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Original article

Molecular profile of aflatoxigenic and non-aflatoxigenic isolates of Aspergillus flavus isolated from stored maize

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ABSTRACT

Maize is a significant staple crop and utilized in Saudi Arabia as food and feed, but maize is often infected with *Aspergillus flavus* in tropical and subtropical climates, especially during storage. This study intended at a polyphasic approach, consisting of microscopic morphological, biochemical, and molecular characterizations that were applied to 29 of A. flavus isolates of stored maize, with the goal of characterization and identification of aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. The technique of real-time PCR (RTi-PCR) was used to detection of *A. flavus* in stored maize samples, the findings have been very accurate. Centered on macroscopic morphological (primarily colony color and morphology of conidia) and microscopic (morphology of conidia and size) characteristics. Results have shown 23 A. flavus isolates (80%) were categorized as the dark green of colonies also all isolates were rough conidia. The isolates have been two different groups, 16 isolates (62%) had sclerotium-forming and the remaining 13 isolates (38%) had no sclerotium-forming isolates. To the identification of aflatoxigenic isolates of *A. flavus* in stored maize, we utilized the qualitative methods (easy and inexpensive) like UV test, yellow pigmentation, and ammonia vapor and quantitative method as HPLC (accurate and expensive). the accuracy methods to the identification aflatoxigenicity isolates, vary, and classified in the following descending order: HPLC (100%) > UV method (81%) > yellow pigmentation (YP) and ammonia vapor (AV) (63%). The profile of Aflatoxigenicity of A. flavus isolates by HPLC has been involved in two types first of 11 isolates (38%) have been aflatoxigenic isolates while 18 isolates (62%) were non-aflatoxigenic isolates. The expression of six aflatoxins (AFs) genes (*aflD*, *aflM*, *aflO*, *aflP*, *aflR*, and *aflQ*) was estimated using PCR and RT-PCR. PCR of all genes did not correspond to the aflatoxigenic isolates. The transcriptional analysis of *aflO* and *aflQ* was a beneficial marker for discriminating aflatoxigenic from non-aflatoxigenic *A. flavus* isolates. Also, qRT-PCR indicated that non-aflatoxigenic isolates had a high incidence of defect or downregulation in late AF-genes contrast with early AF-genes. therefore, these non-aflatoxigenic isolates could be critical factors for an efficient and competent strategy for the control of aflatoxin contamination pre-harvest can be considered.

1. Introduction

Maize (Zea mays L.) is one of the most important cereal crops for human consumption and animal feeds in the world, and Saudi Arabia. Saudi Arabia imported 1.49 million tons valued at $319.23 million in 2016 (Arab Organization for Agricultural Development, 2006). Fungi are normal flora in the maize grains but when conditions favorable for fungal invasion, it constitutes a critical point that may affect the safety, quality, and quantitatively and qualitatively properties of the maize grains (Laca et al., 2006). Fungal invasion of maize grains occurs during crop growth, harvesting, postharvest drying, transportation, and storage, and invasion may be continued which leads to extremely potential fungal risks associated with maize grains (Magan and Aldred, 2006). In fact, maize could be good material for some fungi, especially toxigenic fungi like *Aspergillus* and *Fusarium* species (Queiroz et al., 2012). *Aspergillus* section *Flavi* is one of the most essential sections in the *Aspergillus* genus, also these fungi ordinarily contaminated crops. The

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Aspergillus section *Flavi* consists of two groups of species but the important one includes *A. flavus* and *A. parasiticus* (Cary and Ehrich, 2006; Ehrlich et al., 2007). Another group involves the non producing aflatoxin species *A. oryzae* and *A. sojae* (Kumeda and Asao, 2001). the Taxonomy of *Aspergillus* section *Flavi* have been complicated and ever-evolving. Hence, the rigor and reliability of their identity is critical for maintain an accurate taxonomic system for agricultural, industrial, economic, and regulatory purposes (Pitt and Hocking, 2009). In the past, many researchers have been described Aspergillus section Flavi based primarily on traditional methods based on morphological parameters such as the diameter of a colony, color, and texture, size of conidia and conidiophore structure (Klich, 2002). Classic methods for identifying *A. flavus* in the laboratory were the time-consuming and laborious for followed up morphological characteristics and get accurate results from professional microbiologists (Lievens and Thomma, 2005). PCR technology that has been based on DNA is granted a significant choice for accurate identification of *A. flavus* with high specific, sensitive, and rapid method (Somashekar et al., 2004; Godet and Munaut, 2010).

A. flavus remain the most powerful and representative source of naturally occurring aflatoxins (AFs) in the food chain (Cary and Ehrich, 2006). The mycotoxigenic profile *A. flavus* included many kinds of aflatoxin like AFB1,2 and AFG1,2 have been routinely used for identification aflatoxigenic or non-aflatoxigenic isolates. Based on the development of mycotoxin, the classification of *A. flavus* populations have extremely diverse and included various groups (Vaamonde et al., 2003; Razzaghi-Abyaneh et al., 2006). In the past, molecular-DNA methods have been usually utilized in the differentiating aflatoxigenic and non-aflatoxigenic *A. flavus* isolates through the behaviors of aflatoxin genes (presence or absence) and capacity or incapacity to produce aflatoxins. For example, many PCR systems like Quadruplex and multiplex-PCR were designed for detecting specific genes of AFs (Criseo et al., 2001; Chen et al., 2002).

