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Detection the Gene that Responsible for the Production of OTA from *Aspergillus Niger* and *Aspergillus Ochraceus* in Wheat and Maize Grains

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Abstract :

Ochratoxin A is the main mycotoxin in the group of ochratoxin is produce by several group of fungal species of the genera *Aspergillus* and *Penicillium*, Therefore, the main objective of this study was to investigate the gene that responsible for the production of OTA from *A. niger* and *A. ochraceus* in wheat and maize grains . The results of PCR revealed that 11 isolated classified as *A. ochraceus* . Real-Time PCR results of *A. niger* and *A. ochraceus* indicate the presence of the gene that responsible for production of OTA.

Keyword: Real-time PCR, OTA, *A. niger*, *A. ochraceus*. Wheat, Maize

Introduction:

Mycotoxins are secondary metabolites synthesized by an array of fungal genera, usually *Fusarium*, *Penicillium* and *Aspergillus*. They are natural contaminants which commonly occur in food and feed and pose a threat to animal and human health. These hazards contaminate agricultural commodities either directly or they reach animal tissues, milk and eggs through a “carry-over” mechanism after feeding animals with contaminated feedstuffs (Neši,2018; Emmanuel *et al.*,2020). From regulatory and food safety viewpoints, the most significant and prevailing types of mycotoxins are aflatoxins (AFs), zearalenone (ZEA), fumonisins (FUMs), trichothecenes (TCT) (deoxynivalenol (DON), T-2 toxin (T-2) and HT-2 toxin (HT-2)), ochratoxins (OTA), ergot alkaloids (EAs), patulin and citrinin. If these substances are present in a particularly high amount in feed and food, or in lower dosages but over a long period of time, they can cause a variety of adverse effects, from acute to chronic, both in humans and animals.

Ochratoxins are produced by certain *Aspergillus* species such as *A. ochraceus* or *A. niger* and some *Penicillium* species, especially *P. verrucosum*. The main forms are ochratoxin A, B, and C, which differ in that ochratoxin B (OTB) is a non-chlorinated form of ochratoxin A (OTA) and ochratoxin C (OTC) is an ethyl ester of OTA). OTA ((R)-N-((5-Chlor-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-

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benzo[c]-pyran-7- yl)-carbonyl)-3-phenylalanin) is the most prevalent and relevant form of this group, while OTB and OTC are generally assumed to be of lesser importance(O'Brien and Dietrich ,2004). OTA was isolated in 1965 from a culture of *Aspergillus ochraceus*, but subsequent studies have revealed that a variety of fungal species included in the genera Aspergillus and Penicillium are able to produce ochratoxins (Merve *et al*.,1965). TA is present at all stages of the food chain (cereals, meat, fruits, wine, beer, coffee, *etc.*) (Pfohl-Leszkowicz *et al*.,2002)and based on previous studies its presence may be associated with the chronic tubulo-interstitial kidney disease called Balkan Endemic Nephropathy (BEN) (Bui-Klimke and Wu,2014) .

During the last years several PCR-based assays have been developed to overcome the difficulties of the traditional schemes for identification of toxigenic fungi from food samples. Although conventional PCR has been recognized as a very valuable tool for detecting toxigenic fungi (Massi *et al*., 2016), Real-time PCR does not require gel electrophoresis, and consequently reduces time and manual labor, making it appropriate for large-scale analyses.

Materials and Methods:

Samples Collection:

The Grains (Wheat and Maize) were collected from the Silo and Markets of Babylon and AL-Diwaniyah Governorate, at 3kg per sample. The samples were placed in sterile paper bags and labeled and brought to the laboratory.

Fungi Isolation:

The Fungi associated with Wheat and Maize grains used in this research were isolated by surface sterilization of the grain using a solution of sodium hypochlorate at a concentration of 1 % for a 2-5 minutes , followed by three washes with sterile distilled water. The grains was culture in Petri dishes contained SDA broth medium with 10 grains per dish and in three Repeated and dishes were placed in the incubator at 25˚temperture for a period of 7 days. Then the plates were examined for developing fungi.

Diagnosis of *A. ochraceus* by Using PCR

Primers

PCR and Real Time PCR primers were designed in this study using NCBI-Genbank database and primer 3 plus online. These primers were provided by Macrogen company from Korea as following table 1

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Table 1:PCR and Real Time PCR primers

rgillus ochraceus rRNA gene		**ACCCGTGTATACCGTA**	
		CAAGAGCGGGTGACAA	
ochratoxin A osynthetic gene		**CGCCACGCAGAAAAAG**	
		ACGCCTGTTTGATTTC	
housekeeping gene		**AAACGCGTGTCACTAC**	
		CGAGGTCGAACACTTG	

LN809077.1 *Aspergillus ochraceus* genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, isolate 1012TES1A1

MG701892.1: *A. niger* and *A.ochraceus* culture CBS:513.88 ochratoxin A biosynthetic gene cluster, complete sequence

