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Genetic Variability of Aflatoxigenic and Non-Aflatoxigenic *A. flavus* Isolates by using Aflatoxin Biosynthesis Genes

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The five aflatoxin (AF) biosynthetic pathway genes included regulatory genes *aflR* and *aflS* and the structural genes *aflD*, *aflM*, *aflP* were evaluated in 81 *Aspergillus flavus* isolates isolated from wheat, corn, barley and sorghum. The amounts of aflatoxin B1 (B1) produced by 81 isolates varied from 0.3 to 1.2 ppb. The isolates were placed into seven groups based on their DNA banding pattern and B1 production. Only one group their isolates were able to produce B1 and six groups their isolates were unable to produce aflatoxin B1. All aflatoxinogenic isolates[14] showed in group I, representing more than (17%), amplification DNA fragments that correspond to the complete set of genes. Non-aflatoxigenic isolates showed six groups, group I constituted by twenty eight isolates (34.6%) correspond to the complete set of genes. Group II constituted by sixteen isolates (19.8%) showed four DNA banding pattern clustered in three profiles: *aflD*, *aflM*, *aflP* and *aflS* was the most frequent profile (11.1%) followed by *aflD*, *aflM*, *aflR* and aflS (6.2%) and *aflD*, *aflR* and *aflS* (2.5%). Group III constituted by twelve isolates (13.5%) yielded three DNA banding pattern grouped in two characteristic profiles: *aflD*, *aflP* and *aflS* (7.4%) and *aflD*, *aflM* and *aflR* (6.1%). Group IV seven isolates (8.6%) showed two DNA banding pattern clustered in two profiles. Group V constituted by three strains (3.7%) gave one profile with one DNA fragment specific for *aflD* gene. Finally, a group VI constituted by two isolates (2.5%) gave no DNA bands were found. Our data show a non genetic variability in aflatoxigenic *A. flavus* isolates, whereas a high level of genetic variability in non-aflatoxigenic *A. flavus* isolates.

Key words: *Aspergillus flavus*, Genetic variability, HPLC, Aflatoxin biosynthesis genes.

Aspergillus section *Flavi*, are distributed worldwide. Among the 22 closely related species in *Aspergillus* section *Flavi*, *A. flavus* and *A. parasiticus* are the most contaminate and frequent aflatoxin (AF) producers encountered in many economically important crops, cereal crop, corn, cotton, peanuts, and many tree nuts (Klich, 2007, Godet and Munaut, 2010). About 40% of the naturally occurring isolates of *A. flavus* lack the ability to produce AF and different isolates show large differences in the levels of AFs produced. It seems that *A. flavus* populations are a mosaic of toxigenic and nontoxigenic strains belonging to divergent clades which reproduce in diverse ecological niches throughout warm climates (Cotty *et al*., 1994).

AF gene cluster including more than 18 enzymatic stages and at least 25 genes residing in a 75 kb cluster in the third fungal chromosome (Zeng *et al*., 2011). Nearly all genes involved in the aflatoxin biosynthesis pathway have already been identified and cloned, but there exist some genes

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with unknown functions (Georgianna and G. Payne, 2009, Yu *et al*., 2004). On the other hand, non-aflatoxinogenic *A. flavus* isolates have been found to be extremely diverse in terms of four genes *aflR*, *nor-1*, *ver-1* and *omtA*, whereas aflatoxigenic isolates always show the complete gene set. Non-aflatoxigenic isolates lacking one, two, three or four genes (Criseo *et al*., 2008). The non-aflatoxigenic *A. flavus* isolates probably results from point mutations (Ehrlich *et al*., 2004) or small deletions in genes essential for aflatoxin production, such as those having a regulatory role (Calvo *et al*., 2004) or being involved in the signaling pathway (Hicks *et al*., 1997). A total of 18 strains of *Aspergillus* section *Flavi* isolated from Egyptian peanuts were assessed for AF production during growth on YES medium using HPLC. Thirteen of these strains produced AFB1 and AFB2. All strains were analysed for presence and/or absence of four genes, *aflD (nor-1), aflM (ver-1), aflP* (*omt A*) and *aflR*. PCR revealed that all tested strains contained all four genes (Abdel-Hadi *et al*., 2011). 134 non-aflatoxin producing strains of *A. flavus* isolated from food, feed and officinal plants were examined the presence and/or absence of four genes, *aflD* (nor-1), *aflM* (ver-1), *aflP* (omt A) and *aflR* by Quadruplex PCR. PCR data showed high level of genetic variability among non-aflatoxigenic *A. flavus* isolates (Criseo *et al*., 2008).

