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MOLECULAR DETECTION THE INFLUENCE OF AFLATOXIN BIOSYNTHETIC GENES BY *ASPERGILLUS FLAVUS* BEFORE AND AFTER *BACILLUS SUBTLIS* AND *CANDIDA ALBICANS* BIOCONTROL

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ABSTRACT

The present study was undertaken to evaluate the efficacy of bio-control on structures and regulatory genes in the biosynthetic pathway (aflD and aflO) and the production of aflatoxin B_1 (AFB_1) by aflatoxigenic *A. flavus* that recovered from animal's feeds. A total of 100 samples (25 of each) of consumed animal's feeds, water, litters and walls of animal's houses halls, from a private farm of cattle at Giza Governorate in which the cattle calves suffered from symptoms of toxicosis as vomiting and profuse diarrhea and related environmental factors were investigated for fungal and aflatoxin pollution. The mould of *A. flavus* was recovered from (80%, 20%, 12% and 4%) of environmental factors of diseased animal's (feeds, water, litters and walls), respectively. All *A. flavus* that recovered only from animal's feeds 100% were aflatoxigenic. While, the highest total aflatoxin residues only were detected also in animal's feeds samples (100 %). Whereas, the maximum levels of AFTs were detected in (animal's feeds) (20 ppm) and the minimum amount were (2.0 ppm), with a mean level of (10.4 ± 4.91ppm). The bio-control of *A. flavus* by bacteria (*B. subtillus*) and yeast (*C. albicans*) were evaluated by biochemical and molecular detection of the changes in AFT-s genes production (aflD and aflO). All treated isolates of *A. flavus* were inhibited their ability for AFTs production as detected by chemical chromatographic method. However, the extraction of DNA from these treated isolates showed that the responsible AFTs biosynthesis genes (aflO and aflD) detected by PCR method in control non-treated *A. flavus*. Whereas, the same isolates were negative for AFTs biosynthesis genes (aflO, aflD) and completely eliminated after bio-control. These results indicated the efficacy of bio-control which caused inactivation and removal of regulatory gene in the biosynthetic pathway (aflD and aflO) and the production of AFB_1

Keywords: PCR, Aflatoxin B_1, Biosynthetic Genes, *A. flavus*, Inactivation, *B. subtilis*, *C.albicans*, Bio-control, aflD, aflO.

INTRODUCTION

Colisepticemia is the most important disease in poultry caused by *Aspergillus flavus* was recorded to constitute a public health hazard due to production of aflatoxins which cause some degree of acute toxicity when consumed in high amounts and are potential carcinogen. In developing countries, it appears that there is a direct correlation between dietary aflatoxins intake and the incidence of liver cancer **FDA (2000), Bahtnager *et al.* (2002) and Refai and Hassan (2013).**

Food and feeds are susceptible to fungal contamination that can occur in the field or during storage. In particular, *Aspergillus flavus* that able to produce mycotoxins in kernels **Samapundo *et al*. (2007).** *Aspergillus flavus* and *A. parasiticus,* produce aflatoxins (AFTs), the most toxic naturally occurring fungal compounds, which represent a significant health hazard for humans and animals **(Refai and Hassan, 2013).** It is estimated that 25 to 50% of the crops harvested worldwide are contaminated with mycotoxins. The percentage is highest in tropical regions, where, up to 80% of the crops are reported to contain significant amounts of mycotoxins **(El-Hamaky *et al*., 2016).** The permissible maximum levels of AFB_1 and total aflatoxins (sum of AFB_1, AFB_2, AFG_1 and AFG_2) in cereals grain were (5.0 µg kg-1 and 10 µg kg-1, respectively) as recommended by European Commission Regulation (EC/2006) **Munkvold and Desjardins (1997).** Microbial pollutants are the most dangerous factors and a wide range of diseases resulted from fungal contamination and their mycotoxins which constituted a problem for animal and human health.