XM_001396558.2 *Aspergillus niger* CBS 513.88 actin, mRNA

Molecular study Kits

PCR detection Kits

Table 2: The PCR detection Kits used in this study with their company and country of origin:

		ny and y
1	**G-spin Total DNA extraction kit**	
	CL buffer lysis buffer	
	BL buffer binding buffer	
	Proteinase K	
	WA buffer	
	WB buffer	
	Elution buffer	
	GD column	**iNtRON (Korea)**
	Collection tube 2ml	
2	**Maxime PCR PreMix kit**	
	Taq DNA polymerase	
	dNTPs (dATP, dCTP, dGTP, dTTP)	
	Tris-HCl pH 9.0, KCl, & $MgCl_2$	
	Stabilizer and loading dye	

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Methods

Fungal DNA extraction

Fungal genomic DNA was extracted from *A. Ochraceus* isolates by using (**G-Spin DNA extraction kit with modification**) as and done according to company instructions as following steps:

1-Sample Preparation :

a- 1 ml cultured fungal cells was transferred to 1.5 ml microcentrifuge tube then centrifuged at 10000 rpm for 1 minute then the supernatant was discarded.

2-Cell Lysis steps :

a- 180µl GT buffer and 250mg glass bead were added to the tube and the cell pellet suspended by vortex, then 20 µl of Proteinase K was added and the mixtures were incubated at 60ºC for 30 minutes. During incubation periods the mixtures tubes were inverted every 3 minutes.

b- 200µl GB buffer were added to each tube and mixed by vortex for 10 seconds. Then the tubes incubated at 60ºC for 10 minutes with inverted the tubes every 3 minutes through incubation periods.

3-DNA binding steps :

a- 200µl absolute ethanol were added and immediately mixed by vortex, then precipitates if happen was broken by pipetting.

b- A GD column was placed in a 2 ml collection tube and all mixtures were transferred (including any precipitate) to the GD column. Then centrifuged at 10000 rpm for 1 minute. The 2 ml collection tubes that contains the flow-through were discarded and placed the GD column in a new 2 ml collection tube.

4-Washing steps :

a- 400µl W1 buffer were added to the GD column, then centrifuged at 10000rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.

b-600µl Wash Buffer were added to the GD column. Then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube. and the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

5-Elution steps :

a- The dried GD column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of pre-heated elution buffer were added to the center of the column matrix.

b- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 1 minutes to eluted the purified DNA.

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Estimation of extracted total DNA:

The extracted total DNA was checked by using Nanodrop (**Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer**. **USA**) that measured DNA concentration (ng/µL) and checked the RNA purity at absorbance (260 /280 nm)

PCR master mix preparation

PCR master mix reactions for all genes was prepared by using (Maxime PCR PreMix kit) and this master mix done according to company instructions as following table 3:

Table 3: Standard PCR master mix protocol

Master mix	ne
template 5-50ng	
rd primer (10pmol)	
es primer (10pmol)	
vater	
volume	

After that, these PCR master mix components that mentioned in table above placed in standard Maxime PCR PreMix kit tubes that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$,and loading dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes, and then placed in T100 PCR Thermocycler (BioRad-USA).

PCR Thermocycler Conditions

PCR thermocycler conditions protocol for each gene was calculated by using **Optimase ProtocolWriter™** online application and done by using convention PCR thermocycler following table 4:

Table 4: PCR thermocycler conditions protocol:

	name	denaturation /time	uration /time	ling /time	sion /time		extension /time	/time
	NA gene	4 min	30 sec	30 sec.	60sec.		5min	rever

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PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis method as following steps:

1- 1.5% Agarose gel was prepared in using 0.5X TBE and dissolving in microwave for 5 minutes, and left to cool for 50°C.

2- Then 3µl ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position and left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.

4- The gel tray was fixed in electrophoresis chamber and filled by 0.5X TBE buffer.

5- 10µl PCR product were loaded in to each well with added 5µl (DNA marker Ladder) in first well. Then electric current was performed at 100 volt and 80 AM for 1hour.

Real Time PCR detection Kits

o.	Kit	Company	ountry
	LUE™ Total RNA Extraction Kit	**iNtRON**	**Korea**
	reagent 100ml		
	o RT Prmix kit Hexammer	**Wizbio**	**Korea**
	Script Reverse Transcriptase (200U)		
	ction buffer		
	250µM		
	).25mM		
	Inhibitor (1U)		
	q®Qpcr Master miz	**Promega**	**USA**
	CR mix (1ml)		

Table 5: The Real Time PCR expression Kits used in this study with their companies and countries of origin:

Total RNA extraction

Total RNA were extracted from *A. niger and A.ochraceus* isolates by using (easy-BLUE™ Total RNA Extraction Kit) and done according to company instructions as following steps:

1- Fungal isolates were inoculated on Luria Bertani broth and incubated at 37ºC into reach fungal cells (OD600:0.8-1.0), the fungal cells were harvested by centrifuge at13000rpm for1min then, supernatant removed.

