Swift Journals

Swift Journal of Microbiology and Biotechnology Research
Vol. 2(1) pp. 001-008 January, 2016.
http://www.swiftjournals.org/sjmbr

Original Research Article

Expression of Aflatoxin Genes Nor-1, Omt-B, Omt-A, and Ord-A to Differentiate between Aflatoxinogenic and Non-Aflatoxinogenic Strains of Aspergillus Flavus and Aspergillus Parasiticus

M. H. Moubasher, A. Abu Taleb, and H. M. Aljaeed

Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza, Egypt.

Accepted 4th January, 2016.

ABSTRACT

Eleven strains of Aspergillus flavus and Aspergillus parasiticus, were isolated from Egyptian soil and air. The isolated strains have been screened for their ability to produce aflatoxins (B1, B2, G1 and G2) in three types of media (Czapek's Dox, potato dextrose and glucose peptone yeast). Differentiation between aflatoxinogenic and non-aflatoxinogenic strains was carried out using TLC to detect B1, B2, G1 and G2. The three types of broth media differed in their potentiality to enhance aflatoxins production by the selected strains. Type of produced toxins differed according to the constituent of media and fungal species, as well as its strains. The expression of four aflatoxin biosynthetic pathway genes Nor-1 (Afl-D), Omt-B (Afl-O), Omt-A (Afl-P), and Ord-A (Afl-Q) was evaluated in the eleven strains using RT-PCR. Mostly the results of the conventional method were concomitant with that of RT-PCR. omt- B, omt- A and ord- A are important in differentiation between aflatoxigenic and nonaflatoxigenic strains.

Keywords: *aflatoxinogenic, non- aflatoxinogenic, Aspergillus flavus, Aspergillus parasiticus, RT-PCR.*

INTRODUCTION

Aflatoxins constitute a class of highly toxic, nephrotoxic, carcinogenic, teratogenic and mutagenic secondary metabolites. Aflatoxins are mycotoxins belonging to the group of polyketide-derived furanocoumarins (Šošo et al., 2014). They are polyketide-derived secondary metabolites produced via the following conversion path: acetate → polyketide → anthraquinones → xanthones → aflatoxins (Šošo et al., 2014). The genes involved in the major conversion steps from early precursors to aflatoxins and their functions are discussed by (Yu et al., 2004). Although there are at least 16 structurally different chemical compounds related to aflatoxins, 4 aflatoxins (B1, B2, G1 and G2) are the most often contaminating food and agricultural products.

The list of aflatoxin producing fungi is expanding, and several clades from toxigenic to non-toxigenic, mainly belonging to Aspergillus section Flavi, are distributed worldwide. Among the 22 closely related species in Aspergillus section Flavi, A. flavus and A. parasiticus are the most frequent aflatoxin producers encountered in agricultural products (Razzaghi-Abyaneh et al., 2006; Godet and Munaut, 2010; Varga et al., 2011). The ability of A. flavus strains to produce aflatoxins is reported to be highly variable; several strains are non-aflatoxinogenic because aflatoxin synthesis may become unstable in these fungi (Bennett and Christensen, 1983). Moreover, the production of aflatoxins is regulated by various environmental conditions (Gqaleni et al., 1997; Keller et al., 1997) and nutrient sources (Moss, 1991). Conventional procedures based on microbiological and immunological techniques to differentiate between aflatoxigenic and non aflatoxigenic strains are generally costly, time-consuming and laborious and may lead to false results. Moreover, these methods may fail to detect some aflatoxin-producing strains because of the instability of aflatoxin production may occur in certain aflatoxinogenic strains growing in culture media (Abarca et al., 1988 and Lemke et al., 1989).

Further studies are needed to develop a rapid and more objective technique that permits clear differentiation between aflatoxin-producing and non-producing strains of A. flavus and A. parasiticus. Scientists worldwide has extensively studied the biosynthesis of aflatoxins. Molecular methods have been applied in recent years with different levels of success (Degola et al., 2007, 2009; Leema et al., 2011; Mayer et al., (2003 a,b); Rodrigues et al., 2009; Scherm et al., 2005; Sweeney et al., 2000). PCR-based methods, including monomeric, quadruplex

**Corresponding Author:* Hadel Mosfer Aljaeed Department of Botany and Microbiology Faculty of ScienceGizaCairo, Egypt
Telephone: 00353838022520
Email: aljaeedh@hotmail.com

and multiplex PCR of different aflatoxin biosynthesis genes have been used (Criseo et al., 2001; Geisen, 1996; Manonmani et al., 2005; Shapira et al., 1996; Somashekar et al., 2004; Moubasher et al., 2013). However, PCR detection of aflatoxin biosynthesis genes is not always successful in distinguishing aflatoxigenic from non-aflatoxigenic strains due to inter- and intra-specific genetic mutations within the primers' targeted binding site (Levin, 2012). Criseo et al. (2001) have developed a multiplex PCR protocol to differentiate aflatoxin-producing and -nonproducing strains within the A. flavus group; Mayer et al. (2003a) have used real-time PCR to monitor the expression of an aflatoxin biosynthetic gene of A. flavus in wheat, and to compare the copy numbers of the nor-1 gene to conventional CFU data (Mayer et al., 2003b).