Recently, the function of genomics (RNA) has developed our knowledge of complicated regulatory machinery, and networks have the mapping of controlling the genes of AFs and their behavior for production AFs. Transcriptomic and proteomic analyses together sharing in knowing the differential expressions of the AFs genes and carbon genes to get the comprehensive model of AFs-genes (Mayer et al., 2003; Rodrigues et al., 2009; Rodríguez et al., 2013). Multiplex RT-PCR for aflD, *aflO*, and *aflQ* have also confirmed a very clear relationship between AFs genes and producing and non-producing AFs (Jamali et al., 2013; Mahmoud, 2015).

Since the accurate identification of *A. flavus* may affect prevention and management control strategies of fungi and aflatoxins, the aim of this research was to characterize 29 *A. flavus* isolates (focus on non-aflatoxigenic) recovering from stored maize, in Saudi Arabia, based on a polyphasic approach that involves morphological, biochemical, and molecular methods.

2. Materials and methods

2.1. Fungal isolation and identification

For isolation and identification of seed-borne fungi, thirty corn grain samples, collected from various locations in Riyadh region, were used. The samples were disinfected and put aseptically on an agar petri dish containing Rose Bengal in triplicate and incubated at 25° C (PDA, Difco Laboratories, USA) and the method continues according to (Singh et al., 1991). Isolated fungi were purified either by hyphal tip or single spore methods, and identified according to the methods of (Pitt and Hocking, 1997; Klich, 2002). Designated with the AsFmRnn code, where AsF refers to the Aspergillus flavus genus, M refers to the product (maize), R refers to the area (city of Riyadh) and the isolate number is nn. For subsequent morphological characterization, all isolates were kept on PDA tubes and then kept at 4° C.

2.2. RTi-PCR assay

To help rapid screening for the appearance of *A. flavus* in stored maize samples, two specific primers were used in one reaction mixture for RTi-PCR. RTi-PCR amplifications were performed with a LightCycler 2.0; (Roche Diagnostics GmbH, Germany) using primer Forward CTCCCACCCGTGTTTACTGT, Reverse GCGTTCTTCATCGATGCCT, according to (Rong et al., 2006).

2.3. Morphological characterization

A loop full of spores in 500 μL of 0.2 percent agar was suspended for each isolate and this suspension was used for three-point inoculations on 9 cm diameter Petri dishes containing 20 mL of MEA and CZ. Isolates were incubated at 25° C for 7 days, in the dark, and then examined for colony colour, appearance and scale of sclerotia, and morphology of conids. Identification of isolates by endorsing the available taxonomic keys and guides for the genera of *Aspergillus* (Klich, 2002; Samson et al., 2004).

2.4. Differentiation of aflatoxigenic and non aflatoxigenic A. Flavus isolates

2.4.1. Qualitative methods

2.4.1.1. Ultraviolet light (UV) test and yellow pigmentation. Isolates were grown at 25 °C for 7 days on the coconut agar medium. Petri dishes were observed under long UV light (365 nm) after incubation. The presence of a fluorescent zone around the growing fungal colony and blue/violet fluorescence on the reverse side suggested that only aflatoxin B could be generated by an isolate, while a blue/white fluorescence suggested that aflatoxin B and G were generated by an isolate, the absence of fluorescence revealed that an isolate failed to generate detectable values of aflatoxin B and G (Davis et al., 1987; Dyer and McCammon, 1994). We detected orange-yellow pigmentation on the reverse side of the petri dish during the incubation phase, which makes the isolate harmonious with AF production (Lin and Dianese, 1976).

2.4.1.2. Ammonia vapor test. The isolates were grown in the dark on the yeast extract sucrose (YES) media and incubated at 28 °C for 3–4 days. During incubation, the color of the media transforms pink to red during applying a 500uL ammonia solution (Sigma-Aldrich) was seen by aflatoxigenic isolates. While no color shift for non-aflatoxigenic isolates was observed (Saito and Machida, 1999).

2.4.2. Quantitative method

2.4.2.1. HPLC analysis. All isolates were grown in the medium of YES were examined for the production of aflatoxins. Isolates were inoculated at 25° C to 6 cm in diameter Petri dish for 7 days and continue the technique of (Bragulat et al., 2001). Samples were analyzed using a HPLC PerkinElmer series 200 UV / VIS unit provided with a UV detector, and fluorescence was measured using wavelengths of 365 nm excitation and 430 nm emission. On the C18 column, chromatographic separations were carried out, HPLC conditions such as the mobile phase, the total run time, the amount of injection were applied as (Christian, 1990). A standard HPLC chromatogram of aflatoxins was plotted, and the concentrate samples were quantified.