Hence, the selection of proper decontamination methods that will effectively decompose aflatoxins, while retaining the nutritive quality and palatability of the treated food a continuous challenge.The elimination of environmental pollution becomes the national aim of scientists. Also, the chemotherapeutic agents such as fungicidal drugs are causing residue hazards and deliberating action for human. Various researches are needed to identify alternative methods for human and animal protection, which are less dependent on chemicals. Microbial antagonists are widely used for the biocontrol of fungal diseases. Such microorganisms include bacteria, algae and fungi **Brimner and Boland (2003).**

Recently, the only promising method of control of aflatoxin contamination is to use biological control, which includes applying a toxigenic *A. flavus* strains **Cotty (1994), Dorner (2009) and Abbas *et al.* (2011)**, to outcompete toxigenic strains in the field or spraying a yeast formulation to prevent fungal growth **Hua (2006).** In field tests, these biocontrol approaches have achieved greater than 80 percent reduction in aflatoxin contamination. However, the bio-control by yeast has been shown to inhibit growth and aflatoxin production of *A. flavus* **Hua *et al*. (2011),** such as *Candida albicans* and *Kluyveromyces marxianus* **Gao and Daugulis (2009),** *Saccharomyces cerevisiae* **Wang *et al*. (2011)**.

Also, these yeasts have been demonstrated to have inhibitory properties against *Penicillium italicum* and its toxin citric acid **Liu *et al*. (2014).** Other studies evaluated the effect of *Bacillus megaterium* on aflatoxin pathway gene expression in *A. flavus* and they demonstrated that *B. megaterium* and *B. subtilus* inhibited both fungal growth and aflatoxin biosynthesis and could be used as potential biocontrol agents **Kong *et al*. (2014) and Labeed *et al.* (2016).** Therefore, the present study was undertaken to evaluate the efficacy of biological control on the growth and reduction of aflatoxin production by *A.flavus* using bacterium, *B. subtilis* and *C. albicans.* The molecular detection of changes occurred in some aflatoxin pathway genes expression before and after these biocontrole was detected by polymerase chain reaction.

MATERIAL AND METHODS

Samples:

A total of 100 samples (25 of each) of consumed animal's feeds , water , litters and walls of animal's houses halls, from a private farm of cattle at Giza Governorate in which the cattle calves suffered from symptoms of toxicosis as vomiting and profuse diarrhea. Samples were aseptically collected in sterile swabs for houses walls samples, McCartney bottles for water and clean plastic polyethylene pages for collection of feeds and litters samples. The samples were kept fridge till subjected to mycological examination.

Mycological examination of samples:
The collected samples were prepared and examined for isolation of fungi according to the technique recommended by **(ISO, 2008).**After addition of SDA medium containing chloramfenicol antibiotic (0.05 mg /ml media) to inhibit bacterial growth, the plates were left to solidify at room temperature then incubated at 25-27 ^{o}C for 5 -7 days.

Identification of moulds and yeasts which isolated from samples:
After the end of incubation period, the isolated mould and yeast genera and species were purified by subculture on specific medium of isolation and identified by the authors according to the technique recommended by **(Pitt and Hocking, 2009).**

Production and estimation of aflatoxin B_1 by tested of *A. flavus* in liquid medium (YES) (Gabal *et al*., 1994):
The isolated *A. flavus* that were recovered from the present samples were screened for aflatoxin production in sterile synthetic liquid medium (2% Yeast Extract, 20% Sucrose). The estimation of prepared aflatoxin B_1 was measured by TLC **(Bauer *et al*., 1983).** The detected aflatoxigenic isolates *A. flavus* were purified using Malt Extract Agar (MEA, Oxoid Limited, U.K.). This strain was maintained by regular sub-culturing on MEA at 25 °C for 7 days and then stored at 4 °C until required **(Klich, 2002)**.

Preparation of cells of bacteria or spores suspension of *A. flavus* and *C. albicans* (Gupta and Kohli, 2003):
The tested isolates of *A. flavus, C. albicans* and *B. subtillus* strains cultivated on SDA (for fungi) at 25-27 °C for 5-7 days or nutrient agar (for bacteria) at 30-37°C for 1-3 days. At the end of incubation period the fungal mycelia / spores mat and bacterial colonies were washed off with 6 ml of sterile distilled water by sterile loop, the outer most layer of growth (fungal spores and bacterial colonies) was scraped. The mycelia were removed by filtration through a 2.5 µm pore sieve. This spore suspension was counted by haemocytometer slide considering the dilution factor and the spores count was adjusted to 10^5 spores /ml.