Somashekar et al. (2004) have unsuccessfully tried to differentiate productive from nonproductive strains using specific primers for aflR and omt genes, while other groups had already successfully used PCR to detect A. flavus in grains (Shapira et al., 1996), in figs (Farber et al., 1997) and in the soil and air (Moubasher et al., 2013). These methodologies are relatively rapid and may compete with the existing method, owing to their specificity. Sweeney et al. (2000) reported a significant correlation between aflatoxin production by A. parasiticus 439 and expression of two genes, aflR and aflQ, as determined by RT-PCR in aflatoxins conducive and non-conducive media. Expression of aflD, aflO, aflQ and aflP genes also provided useful differentiation of aflatoxigenic and non-aflatoxigenic strains of A. flavus and A. parasiticus (Rodrigues et al., 2009; Scherm et al., 2005). Multiplex RTPCR for genes aflQ (ord A), aflO (omt B), aflD, aflS, and aflR also showed a good correlation between aflatoxin gene expression and aflatoxin production (Degola et al., 2007; 2009). Mayer et al. (2003 a; b) indicated that significantly higher mean expression levels occurred in aflatoxigenic than in non-aflatoxigenic corneal isolates.

The goal of the present work is to differentiate between aflatoxinogenic and non-aflatoxinogenic strains of A. flavus and A. parasiticus using both conventional and RT-PCR methods.

MATERIALS AND METHODS

Aspergillus isolates

Eleven isolates of A. flavus and A. parasiticus were isolated from Egyptian soil, and air (Moubasher et al., 2013). The isolates were identified according to Raper and Fennel (1973). Identification was confirmed by the Assiut University Mycological Centre (AUMC),(Table3).

Aflatoxin production

Three types of media were used in this study; Czapek's Dox (CD), potato dextrose agar (PDA) and glucose peptone yeast (GPY). Spore suspension (100 µl) of each isolate containing 107 spores/ml, prepared in 0.1% (vol/vol) Tween 20, was added to 100 ml of each tested medium in 250-ml Erlenmeyer flasks. After inoculation, the flasks were incubated on a rotary shaker at 26°C for 7 days (Moubasher et al., 2013).

Extraction, purification and detection of aflatoxin by TLC method

The aflatoxin extraction was carried out according to Soares and Rodriguez (1989). For the determination of aflatoxins production, the mycelial mat was harvested by filtering through Whatman no. 1 filter paper. The filtrate was extracted twice with 100 ml chloroform in a separating funnel; the chloroform layer was filtered through the anhydrous sodium sulfate. The chloroform extracts were combined and evaporated to dryness; hexane was added to remove fatty acids and non-polar compounds from the residue, which was re-dissolved in 1 ml chloroform. Pre-coated silica gel TLC plates (20x20) (Sigma) were used for the aflatoxin detection. Fifty microliters of each sample was spotted onto the TLC sheets. TLC was developed in toluene: ethyl acetate: acetic acid (50: 30: 4) solvent system. Authentic samples of aflatoxins (Sigma) were used as standard. Aflatoxins were visualized under a UV lamp at 365 nm and their presence was confirmed by spraying and with 50% sulphuric acid on the developed plates which reacts with the blue and green fluorescent aflatoxins to give yellow fluorescent derivatives.

Detection of Aflatoxins by (RT- PCR)
Fungal RNA Extraction

Total RNAs were extracted from 250 mg of the harvested mycelia, which was frozen in liquid N2 and ground in a mortar, according to the protocol recommended for the GeneJET RNA Purification Kit #K0731.

RT-PCR amplification

One step RT-PCR THERMO # K0801 was used. Two primers were used separately during the PCR reaction for each isolate (Nor 1+ Omt B) and (Omt 1 + Ord 1). Reverse transcriptase PCR-RT was performed using oligo-dT primers to amplify mRNA. The reaction was assembled in a 20 µl tube as follows: 1 µM Oligo(dT) primer, 1 x reaction buffer, 4U of Reverse Transcriptase, 2 µM dNTPs, 10 U RNase inhibitor , and 40 ng RNA sample in 12 µL H2O (RNase free). The mixtures were incubated at 42 °C for 60 min followed by 94 °C for 5 min in a thermal cycler (Perkin Elmer Gene Amp PCR System 2400), followed by rapid cooling on ice. Each 25 µl PCR reaction contained 700 µM dNTP, 1 x reaction buffer , 1 U Taq polymerase I, 0.2 µM of each primer, 1 µl cDNA mixture, 12 µL H2O (RNase and DNase free).

RESULTS AND DISCUSSION

The conventional methods used to differentiate between aflatoxin-producing and non-producing strains of A. flavus and A. parasiticus, are based on growing the selected isolates on natural and/or artificial media, then toxins production was detected using TLC (Criso et al., 2001; Moubasher et al., 2013). Aflatoxins production (B1, B2, G1 and G2)in growth media (CD, PD and GPY)using the conventional method,usuallydifferentiated the selected strains into two groups, aflatoxigenic and non-aflatoxigenic strains (Table 4).The following strains were nonaflatoxin producers when grown in any one of the selected media: A.flavusvar.columnaisstrains AUMC (9023and 9026) and A. flavusstrain AUMC(9028). The other strains were aflatoxin producers, but their production is affected by broth type as shown in A.flavusvar.columnaris strains AUMC (9022, 9024, 9025, 9030 and 9031), A. flavusstrain AUMC 9027 and A. parasiticusstrains AUMC (9029 and 9032).TLC usually has shown a clear differentiation between aflatoxin-producing and non-producing strains of A. flavus var.columnais, A. flavus and A. parasiticus. This finding is in agreement with that of (Criso et al., 2001; Moubasher et al., 2013).