2.5. Molecular characterization

2.5.1. PCR xxx

The isolation of DNA from 29 A. flavus isolates, as mentioned in (Rodrigues et al., 2009). The parameters of PCR followed those stated by (Rao et al., 2020). The *aflD*, *aflM*, *aflO*, *aflP*, *aflQ*, and *aflR* genes were tested for all isolates using Table 1 have a list of primers. PCR was conducted as follows: 1 stage at 94° C for 5 min; 35 30-sec cycles at 94° C, 60 s at 55° C, and 90 s at 72° C; in the Techne TC-312, UK, with a final extension at 72 8C for 7 min. The β-tubulin encoding housekeeping gene tub1 was used as internal amplification control.

2.5.2. RT-PCr

Twenty-nine isolates of *A. flavus* has been cultivated in the same condition as described above. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), including the RNase-free DNase set (Qiagen) genomic DNA digestion step, as directed by the manufacturer. Fungal RNA was stored at −80 °C. In a 20-μL reaction mixture volume containing 8 μL of the One-Step RT-PCR Pre-Mix (Intron Biotechnology) pack, RT-PCR was conducted with 0.2 μM of each primer. As mentioned above, PCR parameters were carried out using the same set of primers designed for RT-PCR and 1 μg of total RNA as a template.

2.5.3. RNA isolation and real time PCR (qRT-PCR)

For more gene expression studies, each isolate was cultured for 4 days and 28 °C, 150 rpm in the darkness on YES broth. Mycelia used liquid nitrogen to ground them. For RNA extraction, 200 mg of ground mycelium was used by the RNeasy plant mini kit (QIAGEN, Germany), as instructed by the manufacturer. RNA was tested for the consistency and purity of RNA and quantified by a spectrophotometer. cDNA synthesis was carried out by the ProtoScript First Strand cDNA synthesis Kit (New England Biolabs, UK), according to the manufacturer's protocol. qRT-PCR was performed using Luna® Universal qPCR Master Mix (New England Biolabs, UK) with same conditions of (Rao et al., 2020) with primers (Table 1). The relative expression of each specific gene has been evaluated (Scherm et al., 2005; Schmittgen and Livak, 2008). Heatmaps for each gene expression analysis were created using the Heatmapper method (Babicki et al., 2016).

3. Results

3.1. Distribution of isolates of Aspergillus section Flavi

The recovered fungal taxa were greatly influenced by various locations of the samples of maize grains and thus LSD was used to compare the degree of isolation frequency for each fungus (Table 2). Isolation frequencies of *Aspergillus* genera especially *A. flavus* and *Fusarium* genera significantly developed when storage maize grains were collected from eastern and southern regions of Riyadh city. These results may indicate that these fungi affecting storage conditions like different temperatures and humidity also, competition or antagonism between various fungal genera. Hence, the findings showed a significant increase or decrease in their isolation frequencies. The results reported the superiority of *Aspergillus* genera in all the storage maize samples analyzed with the mean of 34% followed by *Fusarium* genera 22.25% (Fig. 1). The other species isolated belonged to the *Aspergillus* section *Nigri* and *Terrei* colonization of maize grains were lower in stored samples (12% and 4.75%). The relative distribution of fungal isolates over samples explained that *A. flavus* was the highest distributed 17.25%.

3.2. A. Flavus specific real-time PCR assay

Melting temperature (Tm) values were 92.58° C for 10 *A. flavus* isolates (Fig. 2). The presence of the one signal of xx only, and not for any other *Aspergillus* genera, like *A. niger* and *A. terreus*, has also confirmed the real-time specificity.

3.3. Morphological analysis

A total of 29 *A. flavus* isolates were grouped into six separate morphology-based chemotype profiles (primarily sclerotia, colony color, and conidia morphology) Table 3. Chemotype I (n = 6), Chemotype II (n = 7), Chemotype 3 (n = 3), Chemotype IV (n = 7), Chemotype V (n = 2) and Chemotype VI (n = 4). The main *A. flavus* isolates was sclerotial production (16 isolates 62%) divided to L-type forming (9 isolates, 31%), and S-type forming (4 isolates,13.8%). No sclerotium-forming (13 isolates, 38%). 23 isolates (79.3%) were categorized as the dark green of colonies also all isolates were rough conidia.

3.4. Differentiation of aflatoxigenic and non aflatoxigenic isolates

3.4.1. Qualitative methods

Among the 11 isolates, 9 (81%) isolates were categorized as potentially aflatoxigenic based on blue fluorescence when exposed to UV light in CAM (Table 4). Yellow pigmentation and ammonia vapour test have the same efficiency in identifying aflatoxigenic isolates (63%), 7 isolates.

3.4.2. Quantitative of aflatoxins by HPLC

29 *A. flavus* isolates were split into three chemotypes (Table 5). Chemotype I was the large preponderance (62%) were non-aflatoxigenic isolates followed by 31% of aflatoxigenic isolates (AB1) belong to chemotype II and finally, chemotype III involved 7% of aflatoxigenic isolates (B1 + B2). AFB1 was produced by eleven isolates (38%) and AB2 was produced by only two isolates Table 6.

Table 1
Primers used in this study, target gene, sequence, and expected PCR/RT-PCR product size.