Biological inhibition of *A. flavus* growth by *B. subtillus* and *C. albicans* (Mohale *et al*., 2013 and Labeed *et al*., 2016):
With some modification, to sterile glass flask containing 100 ml of YES medium, mixtures of 50: 50 (one ml of 10^5 /ml spores suspension of aflatoxigenic *A. flavus* and one ml of 10^5 /ml cell suspensions of *B. subtillus* or *C. albicans*) were added**.** While, one ml of each of tested

aflatoxigenic *A. flavus* strains were inoculated separately without treatments onto YES medium and kept as positive control. A non-aflatoxigenic strain was also separately inoculated onto YES without treatments and kept as a negative strain. The contents of all flasks were mixed vigorously and incubated at 25 °C for 10-15 days. All experiments were done with three replicates per treatment and repeated once.At the end of incubation period, the biomass was harvested for extraction of DNA and genotyping characterization by PCR. While, the YES broth was subjected for detection of aflatoxins **(AOAC, 2000).**

Molecular detection of aflatoxins genes (aflD and aflO) in biologically treated aflatoxigenic *A. flavus* by using polymerase chain reaction (PCR) technique:

For gene expression studies, after 7-10 days of incubation, the fungal biomass was harvested using a Drigalski spatula under sterile conditions, until DNA extraction.

Extraction of DNA:

Total genomic DNA was isolated from fresh mycelium according to a mini-prep protocol described by **Cenis (1992) and Abd-El Salam *et al*. (2003).** In this method Sabauroud dextrose broth medium (Hi-media) was inoculated with fungal mycelium and left at room temperature for three days. After centrifugation at 10,000 rpm for 5 min, mycelial mat was pelleted and washed with 500 µl Tris-EDTA buffer. The mat was then homogenized by hand in 300 µl of extraction buffer for 5 min. One hundred and fifty micro liters of 3 M sodium acetate (pH 5.2) was added and the mixture was cooled to 20°C for 10 min., fungal debris was pelleted by centrifugation at 10,000 rpm for 5 min, the supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. DNA was then pelleted by centrifugation at 10,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was re-suspended in Tris-EDTA.

Amplification of Internal Transcribed Spacer 1 gene of Aflatoxin **(Paterson, 2006).**

PCR amplification: PCR amplification conditions (Mirhendi *et al*., 2007 and Rahimi *et al*., 2008):

DNA samples were subjected to PCR analyses using specific Afla primers. The PCR reaction was carried out in 25 µl volume containing 10 ng of DNA sample, 10X Taq polymerase buffer (AB-gene Housse,

UK), 25 m $MMgCl_2$, 2 m MdNTPs, 20 pmol of each forward and reverse primer and 0.5 µl (3U/µl) of Red Hot Taq DNA polymerase (AB gene House, UK). PCR was performed using AB, (Applied Biosystems) Thermocycler. Samples were heated to 94 ºC for 5 min and then subjected to 35 cycles of 1 min at 94 ºC, 1 min at 58 ºC and 1 min at 72 ºC. The final extension was set at 72°C for 10 min. Ten µl of the PCR product was electrophoresed on 1.5% agarose gel, stained with ethidium bromide, illuminated and documented using Biorad UV Transilluminator.The sequence of ITS gene for Afla primers (Mirhendi *et al.,* 2007).The primers sequences for aflatoxin aflD/ aflO genes (Table 1).

Table (1): Detailed data on targed aflatoxin genes, primer sequences and expected PCR product *of Aspergillus flavus*:

Primer code	**Gene**	**Primer sequence (5' → 3')**	**PCR product**
Nor1-F	aflD	ACG GAT CAC TTA GCC AGC AC	986
Nor1-R		CTA CCA GGG GAG TTG AGA TCC	
omtB(F)-F	aflO	GCC TTG ACA TGG AAA CCA TC	1304
omtB(F)-R		CCA AGA TGG CCT GCT CTT TA	

Statistical analysis

The obtained data were computerized and analyzed for calculation of standard error according to **SPSS 14 (2006).**

RESULTS

The aflatoxin B_1 is a metabolite of *A. flavus* and *A. parasiticus*, the most potent hepato-carcinogenic substance which has been recently proven to be genotoxic and were classified as carcinogenic to humans and animal **Bahtnager *et al.* (2002).**

The current results in (Table, 2) showed that the mould of *A. flavus* which suspected to be aflatoxin producer was recovered from (80%, 20%, 12% and 4%) of (feeds, water, litters and walls), respectively.