Table 1: Sequence of primers and size of RT-PCR

Primer Name	Sequence	Size
Nor1-F (AflD)	ACGGATCACTTAGCCAGCAC (20)	812
Nor1-R	CTACCAGGGGAGTTGAGATCC (21)	
OmtB-F (AflO)	GCCTTGACATGGCCTGCTCTTTA (20)	1131
OmtB-R	CCAAGATGGCCTGCTCTTTA (20)	
OmtA-F(AflP)	GCCTTGCAAACACACTTTCA (20)	1210
OmtA-R	AGTTGTTGAACGCCCCAGT (19)	
OrdA-F(AflQ)	TTAAGGCAGCGGAATACAAG (20)	1088
OrdA-R	GACGCCCAAAGCCGAACACAAA (22)	

Table 2: PCR cycles

Cycle step	Temperature ºC	Duration	No. of cycles
c-DNA (deactivate RTase)	50	30 min	1
Initial Denaturation	95	5 min	1
Denaturation	94	30 sec	40 cycle
Annealing	55	60 sec	
Extension	72	1.5 min	
Final extension	72	7min	1
Incubation	4	∞	

Table (4): RT-PCR results and aflatoxin production in growth media: Czapek's Dox (CD), Potato Dextrose (PDA) and glucose peptone yeast (GPY).

Isolates number	RT-PCR CD	TLC CD				RT-PCR PD	TLC PD				RT-PCR GPY	TLC GPY			
		B1	G1	B2	G2		B1	G1	B2	G2		B1	G1	B2	G2
A. flavus var. *columnaris* AUMC 9022	-	-	-	-	-	+	-	-	+	+	-	-/+	-	-	-
A. flavus var. *columnaris* AUMC 9023	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. flavus var. *columnaris* AUMC 9024	-	-	-	-/+	-/+	+	-	+	-	+	+	+	+	+	+
A. flavus var. *columnaris* AUMC 9025	+	+/-	-	+	+	-	-	-	-	-	+	+	+/-	-	-
A. flavus var. *columnaris* AUMC 9026	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. flavus AUMC 9027	-	-	-	-	-	-	-	-	-	-	+	+/-	+/-	-/+	-/+
A. flavus AUMC 9028	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. parasiticus AUMC 9029	-	-/+	-	-	-	+	+	+	+	+	+	+	+	+	+
A. flavus var. *columnaris* AUMC 9030	-	-/+	-/+	-	-	+	+/-	+	+	+	-	-/+ vl	-/+	-	-/+
A. flavus var. *columnaris* AUMC 9031	+	-	-	+/-	+/-	+	-	-/+ vl	-/+	-/+	-	-	-	-	-
A. parasiticus AUMC 9032	+	+	+/-	-	-	nd	-	-	-	-	+	+	+	-	-/+

Czapek's Dox, CD; Potato dextrose agar, PDA; and Glucose peptone Yeast, GPY; low +/–; very low –/+.
RY-PCR results: - incomplete pattern; + complete pattern
TLC results: -, absence of aflatoxin; +, presence of aflatoxin; +/-, low aflatoxin ; –/+, very low aflatoxin

The three types of broth media differed in their potentiality to enhance aflatoxins production by the selected strains, GPY was the best broth followed by PD and CD, where they induced 5, 4 and 3 strains respectively, to produce aflatoxins. Moubasher et al. (2013) observed the production of aflatoxins in three types of broth (Malt Glucose, Czapek's Dox and Yeast Extract Sucrose) by different strains of A.flavus and A. parasiticus. The authors found that the lowest number of aflatoxin producers was in CD broth, which is in agreement with our results.

Potato dextrose agar (PDA), yeast extract sucrose (YES) broth and white corn grains were suitable for production of aflatoxins and trichothecenes by different Aspergillus and Fusarium strains, respectively (Abu Taleb et al., 2012). Our results proved that type of produced toxins differed according to the constituent of media and fungal species, as well as its strains, which are similar to that of (Abu Taleb et al., 2012). Lacey (1989) reported that fungal growth and production of mycotoxins were dependent upon the physical and chemical properties of growth media. Reddy et al. (1979) concluded that supplementation of growth media with yeast extract, casamino acids, casitone and peptone increased aflatoxin yield.

It was concluded that genes involved in the aflatoxin biosynthetic pathway may form the basis for an accurate, sensitive, and the specific detection system, using PCR, for aflatoxigenic strains (Shapira et al., 1996). DNA extracted from mycelia of the selected strains was used as PCR template for each of the primer pairs. DNA sequences of aflR, nor-1, ver-1, and omt-A genes of the aflatoxin biosynthetic pathway have been done (Moubasher et al., 2013). They reported that non aflatoxinogenic strains gave varying results with two, three, or four banding patterns. Complete banding pattern of DNA in some non aflatoxinogenic strains resulted in non-differentiation between aflatoxin and non-aflatoxin producers by PCR only, so in this work we followed genes expression in the selected strains through RT-PCR.