Primer code	Gene	Primer sequence (5–3)	PCR product size (bp)	RT-PCR product size (bp)
Nor1-F	aflD	ACGGATCACTTAGCCAGCAC	990	815
Nor1-R		CTACCAGGGGAGTTGAGATCC		
Ver1-F	aflM	CCGTTTAGATGGCAAAGTGG	899	756
Ver1-R		CTTTCAGGTGACCGAACGAT		
AflR-F	aflR	CGAGTTGTGCCAGTTCAAAA	999	999
AflR-R		AATCCTCGCCCACCATACTA		
OmtB(F)-F	aflO	GCCTTGACATGGAAACCATC	1333	1131
OmtB(F)-R		CCAAGATGGCCTGCTCTTTA		
Omt1-	aflP	GCCTTGCAAACACACTTTCA	1490	1210
Omt1-R		AGTTGTTGAACGCCCCAGT		
Ord1(P)-F	aflQ	CGACTGTTGGCCTTTTCATT	1088	1088
Ord1(P)-R		ATAGCGAGGTTCCAGCGTAA		

Table 2
Distribution of Fungal genera recovered from stored maize grains.

Percentage of fungal isolates		Riyadh region				Difference[a]
		East zone	West zone	North zone	South zone	
Aspergillus genera	Percentage of Aspergillus section Flavi	23	10	12	24	9.41
	Percentage of Aspergillus section Nigri	10	13	11	14	7.58
	Percentage of Aspergillus section Terrei	5	6	4	4	6.32
	Percentage of Aspergillus flavus	23	10	12	24	9.41
Other fungi	Percentage of Fusarium genera	31	20	17	29	10.93
	Percentage of Penicillium genera	3	1	2	4	3.12
	Percentage of other fungi	28	50	54	25	11.57

LSD ($P \leq 0.05$) for the difference = 7.21; LSD ($P \leq 0.01$) for the difference = 11.78; [a] The difference was significant at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or nonsignificant (NS).

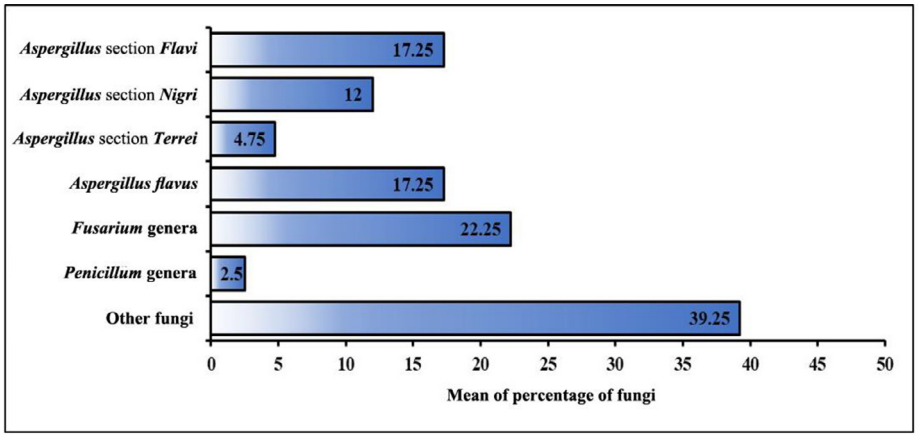

Fig. 1. Relative distribution of fungi recovered from stored maize samples.

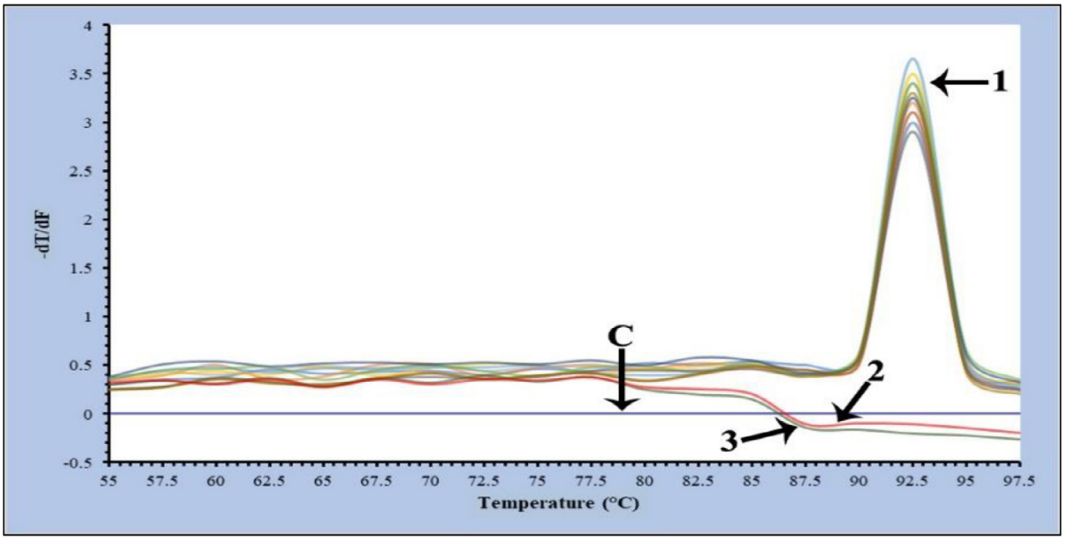

Fig. 2. Real-time PCR melting curve for detection of *A. flavus*,1: ten isolates of *A. flavus*, the melting temperature (Tm) is 92.58 °C., 2: *A. niger* 3. *A. terreus*, 3: C, negative control.