Other genera were also detected in feeds as followed by *Fusarium* spp., *Rhizopus* spp. and *A. niger* (84%, 76% and 72%), respectively all were isolated from animals feeds.

On the other hand, the most isolated mould genera from all samples were *Penicillium* spp*.* (92%, 56% , 64% and 8%) from (animal's feeds, consumed water, litters of animal's houses and animal's houses walls swabs.)*,* respectively. Whereas, the most isolated yeast genera were *Candida* spp. (88%, 60% , 96% and 8%) from animal's feeds, consumed water, litters of animal's houses and houses walls, respectively, while, *Rhodotorulla* spp. was mostly isolated (68%) from consumed water samples.

Recently, the Food and Agriculture Organization **(FAO, 2011)** reported that in areas such as Asia and Africa, 8–18% of cereals commodities, seeds, fruits and vegetables are lost during postharvest handling and storage and about 13.5% of the total value of grain production was lost. The majority of these losses can be attributed to fungal growth and contamination with mycotoxins. In addition, mould spores can disperse in the air with the wind or in combination of wind and rain **Mikulec *et al.* (2005)**. The prevalence of fungi in feed was studied by **Hassan *et al.* (2012)**, who reported that the most common isolated moulds from feed were *Aspergillus spp.* (100%), *Fusarium spp.* (24%), *Mucor spp.* (28%), *Pencillium spp.* (52%), *Cladosporium spp.* (8%) and *Alternaria spp.* (28%). In addition, it has been estimated that 25% of the world's crop production is contaminated with mycotoxins.

In recent work, **El-Hamaky *et al.* (2016)** mentioned that the *Aspergillus* species were recovered from 86% of feed samples with the highest incidence in white corn (95%), followed by yellow corn (90%), poultry feed (85%) and wheat and soya bean (80%). The most predominant isolate was *A. flavus* which was recovered from poultry feed at the rate of (55%) in yellow corn samples from (45%), in white corn, *A. flavus* was yielded from (50%), in wheat samples, *A. flavus* was recovered from (40%) and in Soya beans, *A. flavus* was isolated from (45%).

In general, the breeding factors such as animal housing, feeding on moldy hay and ventilation system or other environmental factors such as temperature, wind and dew increase the odds of contracting the

fungal contamination and infection **Chihaya *et al.* (1991) and Moubasher (1995).**

Table (2): Prevalence of main fungal genera isolated from examined animal's feeds, consumed water, litters and houses walls of diseased cattle.

Isolated fungi	Types of examined samples.									
	Animal's feeds.		Consume dwater.		Litters of animal's houses.		Animal's houses walls swabs.		Total (100).	
	No. of +ve.	%	No. of +ve.	%	No. of +ve.	%	No. of +ve.	%	No. of +ve.	%
A. flavus.	20	80	5	20	3	12	1	4	29	29
A. niger.	18	72	6	24	2	8	2	8	28	28
A. ochraceous.	1	4	7	28	1	4	2	8	11	11
A. parasiticus.	5	20	1	4	1	4	1	4	8	8
A. terrus.	-	-	3	12	2	8	2	8	7	7
Penicillium spp.	23	92	14	56	16	64	2	8	55	55
Fusarium spp.	21	84	-	-	3	12	2	8	26	26
Alternaria spp.	-	-	-	-	12	48	1	4	13	13
Cladosporium spp	-	-	-	-	9	36	2	8	11	11
Mucor spp.	7	28	2	8	-	-	2	8	11	11
Rhizopus spp.	19	76	-	-	-	-	1	4	20	20
Scopulariopsis spp	5	20	2	8	1	4	2	8	10	10
Candida spp.	22	88	15	60	24	96	2	8	63	63
Rhodotorulla spp	1	4	17	68	1	4	1	4	20	20

***Total 100 samples were examined (25 samples of each of animal's feeds, consumed water, litters and houses walls of diseased cattle).