In our investigation a detection system based on reverse transcription PCR (RT-PCR) had been developed to monitor aflatoxin genes expression in A. flavus and A.parasiticus strains. Multiplex PCR was developed using four sets of primer

enclosed in the aflatoxins biosynthetic pathway {norsolorinic acid nor-1 (Afl-D), O-methyltransferase B omt- B (Afl-O), O-methyltransferase I omt-A ((Afl-P)) and oxidoreductaseAord-A (Afl-Q) genes}. RT–PCR was used to detect transcriptional activation (expression) of the previous genes in the selected strains when grown in the three types of broth. (CD, PD and GPY). Bands of the fragments of nor-1, omt-B, omt-A and ord-A genes can be visualized at812, 1131, 1210 and 1088bp, respectively (Fig. 1).

Fig. 1: Gel electrophoresis Quadruplex PCR band for A. flavus and A. parasiticus strainns

Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion path: acetate → polyketide → anthraquinones → xanthones → aflatoxins. Biosynthetic pathway of aflatoxin production involves around 15 different intermediary compounds (Šošo et al., 2014). The aflD (nor-1) gene has a role in monitoring the biosynthetic direction of aflatoxin in A. flavus and A. parasiticus. In A.flavus and A. parasiticus the expression of nor-1, a gene encoding an enzyme that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin (Sambrook and Russel, 2001; Rodríguez, 2012) is a key structural gene in the biosynthetic pathway. The aflO (omtB, dmtA) is involved in the conversion of demethylsterigmatocystin (DMST) and demethyl dihydrosterigmatocystin (DMDHST) to sterigmatocystin (ST) and dihydrosterigmatocystin (DHST), respectively (Yabe et al., 1989). It was found that aflP (omtA) is involved in the conversion of ST to O-methylsterigmatocystin (OMST) and DHST to dihydro-O-methylsterigmatocystin (DHOMST). Finally, aflQ (ordA) is involved in the conversion of OMST to AFB1 and AFG1 and of DMDHST to AFB2 and AFG2 (Yabe et al., 1988; Cleveland, 1989; Bhatnagar et al., 1991).

The results of the conventional method were concomitant with that of RT-PCR in most strains (aflatoxigenic strains expressed four selected genes and showed a complete pattern), when grown in CD, PD or GPY (Table 5 and Fig. 1). However, A.flavus var.columnais strains AUMC 9022 in GPY broth (very low amount of B1 and single pattern of nor- 1 only appeared), 9024 in CD broth (very low amount of B2 and G2 and no bands appeared) and 9030 in the same broth and GYP broth (very low amount of B1, G1and G2 and single band of nor- 1 only appeared) (Tables 4 and 5). It is important to remember that in spite of A.flavus var.columnais strain AUMC 9031expressed the four genes in PD broth, the organism failed to synthesis aflatoxin B1and the production of aflatoxinsB2, G1 and G2was very low (Tables 4 and 5)

It is impossible to synthesis aflatoxin when the selected strains expressed only one gene (nor-1) without the other genes included in aflatoxin biosynthesis pathway (omt-B, omt-A and ord-A. The detection of very low amount of aflatoxin in the absence of quadruplet complete pattern of the four genes, may be attributed to the presence of these genes in very low amounts below the detection level. This result is in agreement with Jamali et al. (2012)

Accinelli et al. (2008) analyzed expression of five aflatoxin genes (aflD, aflG, aflP, flR,andaflS) by RT-PCR. They did not find a correlation between gene expression profiles of aflatoxigenicA. flavus isolates and aflatoxinB1 concentrations in the soil. Although aflD was the most frequently expressed gene, its expression was not detected in two of the 10 samples that contained aflatoxigenicA. flavus isolates. Jamali et al. (2012) found that aflatoxin genes were not expressed when grown in YEP, while transcripts of housekeeping gene tub1 were detected from cultures grown in both YES and YEP.

Our results showed that the expression patterns of the selected genes varied with variation in broth constituents and fungal strains. Abdel-Hadi et al. (2010, 2011) stated that that the expression patterns of aflD were related to changing water activity in stored peanuts.

On the other hand, the correlation between aflatoxins production and RT-PCR results in this work has been confirmed by many authors. Sweeney et al. (2000) observed a significant correlation between aflatoxin production by A. parasiticus 439 and expression of two genes, aflR and aflQ, as determined by RT-PCR in aflatoxins conducive and non-conducive media. Mayer et al. (2003) showed that aflD mRNA levels in wheat artificially contaminated with an aflatoxins producing A. flavus isolate correlated well with the growth kinetics of the fungus and aflatoxinB1 production.

During this investigation, A.flavus var.columnais strains AUMC 9025 and 9031, and A. parasiticusstrainsAUMC9032 showed a complete quadruplet pattern when grown in CD broth (Table 5 and Fig. 2). This result indicated the expression of the four selected genes in aflatoxins biosynthetic pathway, which confirmed the positive data of TLC. A.flavus var.columnais strains AUMC 9022, 9024 and 9030 and A. parasiticus strains AUMC 9029, when grown in PD showed also a quadruplet complete pattern (Fig. 2) Which proved TLC results. Also in case of GPY A.flavus var.columnais strainsAUMC 9024 and 9025, A.flavus isolate AUMC 9027 and A. parasiticus strains AUMC 9029 and 9032, four bands of the previous genes were observed (Fig. 2).Our results are in agreement with that of (Rodrigues et al., 2009; Scherm et al., 2005) who concluded that the expression of aflD, aflO,aflQ and aflP genes provided useful differentiation of aflatoxigenic and non-aflatoxigenic strains of A. flavusand A. parasiticus. Multiplex RT-PCR for genes aflQ, aflO, aflD, aflS, and aflRalso showed a good correlation between aflatoxin gene expression and aflatoxin production (Degola et al., 2007, 2009).