The highest value of B1 97.2 μg/mL was showed by from isolate AsF_mR36, and lowest amount of B1 23.2 μg/mL showed by AsF_mR34. Four isolates were very high B1 producers more than 60 μg/mL. All A. flavus isolates displayed a harmonious between HPLC analysis and aflatoxigenic or non-aflatoxigenic profile.

3.5. Molecular analysis with PCR and RT-PCR

The findings of the PCR revealed that six genes, *aflD*, *aflM*, *aflO*, *aflP*, *aflQ* and *aflR* were found in either aflatoxigenic or non-aflatoxigenic isolates and had a non-diverse at PCR model (data not shown). Consequently, to differentiate between aflatoxigenic and non-aflatoxigenic isolates, such a PCR pattern would not be found useful. A. flavus isolates were analyzed by RT-PCR (gene expression), showing a more diverse pattern and relationship to their ability to produce AB1 (Table 6 and Fig. 3). Some gene the expression of the AF-genes was strongly correlated with AF production. We also categorized aflatoxigenic isolates into two types: type I produced AFB1 in amount of $\geq$ 30 ng/mg fungal dry weight and type II $\leq$ 30 ng/mg fungal dry weight. *AflQ* expression was pos-

Table 3
Morphological characterization of isolates of A. flavus.

Morphotype	n (%)	Sclerotia[a]			Conidia[b]	Colony color[c]	Colony diameter (cm)[d]	
		No	L-type	S-type			1–2	3–4
I	6 (20.7)	+	–	–	r	g	+	–
II	7 (24.1)	+	–	–	r	dg	–	+
III	3 (10.3)	+	–	–	r	dg	+	–
IV	7 (24.1)	–	+	–	r	dg	–	+
V	2 (6.9)	–	+	–	r	dg	+	–
VI	4 (13.8)	–	–	+	r	dg	–	+

+ present, - absence.
[a] absence, L-type sclerotium, and S-type sclerotium.
[b] r: rough.
[c] g: green, d: dark green.
[d] Average of 3 colonies, in cm; Czapek agar.

Table 4
Detection of aflatoxigenic and non aflatoxigenic *A. flavus* isolates by qualitative methods.

No.	Method	Number of isolates		Accuracy to HPLC in identifying the aflatoxigenic isolate
		Aflatoxigenic	Non-aflatoxigenic	
1	UV test	9	20	81.8
2	Yellow pigmentation	7	22	63.6
3	Ammonia vapor test	7	22	63.6
4	HPLC	11	18	100

Table 5
chemotypes of A. flavus isolates based on mycotoxigenic profile by HPLC.

Chemotype	Mycotoxins		Number of isolates of each chemotype (%)
	AfB1	AFB2	
I	+	–	9 (31%)
II	+	+	2 (7%)
III	–	–	18 (62%)

itive in all aflatoxigenic isolates (100%), and aflO expression was positive in 10 aflatoxigenic isolates (90%). *AflD*, *aflM* and aflP expression were positive in 18%, 36% and 45% of aflatoxigenic isolates, respectively. Percent amplification of genes by RT-PCR shown in Fig. 4. *AflR* was the highest percent amplification, there was a total of 29 (100%) positive amplifications *aflD* was the lowest level amplification, with 10 (34.5%) positive amplifications. Positive amplifications of aflM (41.4%) and *aflO* (34.5%), *aflP* (48.3%) and *aflQ* (41.4%).

3.6. Gene expression analysis by qRT-PCR

In this analysis, the AF-genes are divided as early pathway genes (*aflD* and *aflR*), middle pathway genes (*aflM*), and late pathway genes (*aflO*, *aflP*, *aflQ*). There was more variation in the expression pattern of the AF-genes. The findings revealed that the defect/deletion was clear in most of the non-aflatoxigenic isolates in the middle and late stages of aflatoxin synthesis genes Fig. 5. In comparison, in most of the genomic DNA of non-aflatoxigenic A. flavus were amplified by the same primers effectively.

4. Discussion

In Saudi Arabia, climatic conditions are very hot and humid and inadequate storage practices provide significant isolation of various seed-borne fungi are associated with maize grains. In our previous study (Mahmoud et al., 2014), *A. flavus*, *Fusarium*, *Penicillium*, and *Alternaria* species were the major fungal most commonly isolated from maize grains. In the present analysis, Isolation of various species of fungi from maize grains could be assigned to (1) long-term preservation of grains in an adequate climate for the occurrence of moldy (2) ideal nutrient composition of maize grains, making it a very strong fungal substratum (3) During the processing of drying maize grains as well as transport between countries, mechanical damage occurred (Mahmoud et al., 2014).

Real-time PCR technology opens up developing opportunities to identify and study phytopathogenic fungi. it combines the sensitivity of traditional PCR with the production of a unique fluorescent signal, providing a real-time study of reaction kinetics and enabling qualitative and quantitative of specific DNA targets (Schena et al., 2004). RT-PCR enables an accurate, reliable, and high specific of target fungal DNA in mycotoxigenic fungi like *A. flavus* (Mahmoud, 2015), *Fusarium* (Nicolaisen et al., 2009), and *Penicillium* (Rodrigues et al., 2011) in food or feed products.