On the other hand, *Aspergillus flavus* were recorded to constitute a public health hazard due to production of aflatoxins that cause some degree of acute toxicity when consumed in high amounts and are potential carcinogen. The mycotoxins are formed by certain fungal species, whenever environmental factors are conductive during the growth of these frequently occurring mycomycetes on foodstuffs and animal's feeds, the process takes place as a secondary metabolism. The mycotoxin inhibits cell division, RNA/ DNA synthesis and causes apoptosis **Rotter *et al*.** (**1996)**. Aflatoxins (AFTs) are produced mainly

by *Aspergillus flavus* and *Aspergillus parasiticus* **Bennett and Klich (2003)** and usually found in various agricultural commodities **Meissonnier *et al*. (2008),** which are known to be very dangerous mycotoxins. In our study, evaluated the aflatoxigenicity of all isolated *A. flavus* from the present samples on YES medium. All isolated *A. flavus* from animal's feeds only 100% were aflatoxigenic, while, the other tested strains of that recovered from consumed water, litters and animal's houses walls were non-aflatoxigenic *A. flavus* (Table, 3).

Regarding the detection of AFTs residues in the present samples, the tabulated results in (Table 4) showed that the highest total aflatoxins residues were detected only in the examined samples of animal's feeds (100 %). Whereas, the maximum levels of AFTs were detected in (feeds) (20 ppm) and the minimum amount were (2.0 ppm), with a mean level of (10.4 ± 4.91ppm). On the other hand, the AFTs residues not detected in any examined samples of consumed water, litters and animal's houses walls.

Whereas, **Hassan *et al*. (2010)** detected aflatoxins in 30% of feeds samples with the mean value of 3.4 ± 0.1 ppm. Recently, **EL-Hamaky *et al*. (2016)** recorded that a total 47 suspected aflatoxigenic *A. flavus* species were recovered from animal's feeds which 33 isolates produced aflatoxin $B_1$and B_2 at average levels of (170 - 750 ppb).

Table (3): Detection of toxigenicity of isolated *A. flavus* from examined samples using TLC.

A. flavus	Numbers of tested isolates from different types of present samples .							
	Animal's feeds.(20)		Consumed water.(5)		Litters of animal's houses.(3)		Animal's houses walls.(1)	
	No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of +ve	%
Non-Toxigenic	0	0	5	100%	3	100%	1	100%
Toxigenic.	20	100%	0	0%	0	0%	0	0%

Table (4): Prevalence of aflatoxins in animal's feeds, consumed water, litters and houses walls of diseased cattle.

Types of examined samples.	Prevalence of aflatoxins (ppm).				
	%	Max.	Min.	Mean±SE	Types of AFTs.
Animal's feeds.	100	20	2.0	10.4 ±4.91	B_1, B_2, G_1,G_2
Consumed water.	0	0	0	0	0
Litters of animal's houses.	0	0	0	0	0
Animal's houses walls.	0	0	0	0	0

***Total 100 samples were examined (25 samples of each of animal's feeds, consumed water, litters and houses walls of diseased cattle).

The Food and drug administration has established recommended maximum levels for aflatoxins in animals feeds as 20 µg/kg of feeds **FDA (1994),** while the permissible limits of aflatoxin for large ruminants <300 ppb in cattle and <100 in calves **Pitt *et al.*(2012).** Hence, the detected levels of mycotoxins in the present study were over the permissible limits in feeds as a result of continuous feeding of toxiocated feed. The same findings were detected by **Hassan *et al*. (2014),** inassociation with significant high mycotoxin levels in feed and sera of diseased animals. Therefore, frequent testing program of the animal's feeds and their environment for fungi and mycotoxin contamination is a critical demand.The exposure to aflatoxin B_1 can result in suppressed immune response, malnutrition, proliferation of the bile duct, centrilobular necrosis, fatty infiltration of the liver, hepatic lesions and even hepatomas. It is one of the most commonly found metabolites and has highest toxigenic effects **Richard (2007).**

However, not only the emergence of chemical drugs and herbs resistance among different pathogenic strains and their toxins but also their high toxicity had prompted research on new antifungal and antimycotoxins agents **Mukherjee *et al.*(2003).**The biological approaches to antifungal and mycotxins detoxification will be taken as a mean of bio-transformation or degradation of toxin to metabolites that are either nontoxic when ingested by animal or less toxic than that the original toxin and readily extracted from the body.