In this study, we evaluate the presence and the frequencies of the PCR products corresponding to amplification of *aflD*, *aflM*, *aflP*, *aflR* and *aflS* genes in aflatoxigenic and non-aflatoxigenic *A. flavus* isolates isolated from wheat, corn, barley and sorghum.

MATERIALS AND METHODS

Isolates of *A. flavus*

81 isolates of *A. flavus* isolated from wheat (21), corn (23), sorghum (19) and barley (18) were used in this work.

Determination of AFs by HPLC

Isolates were grown in sterile SMKY liquid medium (20 g sucrose, 0.5 g magnesium sulfate, 3 g potassium nitrate, 7 g yeast extract, and 1000 mL distilled water) (Davis *et al*., 1987) . The flasks were inoculated with 6-mm diameter discs of *Aspergillus* spp at 25° ± 2°C for 7 days (Paranagama *et al*., 2003), with three replicates. After incubation, the content of each flask was filtered (Whatman No. 1). For B1 extraction, the filtrate of each flask was treated three times with 50 mL chloroform in a separatory funnel. The chloroform extract was separated, dehydrated with anhydrous sodium sulfate and evaporated to dryness on a water bath at 50°C under vacuum. The residues were dissolved in 10 mL methanol and stored in dark vials. The extract was passed through a 0.45-µm micro-filter. Analysis of compounds was performed by HPLC (Perkin Elmer model series 200 UV/VIS) with a C18 column (300 mm x 3.9 mm, 4 µm). The HPLC system was equipped with a UV detector and fluorescence with 365 nm excitation and 430 emission wavelengths. The mobile phase consisted of methanol: acetic acid: water (20:20:60, v/v/v). The total run time for the separation was approximately 25 min at a flow rate of 1 mL/min (. Christian, 1990).

Isolation of fungal DNA

A. flavus isolates were cultured on double-layer media on 50-mm Petri dishes, one solid and the other liquid. The solid base medium was PDA as a film, and the top medium, liquid, was 1200 µL peptone yeast glucose. Fungi were incubated at 25°C for two days. Fungal mycelia (50 mg) were scraped using slide cover slips and transferred to 1.5-mL sterile Eppendorf tubes for DNA isolation. DNA was extracted from 50 mg fresh mat according to (Amer *et al*., 2011).

PCR reaction

Five pairs of primers were designed on the basis of the sequences of *A. flavus* AF biosynthetic genes *aflD*, *aflM*, *aflP*, *aflR* and *aflS*. Amplification was performed in 25 µL reaction, containing reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3.4 mM $MgCl_2$, 0.25 mM dNTPS, 0.4 mM of each primer, 2 U Taq DNA polymerase (Bio Labs), and 25 ng genomic DNA. Amplification parameters (*aflR*, *aflD*, *aflM*, *aflP*, and *AflQ*) consisted of 1 cycle 95 °C (1 min), 65 °C, (30 s), 72 °C (30 s) and 34 cycles at 94°C (30 s), annealing at 65°C (30 s), and extension at 72°C (30 s). Amplification parameters (*aflS*) 95°C for 10 min, 5 cycles 94°C (1 min), 60°C (1 min), and 72°C (1 min), 30 cycles 94°C (1 min), 55°C (1 min), and 72°C (1 min), with a final extension at 72°C for (6 min). PCR products were separated by 1.5% agarose gel, stained with ethidium bromide in 1X TAE buffer (Tris-acetate EDTA, pH 8.0) at 100 V for 50 min,

using a 100-bp ladder DNA marker (Intron, Korea). The DNA gel was scanned for band Rf using a gel documentation system (AAB Advanced American Biotechnology, Fullerton, CA, USA). Sequences of primers are listed in Table 1.