Studies by Abdel-Hadi et al. (2010, 2011) showed the potential use of nor-1 transcription as a good marker to discriminate between aflatoxigenic and non-aflatoxigenic strains. Also (Iheanacho et al., 2014) concluded that the expression of the nor-1 gene is the main factor responsible for aflatoxins production. However, these findings are in disagreement with our results, asnor-1 was present in several non-aflatoxigenic strains such as: AUMC (9022,9023, 9026, 9029 and 9030 in CD; 9026, 9028 and 9031 in PD; 9022, 9023, 9026,9028 and 9030 and 9031 in GPY), (Table 5). When nor-1 was expressed by some strains in CD, PD and GPY broth, 37.5, 57.1 and 45.5 %, respectively, of these strains produced aflatoxins Many isolates of A. flavus strains are not aflatoxigenic due to the appearance of mutations in one or more genes belonging to the biosynthetic gene cluster (Degola et al., 2007).

It was found that expression of nor- 1 in semisynthetic media was higher than that in synthetic medium. The expression of the majority of aflatoxin biosynthetic genes, including aflR and aflS of all strains varied with regard to the aflatoxin-producing ability and the growth conditions (Scherm et al., 2005). Aflatoxin production could be disrupted if any step in the aflatoxin biosynthetic pathway is completely blocked by a specific inhibitor. Disruption or deletion of the aflD (nor-1) gene leads to the accumulation of norsolorinic acid and blocks the synthesis of all aflatoxins and their intermediates beyond norsolorinic acid (Dheda et al., 2004). However, 100, 80 and 100% of stains which expressed omt- B, omt- A and ord- A were able to synthesize the target toxin when these aflatoxigenic strains were grown in CD, PD and GPY respectively. In this case synthetic and semisynthetic media

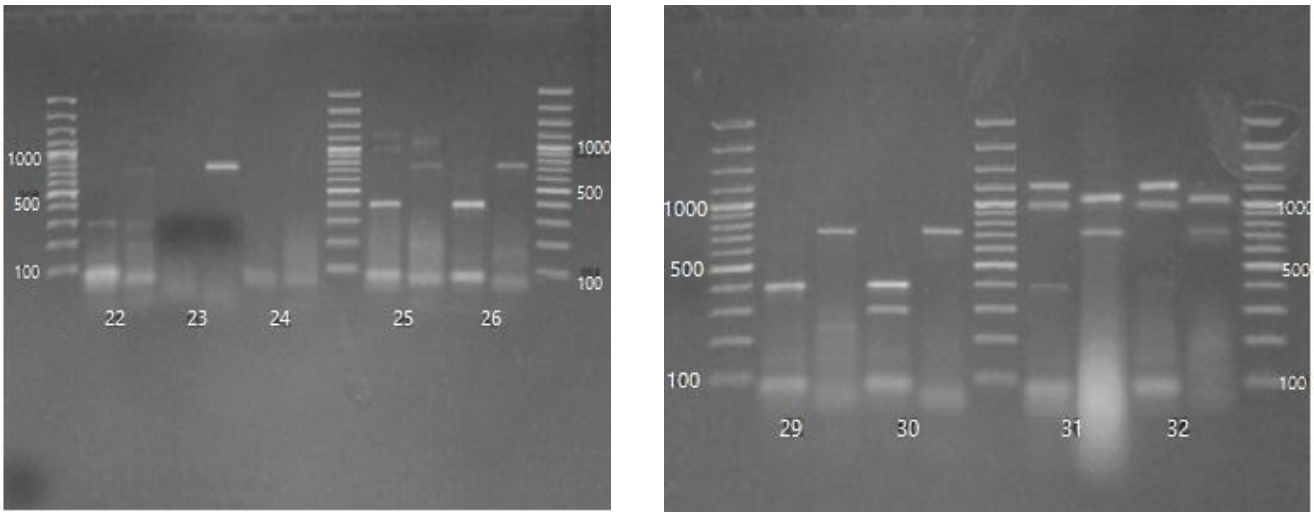
highly activated expression of omt- B, omt- A and ord- A (Table 5 and Fig. 2). These results indicated that these three genes are important in differentiation between aflatoxigenic and nonaflatoxigenic strains. Scherm et al. (2005) concluded that the expression profile of the three genes aflD, aflO, and aflP varied with regard to the aflatoxin-producing ability and the growth conditions including types of media.