In the present study, we aimed to identify and characterize 29 isolates *A. flavus*. Morphological differentiation of *A. flavus* isolates belonging to maize grains may be few but it is difficult to define, due to interspecific significant similarities. Phenotypic characterization (morphological characteristics) is similar and subjective and dubious results limit accurate identification. In general, morphological characteristics are also commonly used to classify *Aspergillus* as this approach is important to categorize the isolates by similar groups, which allows used further methods to confirm identification. Morphological and biochemical characterization are usually complemented with a mycotoxigenic pattern of AFs to get more accurate identification (Rodrigues et al., 2009; Norlia et al., 2018).

Many scholars have attempted to establish a link between the capacity to generate sclerotia and the development of AF, but published evidence is paradoxical. There is a strong association between the presence of small sclerotia and high-level production of AF in different studies (Novas and Cabral, 2002; Pildain et al., 2004) while other researchers reported no relationship between sclerotial production or size and AF production [18,40] or an even a reverse linkage, with large type isolates being the most aflatoxigenic (Abbas et al., 2005). Results can be contradictory because authors have not been establishing a standardized method for fungal growth conditions and media for this purpose (Rodrigues et al., 2009).

Table 6
Presence of Genes aflD, aflM aflO, aflP, and aflQ and aflR PCR) gene Expression (RT-PCR) in Aspergillus flavus Isolates.

No.	Isolate code	Aflatoxin B1 (HPLC; ng/mg mycelia dry weight)[a]	Type[b]	Gene expression					
				aflD	aflM	aflO	aflP	aflQ	aflR
1	AsF-mR11	ND[d]	–	+	+	–	+	–	+
2	AsF-mR12	ND	–	+	+	–	+	+	+
3	AsF-mR13	ND	–	–	–	–	–	–	+
4	AsF-mR14	53.8	+	–	+	+	+	+	+
5	AsF-mR15	76.1[I]	+	+	+	+	–	+	+
6	AsF-mR16	ND	–	–	+	–	+	–	+
7	AsF-mR17	34.5	+	–	–	+	–	+	+
8	AsF-mR18	ND	–	+	–	–	–	–	+
9	AsF-mR19	ND	–	+	–	–	+	–	+
10	AsF-mR20	57.4	+	+	–	+	+	+	+
11	AsF-mR21	ND	–	–	+	–	+	–	+
12	AsF-mR22	ND	–	+	–	–	–	–	+
13	AsF-mR23	23.2	+	–	–	+	+	+	+
14	AsF-mR24	ND	–	–	–	–	–	–	+
15	AsF-mR25	27.7	+	–	+	+	–	+	+
16	AsF-mR26	ND	–	–	+	–	–	–	+
17	AsF-mR27	ND	–	–	–	–	–	–	+
18	AsF-mR28	ND	–	+	–	–	+	–	+
19	AsF-mR29	ND	–	+	+	–	+	–	+
20	AsF-mR30	ND	–	+	–	–	–	–	+
21	AsF-mR31	ND	–	–	–	–	–	–	+
22	AsF-mR32	94.7 [I]	+	–	–	+	+	+	+
23	AsFmR33	50.9	+	–	–	+	–	+	+
24	AsF-mR34	36.4	+	–	–	+	+	+	+
25	AsF-mR35	ND	–	–	–	–	+	–	+
26	AsF-mR36	97.2 [I]	+	–	+	–	–	+	+
27	AsF-mR37	63.8 [I]	+	+	+	+	+	+	+
28	AsF-mR38	ND	–	–	–	–	–	–	+
29	AsF-mR39	ND	–	–	+	–	–	–	+

[c]A. flavus isolates type II were producing AFB1 in amounts ≤ 60.
[a] Fungal culture were grown in YES medium for four days at 28 °C.
[b] *A. flavus* isolates type I classified according to their ability for producing AFB1 in amounts ≥ 60 ng/ mg mycelia dry weight.
[d] ND = Not detected.

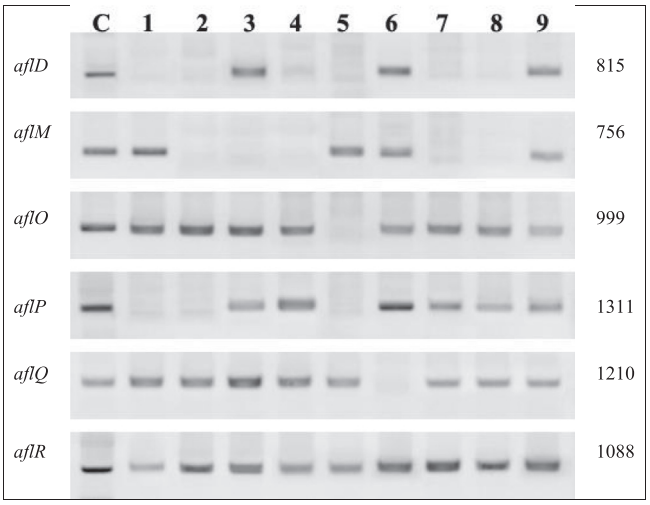

Fig. 3. RT-PCR amplification of 9 RNA of aflatoxigenic *A. flavus* isolates using specific primers of six genes of AF biosynthesis, including *aflD*, *aflM*, *aflO*, *aflP*, *aflQ* and *aflR*, C: Positive control, lane 1: AsFmR14, lane 2: AsFmR15, lane 3: AsFmR17, lane 4 : AsFmR20, lane 5: AsFmR23, lane 6: AsFmR25, lane 7: AsFmR32, lane 8: AsFmR33 and lane 9: AsFmR36.