Currently, the present study evaluated the bacteria of *B. subtillus* and *C. albicans* in inhibition the aflatoxin production by aflatoxigenic strain of *A. flavus* when used in mixed culture at 50: 50 ratios on YES medium. The

aflatoxin production was completely inhibited by all tested strains that treated biologically by *B. subtilus* or yeast of *C. albicans* (Table, 5).On the other hand, yeast cells of *C. albicans* can be used in biological detoxification of AFT producing *A. flavus* where there are two components of the yeast cell wall that has greatest impact (the phosphorylated mannan oligosaccharide protein (MOS) and Beta glucan) **Reed and Nagodawithana (1991).** It is interesting to report here that **Hashmi *et al.* (2006) and Awad *et al.* (2011) and Hassan *et al.* (2011)**, found that if 100 ml yeast sludge contains 8.96 gm yeast cells, it contains 0.26% mannan oligosaccharide, which is the principal compound that bind the aflatoxins and ochratoxin A and improve the survival, yield and economics of commercial poultry production.

Recently**, Kong *et al*. (2014)** studied the effect of *Bacillus megaterium* on aflatoxin pathway gene expression in *A. flavus* and they demonstrated that *B. megaterium* inhibited both fungal growth and aflatoxin biosynthesis through altering gene transcription, especially of the regulatory afls gene. While, **Li *et al*. (2013) and Labeed *et al*. (2016)** reported that strains of *B. subtilis* and *P. fluorescens* have been used as potential bio-control agents to control a range of root pathogens.

Table (5): Influence of aflatoxins production by *A. flavus* before and after biological detoxification with bacteria of *B. subtilus* or and yeast of *C. albicans*.

***A. flavus* strains (10^5/ ml) that isolated from present feeds.**	**Before treatment**	**After treatment.**
	Mean levels of produced AFTs (ppm) (mg/l).	**Mean levels of produced AFTs (ppm) (mg/l).**
1	6.4	0
2	24.0	0
3	4.8	0
4	14.0	0
5	6.0	0
6	4.0	0
7	4.0	0

In the present work, the bio-control of *A.flavus* by bacteria of *B. subtilus* or and yeast of *C. albicans* was evaluated by molecular detection of the changes in AFTs gens biosynthesis. All treated isolates of *A. flavus* were inhibited their ability for Afs production as detected by chemical chromatographic method.

However, the extraction of DNA from these treated isolates showed that the responsible AFTs biosynthesis genes (aflO and aflD) detected by PCR method in control non-treated *A. flavus* (Lane 2-3 in photo 1, 2). Whereas, the same isolates were negative for AFTs biosynthesis genes (aflO, aflD) and completely eliminated after bio-control. These results indicated the efficacy of bio-control which caused inactivation and removal of regulatory gene in the biosynthetic pathway (aflD and aflO) and the production of AFB_1 (Table, 5 and Photo 1, 2).

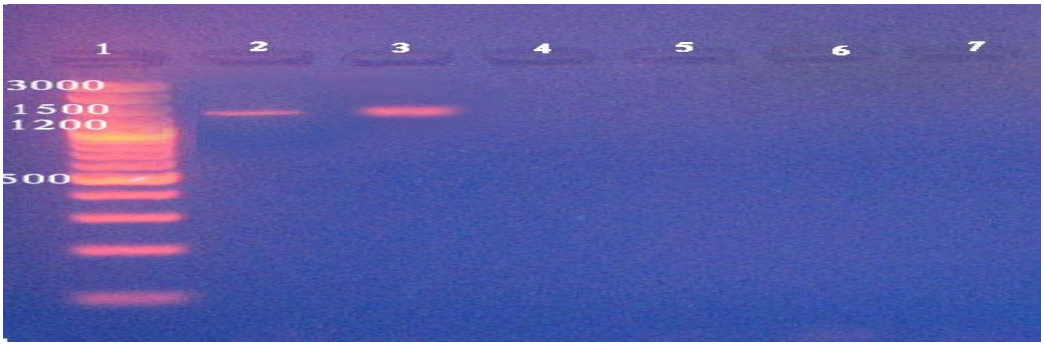

Photo (1): Electrophoretic band pattern of PCR products for aflO gene of AFT.
Lane 1:100 bp DNA Ladder-
Lane 2- 3 positive aflatoxigenic *A. flavus* for aflO gene.
Lane 4- 5 bio-controlled *A. flavus* by *C. albicans.*
Lane 6- 7 bio-controlled *A. flavus by B. subtilus.*