Indeed, RT-PCR enables the study of gene expression by allowing the detection of mRNAs transcribed by specific genes owing to PCR amplification of cDNA intermediates synthesized by reverse transcription. The presence or lack of mRNAs could permit direct differentiation between aflatoxinogenic and non-aflatoxinogenic strains. In addition, several specific mRNAs may be detected simultaneously in a single RNA sample by multiplex RT-PCR, with the advantage of having a unique response to the expression of several genes enclosed in the aflatoxin biosynthetic pathway (Criseo et al., 2001)

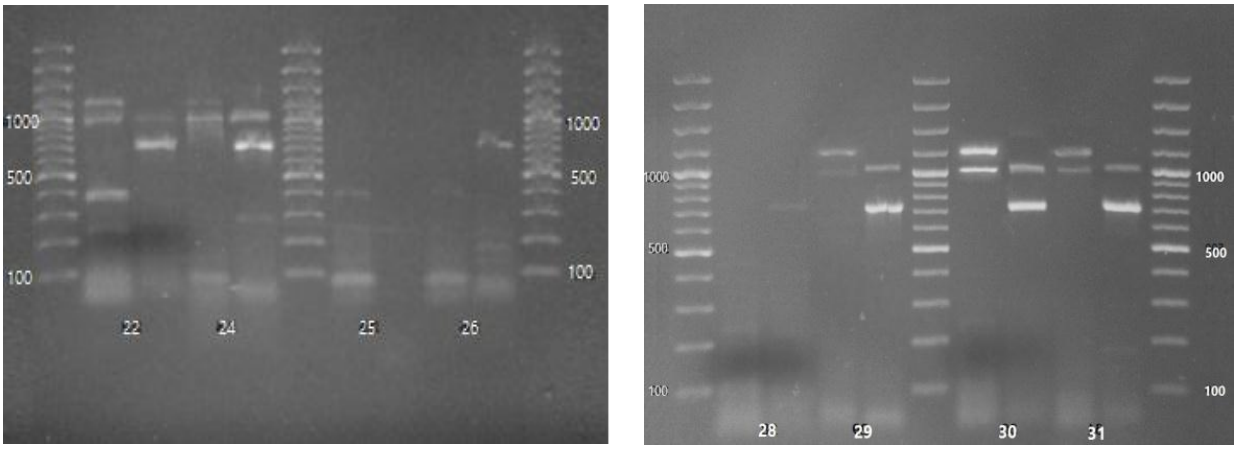
Table 5: Genes expression and aflatoxins production in Czapek's Dox (CD), Potato Dextrose (PD) and glucose peptone yeast (GPY) by different strains of *A. flavus* and *A. parasiticus*

Strains AUMC	*Nor- 1*/ complete pattern/TLC			*Omt- B*/ complete pattern /TLC			*Omt- A*/ complete pattern /TLC			*Ord A*/ complete pattern /TLC		
	CD 8/3/3	PD 7/5/4	GPY 11/5/5	CD 3/3/3	PD 5/5/4	GPY 5/5/5	CD 3/3/3	PD 5/5/4	GPY 5/5/5	CD 3/3/3	PD 5/5/4	GPY 5/5/5
9022	+/-/-	+/+/+	+/-/vl	-/-/--	+/+/+	-/-/vl	-/-/--	+/+/+	-/-/vl	-/-/--	+/+/+	-/-/vl
9023	+/-/-	-/-/--	+/-/-	-/-/--	-/-/--	-/-/--	-/-/--	-/-/--	-/-/-	-/-/--	-/-/--	-/-/--
9024	-/-/vl	+/+/+	+/+/+	-/-/vl	+/+/+	+/+/+	-/-/vl	+/+/+	+/+/+	-/-/vl	+/+/+	+/+/+
9025	+/+/+	-/-/--	+/+/+	+/+/+	-/-/--	+/+/+	+/+/+	-/-/--	+/+/+	+/+/+	-/-/--	+/+/+
9026	+/-/-	+/-/-	+/-/-	-/-/--	-/-/--	-/-/--	-/-/--	-/-/--	-/-/--	-/-/--	-/-/--	-/-/--
9027	-/-/--	-/-/--	+/+/+	-/-/--	-/-/--	+/+/+	-/-/--	-/-/--	+/+/+	-/-/--	-/-/--	+/+/+
9028	-/-/--	+/-/-	+/-/-	-/-/--	-/-/--	-/-/--	-/-/--	-/-/-	-/-/--	-/-/--	-/-.--	-/-/--
9029	+/-/vl	+/+/+	+/+/+	-/-/vl	+/+/+	+/+/+	-/-/vl	+/+/+	+/+.+	-/-/vl	+/+/+	+/+/+
9030	+/-/vl	+/+/+	+/-/vl	-/-/vl	+/+/+	-/-/vl	-/-/vl	+/+/+	-/-/vl	-/-/vl	+/+/+	-/-/vl
9031	+/+/+	+/+/vl	+/-/-	+/+/+	+/+/vl	-/-/--	+/+/+	+/+/vl	-/-/--	+/+/+	+/+/vl	-/-/--
9032	+/+/+	-/nd/-	+/+/+	+/+/+	/nd/--	+/+/+	+/+/+	-/nd./-	+/+/+	+/+/+	/nd/--	+/+/+