RESULTS

B1 production by *A. flavus* isolates

81 isolates were analyzed for the levels of AFB1 (B1) by HPLC (Table 2). B1 production showed that 14 (17.3%) of 81 tested *A. flavus* isolates produced detectable levels of B1. Four isolates of (wheat and corn) and three isolates of (barley and sorghum) were produced detectable levels of B1 at concentrations ranging from 0.3 to 1.2 ppb.

PCR of AF biosynthesis genes

All *A. flavus* isolates examined using PCR to amplification the AF biosynthetic genes. DNA fragments amplicons of 400 bp, 537 bp, 797 bp, 1032 bp and 1399 were obtained with *aflD*, *aflM*, aflP, *aflR* and *aflS* genes respectively (Figure 1).

Amplification patterns of aflatoxin biosynthesis genes and B1 production

Primers pairs were designed for this study to target five AF biosynthetic genes: the two regulatory genes *aflR* and *aflS*, and the structural genes *aflD*, *aflM*, *aflP* and *aflS*. As shown in (Table 3). The presence or absence the AF biosynthetic genes in the genomes of all *A. Flavus* isolates was separated into seven groups on the basis of PCR products and AFB1 production. Only one group their isolates were able to produce AFB1 and six groups their isolates were unable to produce AFB1. Group I of 12 isolates was characterized by amplification of all five biosynthetic genes and the isolates were able to produce B1.

Group II of 28 was characterized by amplification of all five biosynthetic genes and the isolates were unable to produce B1. Group III, consisting of sixteen isolates was characterized by amplification four of five AF biosynthetic genes clustered in three profiles, 1) *aflD*, *aflM*, *aflP*, and aflS (nine isolates), 2) *aflD*, *aflM*, *aflR* and *aflS* (five isolates), 3) *aflD*, *aflP*, *aflR* and aflS (two isolates). Group IV constituted by eleven isolates was characterized by amplification three of genes clustered in two profiles, 1) *aflD*, *aflP*, and *aflS* (six isolates), 2) *aflD*, *aflM*, and *aflR* (five isolates). Group V consisting of eight isolates was characterized by amplification two genes clustered in two profiles, 1) *aflM* and *aflP* (four isolates), 2) *aflD* and *aflP* (three isolates). Group VI consisting of three isolates gave one profiles with one DNA fragment specific for *aflD* gene. Group VII consisted of two isolates that lacked all of the biosynthetic genes tested.

Genetic pattern of *A. flavus* isolates

All *A. flavus* isolates yielded different DNA banding patterns with a number of bands ranging from zero to five (Table 4). All aflatoxinogenic isolates (14 isolates) showed a complete set pattern, indicating the presence of the five genes of the AF biosynthetic pathway, whereas non-aflatoxinogenic isolates (67 isolates) presented varying patterns.

Non- aflatoxinogenic *A. flavus* isolates divided into six groups on the basis of their DNA banding patterns: twenty eight isolates displayed

Table 1. Sequences of the nucleotide primers used in this study

Primer code	Target gene	Primer sequences	PCR product size (bp)
nor-1	*aflD* (*nor-1*)[a]	5' -ACCGCTACGCCGGCACTCTCGGCAC'	400
nor-2		5' -GTTGGCCGCCAGCTTCGACACTCCG'	
ver-1	*aflM* (*ver-1*)	52 -GCCGCAGGCCGCGGAGAAAGTGGT'	737
ver-2		5' -GGGGATATACTCCCGCGACACAGCC'	
omt-1	*aflP* (*omt-1*)	5' -GTGGACGGACCTAGTCCGACATCAC'	799
omt-2		5' -GTCGGCGCCACGCACTGGGTTGGGG'	
aflR-1	*aflR*	5' -TATCTCCCCCCGGGCATCTCCCGG'	1032
aflR-2		5' -CCGTCAGACAGCCACTGGACACGG'	
AflJ-gF	*aflS* (*aflJ*)	5' -GAACGCTGATTGCCAATGCC'	1399
AflJ-giR		5' -CGGTCAGGATGTTACTAAGC'	

