bands were observed at the size of 674 bp for the fungal DNA, Lane 51 to 53, 55 to 62, 64, 66 to 73, 75: bands were observed at the size of 674 bp in these fungal DNA, Lane W: negative control.

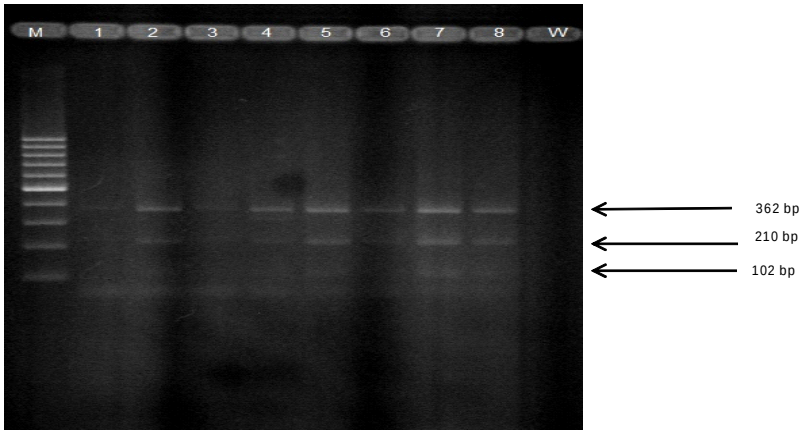

Fig 2: A total of 2% gel electrophoresis showing PCR products amplified with IGS-F/R primers and also digested with *Bgl*II. Lane M: 100 bp DNA marker; Lane 1 to 8: *A. flavus*; Lane W: negative control.

In total, soil samples taken during planting (58) and harvesting (62) periods as well as from infected peanut plants (134) were observed to have *aflJ-aflR* aflatoxin biosynthesis genes in total 254 isolates (Table 2). In the PCR products performed with IGS primers, no DNA bands were observed from air isolates in planting (43) and harvesting (28) periods and, it was also determined that they did not have *aflJ-aflR* aflatoxin biosynthesis genes (Table 2, Fig 1B).

A total of 254 (78.2%) PCR products of *aflJ-aflR* intergenic region were cleaved into 3 fragments of 362, 210 and 102 bp using *Bgl*II restriction enzyme by PCR-RFLP (Fig 2). So, PCR products targeting the *aflJ-aflR* intergenic spacer of the aflatoxin biosynthesis genes had been identified as *A. flavus*. Similar to our study, 202 food and poultry feeds collected from different agricultural regions of Cameroon were analysed by PCR-RFLP using the IGS for aflatoxin

Table 2: PCR products were obtained from infected peanut plants, soil and air samples during planting and harvesting periods.

Primer code	Planting period		Infected peanut plants	Harvesting period		
	Soil	Air		Soil	Air	Total
			Units			
			2015			
β-tubulin	41	11	60	32	8	152
IGS	41	-	60	32	-	133
			2016			
β-tubulin	17	32	74	30	20	173
IGS	17	-	74	30	-	121
			Total			
β-tubulin	58	43	134	62	28	325
IGS	58	-	134	62	-	254

biosynthesis to confirm all fungi identified as *A. flavus* based on morphology. The restriction enzyme *Bgl*II digested with IGS-PCR products of *A. flavus* separated fragments of 362, 210 and 102 bp (Raphaël *et al.* 2013).

As shown in Fig 3, selected 100 *Aspergillus* isolates were defined as *A. flavus* with respect to the sequence analysis of β-tubulin genes. Isolate 14 and isolate 7 from soil during harvesting period showed similarity of 99% to *A. flavus* partial tubb gene for β-tubulin-strain Kw 2989 and 96% to *A. flavus* NRRL 1957 β-tubulin gene partial cds. Isolate 89 from soil during planting period and isolate 124, 132, 133, 145 and 153 from soil during harvesting period showed 98%, 100%, 98%, 98%, 99% and 100% similarity, respectively, to *A. flavus* NRRL 3357 tubulin beta patative mRNA. Isolate 87 and 91 from soil during planting period showed similarity of 98% and 97%, respectively, to *A. flavus* NRRL β-tubulin gene partial cds. Particularly, total 90 isolates from infected peanut plant, soil, and air were found to range from 96 to 100 as *A. flavus* beta-tubulin gene complete cds. The Fig 3 shows high similarity between DNA sequences of the β-tubulin gene regions of all isolates and sequences in the database. Analysis of the phylogenetic tree showed that the isolate 54 isolated from air was different from other isolates and found to be in a different group. The remaining 99 out of 100 isolates belonged to a different group. In addition, isolate 7, isolate 87 and isolate 91 were seen dramatically to be different in the group of 99 isolates (Fig 3).

In the experimental area in 2015-2016, 213 (65.5%) out of 325 isolates of *A. flavus*, which were isolated from soil samples (24.3%) during planting-harvesting period and infected peanut plants (41.2%), were described aflatoxin production (Table 3). In this study, more than half of *A. flavus* isolates are indicated aflatoxigenic properties.