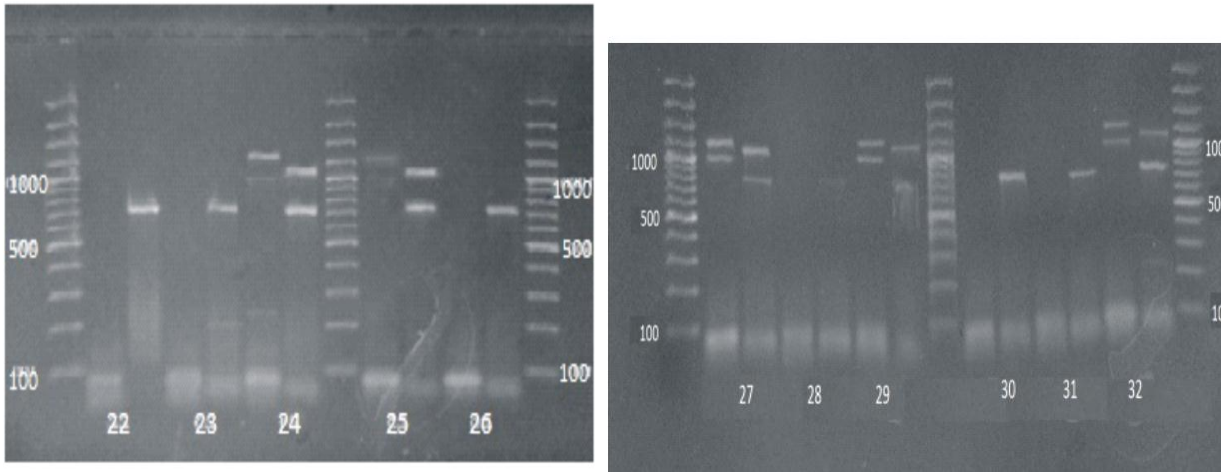
+, detected; -, not detected; +/- low; vl, very low



A: Czapek's Dox medium



B: Potato dextrose agar medium



C: glucose peptone yeast medium

Fig 1: Gel electrophoresis Quadruplex PCR band for *A. flavus* and *A. parasiticus strains*

REFERENCES

Abarca ML, Bragulat MR, Bruguera MT, Cabanes, FJ (1988). Comparison of some screening methods for aflatoxigenic moulds. Mycopathol., 104: 75–79.

Abdel-Hadi A, Carter D, Magan N (2010). Temporal monitoring of the nor-1 (aflD) gene of Aspergillus flavus in relation to aflatoxin B1 production during storage of peanuts under different environmental conditions. J. Appl. Microbiol. 109, 1914–1922 10.1111/j.1365-2672.2010.04820.x

Abdel-Hadi A, Carter D, Magan N (2011). Discrimination between aflatoxigenic and non-aflatoxigenic strains of Aspergillus section flavi group contaminating Egyptian peanuts using molecular and analytical techniques.World Mycotoxin J. 4:69–77. doi: 10.3920/WMJ2010.1223.

Abu-Taleb A M, Al-Julif M Z, Al-Arjani AF (2012). Toxigenic fungi isolated from some food commodities and their pyhtotoxicity. Egypt. J. Exp. Biol. (Bot.), 8(1): 141 – 149.

Accinelli C, Abbas HK, Zablotowicz RM, Wilkinson JR (2008). Aspergillus flavus aflatoxin occurrence and expression of aflatoxin biosynthesis genes in soil. Can.J. Microbiol. 54, 371e379.

Bennett J W, and Christensen SB (1983). New perspectives on aflatoxin biosynthesis. Adv. Appl. Microbiol., 29: 53–92.

Bhatnagar D, Cleveland TE, Kingston DGI (1991). Enzymological evidence for separate pathways for aflatoxin B1 and B2 biosynthesis. Biochemistry 30:4343-4350.

Criseo G, Bagnara A, Bisignano G (2001). Differentiation of aflatoxin-producing and non-producing strains of Aspergillus flavus group. Letters in Applied Microbiology. 33(4):291-295.

Degola F, Berni E, Dall'Asta C, Spotti E, Marchelli R, Ferrero I, Restivo FM (2007). A multiplex RT-PCR approach to detect aflatoxigenic strains of Aspergillus flavus. J Appl Microbiol 203:409–427.

Degola F, Berni E, Spotti E, Ferrero I, Restivo FM (2009). Facing the problem of "false positives". Re-assessment and improvement of a multiplex RT-PCR procedure for the diagnosis of A. flavus mycotoxin producers. Int. J. Food Microbiol. 129, 300e305.

Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques, 371, pp. 112–114

Färber P, Geisen R, Holzapfel W H (1997). Detection of aflatoxinogenic fungi in figs by a PCR reaction. Int. J. Food Microbiol., 36: 215–220.

Geisen R (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi, Syst. Appl. Microbiol., 19: 388–392.

Godet M. and Munaut F (2010). Molecular strategy for identification in Aspergillus section Flavi. FEMS Microbial lett, 304, 157-68.

Gqaleni N, Smith J E, Lacey J. Gettinby G (1997). Effects of temperature and water activity, and incubation time on production of aflatoxins and cyclopiazonic acid by an isolate of Aspergillus flavus in surface agar culture. Appl. Environ. Microbiol., 63: 1048–1053.

Jamali M, Ebrahimi MA, Karimipour M, Shams-Ghahfarokhi M, Dinparast- Djadid N, Kalantari S, Pilehvar-Soltanahmadi Y, Amani A, Razzaghi-Abyaneh M (2012). An insight into the distribution, genetic diversity, and mycotoxin production of Aspergillus section Flavi in soils of pistachio orchards. Folia Microbiol. 57, 27e36.