[a] Aflatoxin biosynthetic genes are named as proposed by Yu *et al*. (2004), old names are reported in brackets.

a complete set pattern, sixteen isolates displayed four-banded pattern, eleven sixteen isolates displayed three-banded pattern, seven isolates displayed two-banded pattern, three isolates displayed one-banded pattern and finally two isolates displayed no-banded pattern.

Table 2. B1 production by *Aspergillus flavus* isolates isolated from wheat, corn, barley and sorghum grains

Code of *A. flavus* isolates	B1
Wheat	
W101	0.0
W102	0.9
W103	0.0
W104	1.1
W105	0.0
W106	0.0
W107	0.0
W108	0.0
W109	0.0
W110	0.0
W111	0.0
W112	0.0
W113	0.0
W114	0.0
W115	0.0
W116	0.7
W117	0.0
W118	0.6
W119	0.0
W120	0.0
W121	0.0
Corn	
C122	1.2
C123	0.0
C124	0.0
C125	0.0
C126	0.0
C127	0.0
C128	0.0
C129	1.1
C130	0.0
C131	0.0
C132	0.0
C133	0.0
C134	0.0
C135	0.9
C136	0.0
C137	0.0
C138	0.0
C139	0.0
C140	0.0
C141	0.9
C142	0.0
C143	0.0
C144	0.0
Barley	
B145	0.0
B146	0.0
B147	0.0
B148	0.0
B149	0.5
B150	0.0
B151	0.0
B152	0.0
B153	0.0
B154	0.0
B155	0.0
B156	0.0
B157	0.3
B158	0.0
B159	0.0
B160	0.0
B161	0.7
B162	0.0
Sorghum	
S163	0.0
S164	0.0
S165	0.5
S166	0.0
S167	0.0
S168	0.0
S169	0.0
S170	0.0
S171	0.0
S172	0.0
S173	0.4
S174	0.0
S175	0.0
S176	0.0
S177	0.0
S178	0.0
S179	0.0
S180	0.5
S181	0.0

Table 3. Amplification patterns of aflatoxin biosynthesis genes and aflatoxin B1 production in *A. flavus* isolates[a]

Code of *A. flavus* isolates	AB1[b]	*aflD*	*aflM*	*aflP*	*aflR*	*aflS*	Group
W102	+						I[c]
W104	+						
W116	+						
W118	+						
C122	+						
C129	+						
C135	+						
C141	+						
B149	+						
B157	+						
B161	+						
S165	+						
S173	+						
S180	+						
W103	-						II
W105	-						
W106	-						
W107	-						
W111	-						
W112	-						
W114	-						
W117	-						
W123	-						
C127	-						
C128	-						
C131	-						
C133	-						
C139	-						
B145	-						
B146	-						
B147	-						
B153	-						
B154	-						
B159	-						
S163	-						
S164	-						
S170	-						
S171	-						
S172	-						
S178	-						
S179	-						
S181	-						
W101	-						
W108	-						
W109	-						

C125	-	
C126	-	
C130	-	
B150	-	
B151	-	
S166	-	
C136	-	
C138	-	
B160	-	
B162	-	
S167	-	
C132	-	
C137		
W110	-	III
C134	-	
C140	-	
C142	-	
S168	-	
S169	-	
W119	-	
W121	-	
B152	-	
B155	-	
S177	-	
W120	-	IV
C143	-	
S174	-	
S175	-	
W115	-	
C144	-	
B156	-	
W113	-	VI
C124	-	
B158	-	VII
B148	-	
S176		

a Presence (black box) or absence (grey box) of PCR products of the tested AFs genes
b B1 (+/-): B1 and no B1 production
c Distribution of isolates in seven amplification patterns.