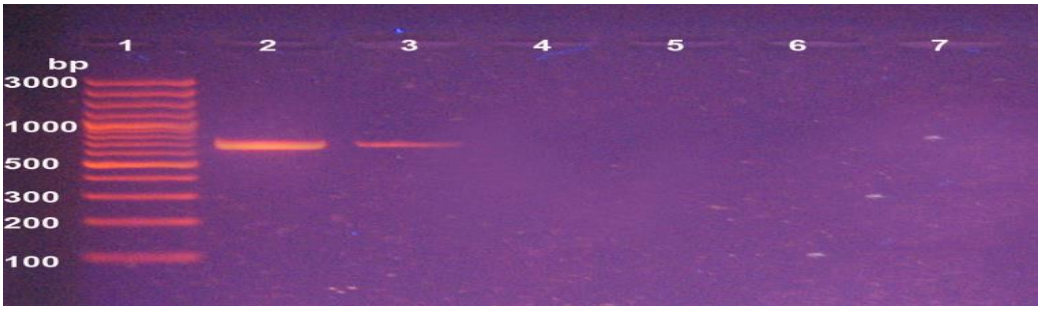

Photo (1): Electrophoretic band pattern of PCR products for aflO gene of AFT.
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Lane 4- 5 bio-controlled *A. flavus* by *C. albicans.*
Lane 6- 7 bio-controlled *A. flavus by B. subtilus.*

Recently, **El-Hamaky *et al*. (2016),** detected that the traditional screening of *A. flavus* that recovered from feeds by chemical chromatographic method of aflatoxigenicity revealed that, *A. flavus* isolates No.1 and 2 were negative producers of aflatoxins, while isolates No. 3, 4 and 5 were positive producer. But the amplification plot of the DNA samples indicated the presence of nor-1 gene in all aflatoxins-producing isolates (3, 4, 5) and only one isolate of the negative aflatoxins-producing *A. flavus* (isolate 2).

Whereas, **Geisen (1996) and Scherm *et al*. (2005)** used multiplex PCR with three sets of primers specific for three structural genes of the AFT biosynthetic pathway aflD, aflM and aflO and was able to differentiate aflatoxigenic *A. flavus* and *A. parasiticus* from other food borne fungi. While, **Färber *et al*. (1997)** detected aflatoxigenic strains of *A. flavus* in contaminated figs by performing a monomeric PCR with the same sets of primer used by **Geisen (1996)**.

Also, **Erami *et al.* (2007)**The aflatoxins detection using TLC and PCR with nor-1, ver-1, omt-1 and aflR primers resulted that three samples of fourteen strains of *A. flavus* were positive using TLC technique and totally twelve samples with the four mentioned primers using in PCR technique showed positive results. None of the other fungal strains using TLC and PCR did show any positive results.
Furthermore, one had to beaware that some genes are not exclusive of the aflatoxin biosynthetic pathway, which could create false-positives from sterigmatocystin producing fungi **Paterson (2006).**

Recently, it has been found and reported that some aflatoxin non-producing *A. flavus* strains show a complete set of genes. Because less is known about the incidence of structural genes aflR, nor-1, ver-1 and omt-A in aflatoxin non-producing strains of *A. flavus*, they decided to study the frequencies of the aflatoxin structural genes in non-aflatoxigenic *A. flavus* strains isolated from food and feed commodities **(Criseo *et al.*, 2008).** Other studies reported that the presence of AFTs biosynthetic genes did not correlate with aflatoxigenicity, where, aflD expression was not considered a good marker for differentiating aflatoxigenic from non-aflatoxigenic isolates, but aflQ showed a good correlation between expression and aflatoxin-production ability **(Dangwa *et al.*, 2014).**

Hence, the differences in detection of AFTs biosynthetic genes by PCR in some biologically treated aflatoxigenic *A. flavus* which not produced any AFTs as measured by chromatographic analysis may be due to the

detected genes lost their ability, lost its role in AFTs biosynthesis pathway and inactivation of these genes may occur. While, other aflatoxigenic isolates after bio-control, no genes were detected by PCR and no levels of AFTs detected by chemical chromatographic method indicated a successful bio-control (Table 5 & Photo 1, 2). Similar results were reported by **Labeed *et al*. (2016),** who reported that the gene expression of some biosynthetic genes of AFTs in *A. flavus* after bio-control may be inhibited when compared with the control and there were markedly different impacts on AFB_1 production. They added that the molecular detection should give critical information on the activation or inactivation of specific genes involved in toxin biosynthesis.