As can be seen from Fig 3, *A. flavus* isolates having aflatoxin biosynthesis genes and not having aflatoxin biosynthesis genes have been identified in the same group. In our study, it was determined that *A. flavus*, which was isolated during planting (41) in 2015, did not produce

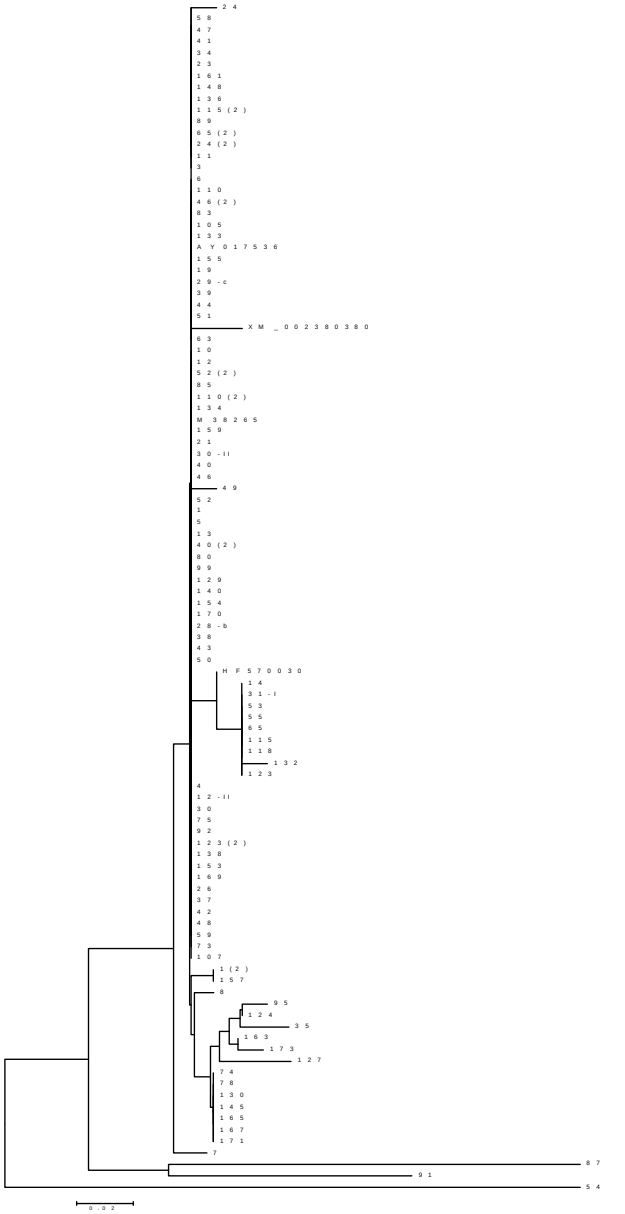

Fig 3: Phylogenetic tree showing the relationship between *A. flavus* isolates and NCBI GenBank.

Table 3: Occurrence of aflatoxins in *A. flavus* (n=325) isolated from infected peanut plants, soil and air samples during planting and harvesting periods and analyzed by HPLC.

A. flavus	Samples (positive samples)	Percentage (%)	Range of AFs (µg/kg)
		2015-2016	
Infected peanut plant	134(134)	41.2	0.42-5621.74
Soil from planting-harvesting	79(79)	24.3	0.30-536.70
Air and soil from planting-harvesting	112(0)	-	0.00
Total	325 (213)	65.5	0.30-5621.74

aflatoxin but they had an aflatoxin biosynthesis gene, *aflJ* and *aflR*. (Table 2, Table 3). This situation, lack of amplification of aflatoxin biosynthetic genes may be related to non-aflatoxigenic isolates. It is thought that the biosynthetic genes within the non-aflatoxin producing isolates have undergone deletion or mutation. Chang *et al.* (2005) studied and identified deletions of a fragment or the whole aflatoxin gene cluster in non-aflatoxigenic *A. flavus* isolates corroborating the theory that the loss of aflatoxin-producing capability might be associated with deletions or mutations in the concerned genes. Aflatoxin contamination due to the changing structures of fungal populations in or around plants are reduced by applications of non-aflatoxigenic (Antilla and Cotty, 2002). It is probably that one or more of the other genes included in aflatoxin biosynthesis are lacking or carry some deletions in these 41 isolates (Table 2, Table 3).

The results obtained in our study corroborated that lack of amplification of aflatoxin biosynthetic genes is related with not having aflatoxin biosynthetic genes. Mohankumar *et al*. (2010) also reported that the dendrogram, which showed the *A. flavus* isolated from corn in India, consisted of five different groups and group V was divided into two subgroups. It has been reported that more or less toxigenic *A. flavus* isolates in two different groups have no relationship between ITS-RFLP and gene cluster based on aflatoxin production. In the same way, the genetic similarity between 20 *A. flavus* and 15 *A. parasiticus* isolates included in *A.* section *Flavi* group was studied by RAPD and Neighbour Joining Analysis. In this study, *A. flavus* isolates were divided into two groups as having toxigenic and non-aflatoxigenic isolates in both groups. Besides that, five non-aflatoxigenic *A. parasiticus* isolates were found in the group of toxigenic *A. parasiticus* isolates. Our result agrees with the related literature and it points out the genetic diversity within *A. flavus* isolates.

CONCLUSION

Results of the available study indicated that PCR-RFLP is as quick and correct method to identify genes (*aflR-alfJ*) regulating toxin production using the IGS region of the *A. flavus* isolates. It is also shown in this study that 254 out of 325 (78.2%) *A. flavus* isolates are identified as *aflJ-aflR* intergenic spacer for aflatoxin biosynthesis genes. This is the first study about toxigenic and non-aflatoxigenic *A. flavus* isolates in peanut field in our country. The presence of *A. flavus*, which has aflatoxin biosynthesis genes in secondary peanut growing areas, is a hint that aflatoxin may be produced at the same time. This study will be provided for the determination of targeting genes that play a role in toxin production in our country. This result could be contributed to the improvement of credible molecular analysis for the identification of aflatoxigenic fungi besides determination of territorial *A. flavus*. In addition, molecular analysis was conducted to identify non-aflatoxigenic *A. flavus* isolates that could potentially be used in biological control for aflatoxin contamination. For this reason, PCR-RFLP has been used to determine the aflatoxin biosynthesis genes safely and effectively in non-aflatoxigenic fungal species to be used in the biological control of aflatoxin contamination.

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