Keller NP, Nesbitt C, Sar B, Phillips TD, Burrow GB (1997). pH regulation of sterigmatocystin and aflatoxin biosynthesis in Aspergillus spp. Phytopathol., 87: 643–648.

Lacey J, (1989). Pre and post harvest ecology of fungi causing spoilage of foods and other storage product, Journal of applied bacteriology 67: 11-25.

Leema G, Chou DS, Christadoss A, Jesudasan N, Geraldine P, Thomas PA (2011). Expression of genes of the aflatoxin biosynthetic pathway in Aspergillus flavusisolates from keratitis. Molecular Vision. 17:2889-2897.

Lemke P A, Davis ND, Creech GW (1989). Direct visual detection of aflatoxin synthesis by minicolonies of Aspergillus species, Appl. Environ. Microbiol. 55:1808–1810.

Levin RE, (2012). PCR detection of aflatoxin producing fungi and its limitations. Int. J. Food Microbiol. 156, 1; 1-6.

Manonmani H K, Anand S, Chandrashekar A, Rati E R (2005). Detection of aflatoxigenic fungi in selected food commodities by PCR. Process Biochem. 40: 2859–2864.

Mayer Z, Bagnara A, Fa¨rber P, Geisen R (2003a). Monitoring the production of aflatoxin B1 in wheat by measuring the concentration of nor-1 mRNA. Appl Environ Microbiol. 69: 1154–1158.

Mayer Z, Bagnara A, Fa¨rber P, Geisen R (2003b). Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real time PCR, and its correlation to the cfu of Aspergillus flavus in foods. Int. J. Food Microbiol., 82: 143–151.

Moss M O, (1991). Economic Importance of Mycotoxins Recent Incidence. Int. Biodeterior. 27: 195–204.

Moubasher H, Abu Taleb A, and Senousy H (2013). Molecular differentiation between aflatoxinogenic and non- aflatoxinogenic strains of Aspergillus flavus and Aspergillus parasiticus. Microbiol. 82(5): 642-646.

Raper K B, Fennell DI (1973). The Genus Aspergillus, Hungtington, R.E., Ed., New York: Krieger, 1973.

Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Allameh A, Kazeroon-Shiri M, Ranjbar-Bahadori S, Mirzahoseini H, Rezaee MB (2006). A survey on distribution of Aspergillus section Flavi in corn field soils in Iran: population patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. Mycopathologia. ;161:183–192.

Reddy TV, Viswanathan L, Venkitasubramanian TA,

(1979). Factors affecting aflatoxin production by Aspergillus parasiticus in a chemically defined medium. J. Gen. Microbiol. 114, 409–413.

Rodrigues P, Venencio A, Kozakiewicz Z, Lima N (2009). A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus section Flavi isolated from Portuguese almonds. International Journal of Food Microbiology. 129:187-193.

Rodríguez A, Rodríguez M, Luque MI, Martín A, Córdoba J (2012). Real-time PCR assays for detection and quantification of aflatoxin-producing molds in foods. Food Microbiol. 31: 89-99.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual, the Third edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor. New York, 1.124-1.125, A1.27.

Scherm B, Palomba M, Serra D, Marcello A, Migheli Q (2005). Detection of transcripts of the aflatoxin genes aflD, aflO, and aflP by reverse transcriptionpolymerase chain reaction allows differentiation of aflatoxin-producing and nonproducing isolates of Aspergillus flavus and Aspergillus parasiticus. International Journal of Food Microbiology 98:201-210.

Shapira R, Paster N, Eyal O, Menasherov M, Mett A, Salomon R (1996). Detection of aflatoxigenic molds in grains by PCR. Appl. Environ. Microbiol. 62(9): 3270-3273.

Soares LM, and Rodriguez-Amaya DB (1989). Survey of aflatoxins, ochratoxin A, zearalenone, and sterigmatocystin in some Brazilian foods by using multi-toxin thin-layer chromatographic method. J. Assoc. Off. Anal. Chem., 72(1): 22-26.

Somashekar D, Rati ER, Anand S, Chandrashekar A (2004). Isolation, enumeration and PCR characterization of aflatoxigenic fungi from food and feed samples in India. Food Microbiol. 21: 809–813.

Šošo VM, Marija M, Škrinjar M M, Nevena T, Blagojev NT, Slavica M, Vesković Moračanin SM (2014). IDENTIFICATION OF AFLATOXIGENIC FUNGI USING POLYMERASE CHAIN REACTION-BASED ASSAY. Original scientific paper. 45, 259-269

Sweeney M J, Pamies P, Dobson ADW (2000). The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in Aspergillus parasiticus 439. Int. J. Food Micriol. 56(1): 97-103.

Varga J, Frisvad JC, and Samson RA (2011). Two new aflatoxin producing species, and an overview of Aspergillus section Flavi Studies in Mycology, pp. 57–80.

Yabe K, Ando Y, Hamasaki T (1988). Biosynthetic relationship among aflatoxins B1, B2, G1, and G2. Appl. Environ. Microbiol. 54:2101-2106.

Yabe K, Ando Y, Hashimoto J, Hamasaki T (1989). Two distinct O-methyltransferases in aflatoxin biosynthesis. Appl. Environ. Microbiol. 55:2172-2177.

Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW (2004). Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ. Microbiol. 70: 1253–1262.