Frequency of single genes and genetic pattern in *A. flavus* isolates

Frequency of single genes in all *A. flavus* isolates were showed in Table (5). All aflatoxinogenic isolates (14) showed in one group, representing more than (17%), amplification DNA fragments that correspond to the complete set of genes. Non-aflatoxigenic isolates showed six groups, first group constituted by twenty eight isolates (34.6%) correspond to the complete set of genes. Second group constituted by sixteen isolates (19.8%) showed four DNA banding pattern clustered in three profiles: *aflD*, *aflM*, *aflP* and *aflS* was the most frequent profile (11.1%) followed by *aflD*, *aflM*, *aflR* and aflS (6.2%) and *aflD*, *aflR* and *aflS* (2.5%). Third group constituted by twelve isolates (13.5%) yielded three DNA banding pattern grouped in two characteristic profiles: *aflD*, *aflP*

Table 4. Genetic pattern of *A. flavus* isolated from different crops

Crop	Aflatoxigenic isolates						
	No. of isolates	Complete set	Four bands	Three bands	Two bands	One band	No band
Wheat	4	4	0	0	0	0	0
Corn	4	4	0	0	0	0	0
Barley	3	3	0	0	0	0	0
Sorghum	3	3	0	0	0	0	0
Total	14	14	0	0	0	0	0
Non-aflatoxigenic isolates							
Wheat	17	8	3	3	2	1	0
Corn	19	6	7	3	2	1	0
Barley	15	6	4	2	1	1	1
Sorghum	16	8	2	3	2	0	1
total	67	28	16	11	7	3	2

Table 5. Frequency of single gene and genetic pattern in *A. flavus* isolates[a]

No of	Aflatoxigenic isolates					
isolates	*aflD*	*aflM*	*aflP*	*aflR*	*aflS*	Genetic pattern %
14						17.3
	Non-aflatoxigenic isolates					
28						34.6
9						11.1
5						6.2
2						2.5
6						7.4
5						6.1
4						4.9
3						3.7
3						3.7
2						2.5
81	92.6%	80.2%	81.5%	66.7%	79.2%	

[a] Presence (black box) or absence (grey box) of PCR products of the tested AFs genes

and *aflS* (7.4%) and *aflD*, *aflM* and *aflR* (6.1%). Fourth group seven isolates (8.6%) showed two DNA banding pattern clustered in two profiles. Fifth group constituted by three strains (3.7%) gave one profile with one DNA fragment specific for *aflD* gene. Finally, a sixth group constituted by two isolates (2.5%) gave no DNA bands were found. The *aflD* gene was the most representative more than (92%) between the five AF assayed genes followed by *aflP* (81.5%) and *aflM* (80.2%) and *aflS* (79.2%). Lower incidence (66.7%) was found for *aflR* gene. The frequencies of banding pattern and respective profiles are showed in (Fig. 2).

DISCUSSION

In present work, *A. flavus* including both aflatoxigenic and non-aflatoxigenic isolates were screened for the presence of five genes (*aflD*, *aflM*, *aflP*, aflR and *aflS*) of the aflatoxin biosynthesis. The result was the grouping of aflatoxigenic isolates into one group, complete set of genes, whereas non-aflatoxigenic isolates lacking one, two, three and four genes or no DNA band. Our data show a low level genetic variability in aflatoxigenic *A. flavus* isolates, a high level of genetic variability in non-aflatoxigenic *A. flavus* isolates.