CAM fluorescence did not allow unequivocal discrimination of aflatoxigenic from non-aflatoxigenic *A. flavus* isolates, leading to false positives or false negatives in some of the tested isolates. Nonetheless but, CAM fluorescence a simple method for a fast screening for the isolates were potentially aflatoxigenic. Many authors have stated that CAM fluorescence is not reliably consistent with HPLC aflatoxin detection (Scherm et al., 2005; Giorni et al., 2007). Some authors have another opinion, detection of AF production by CAM fluorescence showed a good correlation with the HPLC results (Rodrigues et al., 2009; Norlia et al, 2018). Qualitative techniques like CAM fluorescence, ammonium vapor yellow pigment for the detection of AF production by *A. flavus* have been stated not to be reliable or accurate. Thus, besides traditional techniques, we need to use analytical or molecular techniques (Adetunji et al., 2019). From different biological sources, 166 *A. flavus* isolates were collected and tested by yellow pigmentation. The findings of analysis reported152 isolates (92.5%) of these isolates produced AFs and 144 isolates (87%) were harmonious to HPLC analysis (Rao et al., 2020). *A. flavus* isolates were collected from four agroclimatic zones of India and exposure cultures to ammonia vapour. 22% of isolates were highly aflatoxigenic isolates displayed plum red color, 12% moderately aflatoxigenic shown pink color, and 10% toxic isolates indicated to red color. The ammonia vapor test is simple, low-cost, accurate, and time-saving. The method that can be used to segregate or pre-screen contaminated samples from staple food or feedstocks (Shekhar et al., 2017). The ammonia vapor and yellow pigmentation have been color reaction is attributed only to intermediate anthraquinone metabolites such as norsolonic acid and other unique aflatoxin-related chemicals (Shier et al., 2005).

The culture methods are not as dependable as HPLC analysis, but they are easy, inexpensive. Cultural methods have been used for the detection of AFs production. They are more appropriate for the first screening with less time and fewer resources in a large population in potentially extreme A. flavus isolates. In some cases, it is not possible to depend on culture methods only, but we need a reliable method as HPLC analysis. Methods for the detection of aflatoxin production. They are most suitable for the first screening

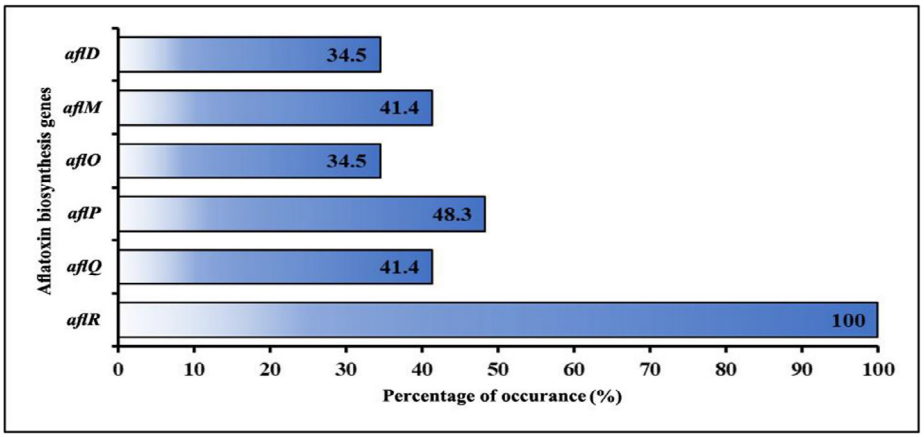

Fig. 4. Percent amplification of six aflatoxin biosynthesis pathway genes by RT-PCR in A. flavus isolates.