CONCLUSION

In this study, mould were recorded to produce aflatoxins caused some degree of acute toxicity when consumed in high amounts and are potential carcinogens.Therefore, frequent testing program of the animal feeds and other environmental factors for fungal and mycotoxins contamination must be undertaken. Hence, the regulation of aflatoxin biosynthetic gene expression of aflatoxigenic *A. flavus* by successful method of control to prevent its activity and inhibited aflatoxins production become critical demand. The bio-control in this study yielded desirable results in manipulation of genetic factors, biotic and abiotic elements that affect aflatoxin biosynthesis. The genetic and genomic resources will significantly enhance our understanding of the mechanisms of aflatoxin production, pathogenicity of the fungus, and crop-fungus interactions.

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الكشف الجزيئى للتغيرات الحادثة فى الجينات المسؤولة عن تخليق الأفلاتوكسين بفطر الأسبرجيللس فلافس قبل وبعد المعالجة البيولوجية بالباسيبلس سابتيلس و الكانديدا البيكانز.

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الملخص العربى

أجريت هذه الدراسة لتقييم فعالية السيطرة الحيوية على التحكم فى الجينات الوظيفية والتنظيمية (aflD and aflO) في المسار الحيوي لتخليق وإنتاج الأفلاتوكسين B_1 (AFB_1) بواسطة عترات *الأسبرجيللس فلافس* السامة التي تم عزلها من الأعلاف الحيوانية. وقد تم إجراء الفحوص على مائة عينة (25 عينة من كل) من (الأعلاف والماء والفرشة والجدران) من مزرعة خاصة بالماشية بمحافظة الجيزة التي عانت فيها عجول الماشية من أعراض التسمم كالقئ والإسهال الشديد وقد تم التحقق من العوامل البيئية ذات الصلة بالتلوث الفطري وإفراز سموم الأفلاتوكسين. وقد تم عزل فطريات *الأسبرجيللس فلافس* (80 ٪، 20٪، 12٪، 4٪) من العوامل البيئية المختلفة المعزولة من أمام الحيوانات المريضة ، على التوالي. وكانت جميع عترات *الأسبرجيللس فلافس* المعزولة فقط من الأعلاف الحيوانية 100% مفرزة لسموم الافلاتوكسين، وقد وجدت بقايا الأفلاتوكسين موجودة بأعلى معدل فقط أيضا في الأعلاف (100٪) في العينات التي تم تجميعها. حيث كانت أعلى المستويات لسموم الأفلاتوكسين فقط في عينات (الأعلاف) (20 جزء في المليون) وكان الحد الأدنى (2.0 جزء في المليون)، مع مستويات متوسطة (10.4 ± 4.91 جزء في المليون).

وقد تم تقييم السيطرة الحيوية على نمو عترات *الأسبرجيللس فلافس* بواسطة بكتيريا *الباسيلس سابتيلس* وخميرة *الكانديدا البيكانز* باستخدام طرق الكشف البيوكيميائية والجزيئية للكشف عن التغيرات في إنتاج الجينات المسؤلة عن إفراز سموم الافلاتوكسين حيث قد أظهرت النتائج أن قدرة جميع معزولات فطر *الأسبريجللس فلافس* التي تمت معالجتها حيويا على إفراز سموم الأفلاتوكسين قد عطلت وهذا ما أظهرته نتائج طريقة الكروماتوغرافي الكيميائية, فى حين أنه عند إستخلاص الحمض النووي من هذه المعزولات المفرزة للأفلاتوكسين الغير المعالجة أن الجينات المسؤولة عن إنتاج سموم الأفلاتوكسين التي تم الكشف عنها بطريقة إختبار البلمرة المتسلسل كانت واضحة، فى حين أن هذه المعزولات كانت سلبية للجينات المختبرة بعد المعالجات البيولوجية. وأظهرت هذه النتائج فعالية السيطرة البيولوجية التي تسببت في تعطيل حيوية وإزالة الجينات التنظيمية في المسار الحيوى وإنتاج سموم الافلاتوكسين B_1 (aflD and aflO).