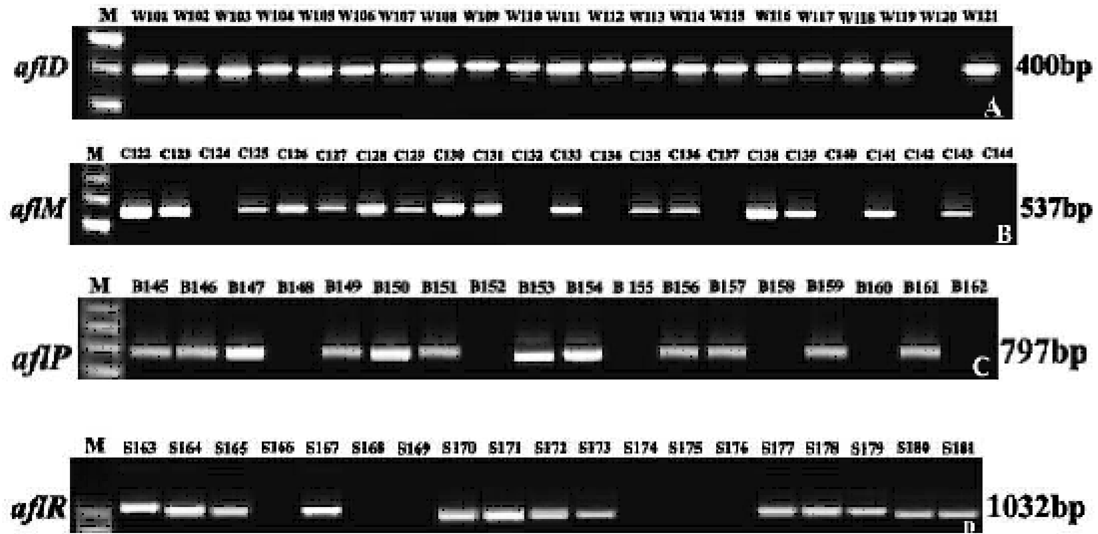
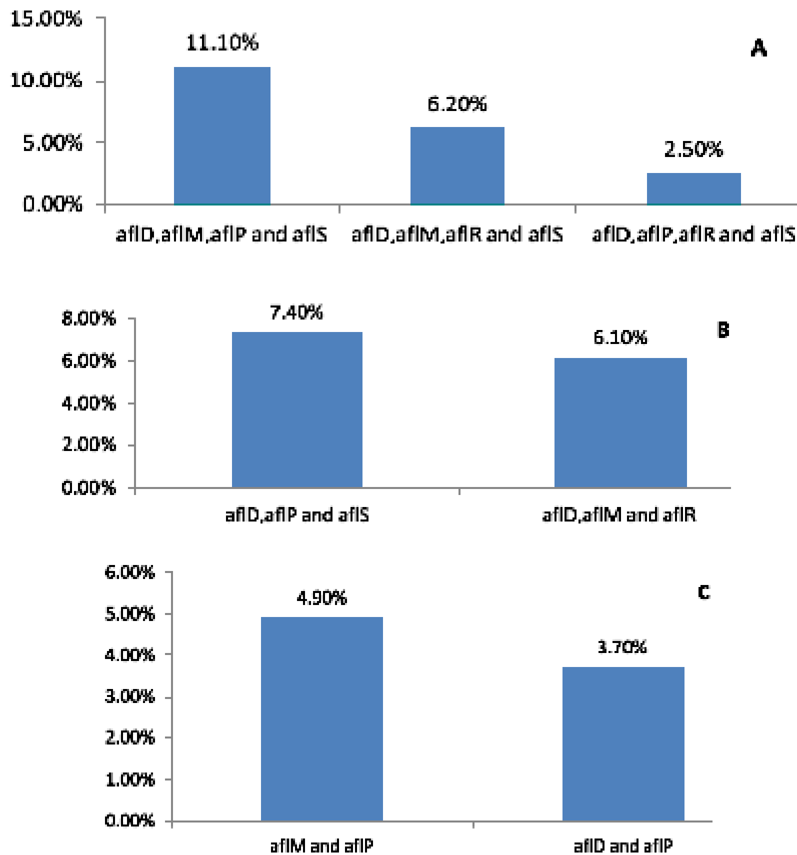