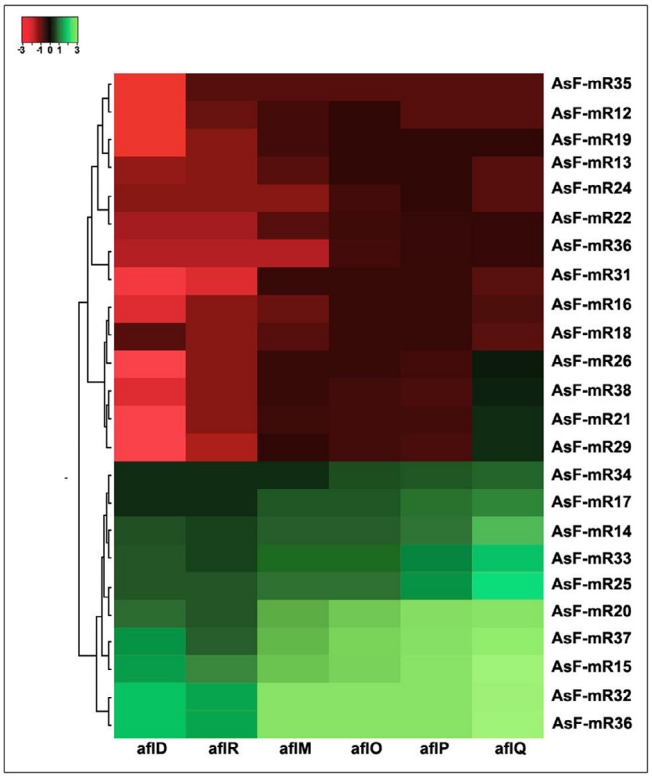

Fig. 5. Gene expression pattern of aflatoxin synthesis gene cluster depicted using heatmap.

of a large community of potentially serious Aspergillus isolates in less time and limited resources (Rao et al., 2020). The HPLC system used for detection and measurement of AFs is specific, highly sensitive, and accurate in *A. flavus* isolates and well known in many agricultural crops, such as maize (Sserumaga et al., 2020), wheat (Al-Wadai et al., 2013) peanut, (Norlia et al., 2018) and spices (El-Dawy et al., 2019).

Assessment of four AF-genes PCR, *aflD*, *aflO*, *aflP*, and *aflQ*, were did not correspond with the aflatoxigenicity profile of *A. flavus* isolates (Abdel-Hadi et al., 2011; Jamali et al., 2013; Mahmoud, 2015). Multiplex RT-PCR molecular results for 11 genes (structural and regulation) in 13 *A. flavus* isolates concluded that the best correlation for aflatoxigenicity character was for three expression genes. The expression profile of the three *aflD*, *aflO*, and aflP genes was consistently correlated with the capacity to produce AFs (Scherm et al., 2005). 31 *A. flavus* isolates were analyzed by the expression of two AF-genes, *aflD*, and *aflQ*. *AflQ* expression demonstrated a significant correlation between the expression gene and the capacity to produce AFs, but *aflD* was a failure in this assignment (Rodrigues et al., 2009). The expression of four AF-genes (*aflD*, *aflO*, *aflP*, and *aflQ*) was estimated in twenty-four *A. flavus* isolates. Transcriptional profile of *aflD* and *aflP* genes provided these genes has been not associated with AFB1 production but, the transcriptase patterns of *aflO* and *aflQ* have been set to be perfectly correlated with the values of AFB1 produced (Jamali et al., 2013). Four AF-genes *aflD*, *aflM*, *aflP*, and *aflQ* were evaluated employing RT-PCR in 22 A. flavus isolates from peanut kernels. The transcriptic analysis of *aflD* and *aflQ* displayed a helpful marker for discrimination between toxigenic and atoxigenic *A. flavus* isolates (Mahmoud, 2015). Finally, there is currently no consensus for transcription data where a single gene should be used or more than one gene to differentiate entirely between producers of AF and non-AF, however many researchers have been needed to transcription analysis for more than one gene to get fully discriminate (Abdel-Hadi et al., 2011; Jamali et al., 2013; Mahmoud, 2015).

The expression of the aflR gene was analyzed by qRT-PCR and it appeared that the expression of the aflR gene was a gradual increase in A. flavus from maize and without non-specific expression from rice grain. The findings of qRT-PCR indicate an effective molecular tool for detecting and dominating *A. flavus* distribution in grain stores but the expression gene is highly related to the crop (Imran et al., 2014). SYBR Green qPCR of three AF-genes (*aflM*, *aflR*, and *aflD*) utilized for Early detection of maize contamination by AB1. qPCR protocol founded on these genes could be developed significantly related to the detection and determination of AB1 content in food or feed samples. qPCR is an advanced, accurate, and sensitive, a method for discovering expression gene in *A. flavus* (Anong et al., 2016). Transcriptional analysis was completed by qRT-PCR by utilizing specific AF-genes aflR, *aflD* and *aflP* to control the production of AB1. aflatoxigenic A. flavus isolates exhibited a higher expression gene of *aflR* and *aflD* genes ($p < 0.05$). Our findings proposed that the expression of *aflR* and *aflD* is related to AB1 production in *A. flavus* and that overexpression of aflR could affect the toxigenic and transcriptional pattern (Baquião et al., 2016).

5. Conclusions

To classify and recognize aflatoxigenic and non-aflatoxigenic *A. flavus* isolates, a polyphasic approach involving morphological, biochemical, and molecular characterization was applied to 29 *A. flavus* isolates collected from stored maize in Riyadh City, Saudi

Arabia. In two classes, the aflatoxigenicity profile of *A. flavus* isolates by HPLC was involved, first with 38% being aflatoxigenic isolates and second with 62% being non-aflatoxigenic isolates. The expression pattern of *aflO* and *aflQ* was a beneficial marker for discriminating aflatoxigenic from non-aflatoxigenic A. flavus isolates. Also though the pathway of AF biosynthesis is complex, but a strong link has been observed between the expression of the gene and production of aflatoxin, qRT-PCR of AF-genes present an important role in the field.

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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