Fig. 1. Agarose gel electrophoresis analysis PCR products using primers and DNA extracted from of *A. flavus* isolates, M: 100 bp DNA ladder (A): Lanes 1-21, *A. flavus* isolates isolated from wheat using *aflD* primer with 400bp. (B): Lanes 1-23, *A. flavus* isolates isolated from corn using *aflM* primer with 537bp. (C): Lanes 1-18, *A. flavus* isolates isolated from barley using *aflP* primer with 797bp. (D): Lanes 1-19, *A. flavus* isolates isolated from sorghum using *aflR* primer with 1032bp



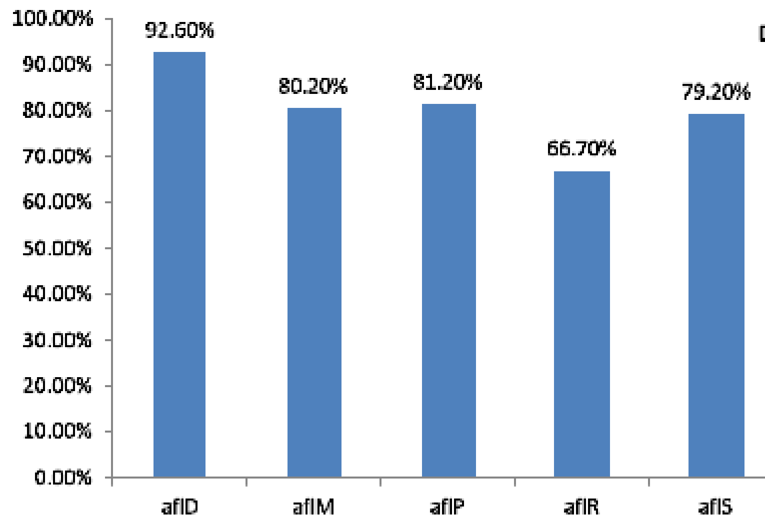


Fig. 2. Frequencies of four (A), three (B), two (C) and one (D) genes pattern in *A. flavus* isolates

In the last few years, several PCR-based systems have been developed to revealed genetic variation in *A. flavus* isolates; PCR-based systems included molecular marker (APLP, DAF, RAPD and ISSR) and the aflatoxin biosynthesis gene cluster. Molecular markers are useful for genetic variation in fungi (Majer *et al*., 1996).

Several molecular methods were employed in an attempt to study genetic variability for aflatoxigenic and non aflatoxigenic isolates of *A. flavus*. (Montiel *et al*., 2003) who analysed 24 isolates of *A. sojae*, *A. parasiticus*, *A. oryzae* and *A. flavus* using the AFLP technique, and could clearly separate the *A. sojae* D *A. parasiticus* isolates from the *A. oryzae* D *A. flavus* isolates. Barros *et al*., 2007 reported that AFLP analysis was applied to compare 82 isolates of *A. flavus* and *A. parasiticus*. AFLP analysis indicates that no genotypical differences can be established between aflatoxigenic and nonaflatoxigenic producers in both species analysed. Ribosomal sequence ITS for 24 isolates of *Aspergillus sojae*, *A. parasiticus*, *A. oryzae* and *A. flavus* (aflatoxigenic) found some variation between *A. oryzae* and *A. flavus* isolates, but it is difficult to use molecular data to separate the two species (Montiel *et al*., 2003). Using DNA amplification fingerprinting (DAF) to differentiate aflatoxigenic from nonaflatoxigenic isolates of *A. flavus* but, could not be separated the isolate types (Baird *et al*., 2006). The ISSR primers produced differential amplification products, varying both in size and band intensity. ISSR revealed higher genetic variability in several strains of *A. flavus*, even though high interspecific variation was observed (Yin *et al*., 2009, Tran-Dinh *et al*., 2009, Wang *et al*., 2012)). The utility of DNA markers as RAPD-DNA employ it as well established sample molecular marker tool for detecting genetic variability for many phytopathogenic fungi (Megnegneau *et al*., 1993) especially *Aspergillus* genera and related species (Wostemeyer and Kreibich *et al*., 2002, Gashgari *et al*., 2010, Sepahvand *et al*., 2011) . Furthermore, several researches have adopted detect an AF biosynthetic gene and differentiate AF-producing from non-producing strains of *A. flavus* (Criseo *et al*., 2001). Who combined sets of primers for *aflR*, *nor-1*, *ver-1* and *omt-A* genes of the AF biosynthetic pathway, Quadruplex-PCR showed that aflatoxinogenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the AF biosynthetic pathway which encode for functional products. Non-aflatoxinogenic strains gave varying results with one, two, three or four banding patterns.

Aspergillus flavus (14 isolates) isolated from maize. The isolates were analysed for the presence of five AF biosynthesis genes in relation to their capability to produce AFB1, targeting five genes of the AF gene cluster of *A. flavus*, two regulatory (*aflR* and *aflS*) and three structural (*aflD*,

aflO and *aflQ*). The isolates were placed into four groups based on their patterns of amplification products: group I (5 isolates) characterized by presence of all five amplicons; groups II (one isolates) showing all five amplicons, group III (three isolates) and group IV showing three (*aflO*, *aflQ* and *aflR*) and two (*aflO* and aflQ) amplicons, respectively. Only group I isolates able to produce aflatoxin B1 (Degola *et al*., 2007). Fourteen strains of *A. flavus* were examined using TLC and PCR with *nor-1*, *ver-1*, *omt-1* and *aflR* primers. The results showed that three isolates fourteen strains of *A. flavus* were positive aflatoxin production and complete pattern of fragment genes. Other eleven isolates were negative aflatoxin production and presenting varying pattern of fragment genes (Erami *et al*., 2007). 134 non-aflatoxin producing strains of *A. flavus* isolated examined using a multiplex PCR-system, four DNA fragments specific for *aflR*, *nor-1*, *ver-1*, and *omt-A* genes. Forty nine (36.5%) of the examined non-aflatoxigenic *A. flavus* strains showed DNA fragments that correspond to the complete set of genes (quadruplet pattern). Forty three strains (32%) showed three DNA banding patterns grouped in four profiles where *nor-1*, *ver-1* and *omt-A*. Twenty five (18.7%) yielded two DNA banding pattern whereas sixteen (12%) of the strains showed one DNA banding pattern. In one strain, isolated from poultry feed, no DNA bands were found (Criseo *et al*., 2008).

Using PCR showed that all aflatoxin and non-aflatoxin producers of *A. flavus* harbor 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 (Hicks *et al*., 1997). The PCR of four aflatoxin (AF) biosynthetic pathway genes (*aflD*, *aflO*, *aflP* and *aflQ*) was evaluated in 24 *Aspergillus flavus* strains (toxigenic and non-toxigenic) isolated from soils of pistachio orchards. The PCR results obtained with genomic DNA as the template indicated that four genes, *aflD*, *aflO*, *aflP* and *aflQ*, were present in all strains either aflatoxigenic or non-toxigeni (Jamali *et al*., 2013). Our results are in agreement with the results reported by (Criseo *et al*., 2001, Degola *et al*., 2007, Erami *et al*., 2007) and disagreement with (Abdel-Hadi *et al*., 2011, Jamali *et al*., 2013).

In our study, we found that 27 of aflatoxin non-producing *A. flavus* isolates lacking the *aflR* PCR amplicon because in these strains the *aflR* gene has been lost or mutations occurs within the primer binding sites. This could be due to the location of the aflatoxin gene cluster in the telomeric region of *A. flavus* that would facilitate gene loss as well as recombination, DNA inversions, partial deletions, translocations and other genomic rearrangements (Carbone *et al*., 2007).

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