2- The fungal pellets were suspended by added 1ml easy-BLUE (Trizol reagent) and vigorously vortex in room temperature for 10 sec.

3- 200µl chloroform was added to each tube and shaken vigorously for 1 min.

4- The mixture was incubated on ice for 5 minutes. Then centrifuged at 13000 rpm, 4C°, for 15 minutes.

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5- Supernatant was transferred into a new 1.5ml microcentrifuge tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 13000 rpm , 4C° for 10 minutes.

6- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 13000 rpm, 4C° for 5 minutes.

7- The supernatant was discarded and the RNA pellet was left to air to dry.

8- 100µl Free nuclease water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at -80.

Estimation of extracted total RNA:

The extracted total RNA was checked by using Nanodrop (**Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer**. **USA**) that measured RNA concentration (ng/µL) and checked the RNA purity at absorbance (260 /280 nm)

DNase I treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions as follow:

Table 6: DNase I enzyme kit

Mix	Volume
Total RNA 1µg	
DNase I enzyme	
10X buffer	
water	

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

cDNA synthesis

The DNase treated total extracted RNA samples were used in cDNA synthesis step from mRNA transcripts by using **(AccuPower® RocketScript™ RT PreMix)** and this kit was done according to company instructions as following:

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Table 7: cDNA synthesis kit

RT mix	Volume
Total RNA 100µg	10µL
Random Hexamer primer (50pmol)	1µL
DEPC water	9µL
Total	10ul

After that, these RT mix components that mentioned in table above placed in AccuPower® RocketScript™ RT PreMix kit strip tubes that containing all other components which needed to cDNA synthesis such as (Reverse Transcriptase, 5 x Reaction Buffer, DTT, dNTP, and RNase Inhibitor). Then, all the strip tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes, and then incubated in Thermocycler (BioRad-USA) as following thermocycler conditions protocol:.

Table 8: thermocycler conditions protocol

Step	Temperature	Time
synthesis (RT step)		
nactivation		ıtes

Real-Time PCR (qPCR) master mix preparation

qPCR master mix was prepared by using **(RealMODTM Green SF 2X qPCR mix Kit)** based on SYBER green dye amplification in Real-Time PCR system and the qPCR master mix was prepared as following:

qPCR master mix for target genes and housekeeping gene:

` qPCR master mix for target genes and housekeeping gene was prepared as following table 9

Table 9: qPCR standard master mix protocol

qPCR master mix	volume
cDNA template (10ng)	5µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL
qPCR Master Mix	10 µL
Nuclease free water	3 µL
Total	20 µL

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After that, these qPCR master mix component that mentioned above placed in qPCR white plate strip tubes and mixed by Exispin vortex and centrifuge for 5 minutes, then placed in MiniOpticon Real-Time PCR system.

- **qPCR Thermocycler conditions**

qPCR Thermocycler conditions was done according to qPCR kit instruction and used by using **Optimase ProtocolWriter™** online for primers annealing calculation as following table 10

Table 10: qPCR Thermocycler conditions:

qPCR step	Temperature	Time	epeat cycle
Initial Denaturation	95 °C	10min	1
Denaturation	95 °C	20 sec	40
Annealing\Extension Detection(scan)	58 °C	30 sec	
Melting	65-95°C		1

Data analysis of qPCR

The data results of qPCR for target and housekeeping gene were collected and the expression analysis (fold change) used analyzed by using (Livak method) that described by (Livak and Schmittgen, 2001) as following equations:

ΔCT (Test) = CT (target gene, test) – CT (HKG gene, test)

ΔCT (Control) = CT (target gene, control) – CT (HKG gene, control)

ΔΔCT= ΔCT (Test)– ΔCT (Control)

Fold change (target / HKG) = 2^{-CT} ΔΔCT

Results and Discussion:

Molecular Identification of *A.ochraceus* by using PCR

After the phenotypic diagnosis of the two *Aspergillus* species, the method polymerase Chain Reaction (PCR) was used to support the diagnosis of *A. ochraceus*. The results of extracted DNA proved in figure (1) which contain 11 isolate of *A .ochraceus*, the extracted genomic DNA of these isolates were used as a template for amplification with primers of internal transcribed spacer (ITS), the results revealed that 11/11 isolates were classified as *A. ochraceus* after band electrophoresis and UV-trans illuminated of the product . The internal transcribed spacer (ITS) regions of the fungal ribosomal DNA (r RNA) has been used as one of the techniques for species identification because it is faster, specific, accurate species determination and less feasible to be affected by exterior effects such as

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temperature and change chemotherapy (Girgis *et al*.,2006; Kong *et al*., 2008). This results agree with many studies , in one study, Patiño *et al*., (2005) developed specific PCR assay for detection of *A. ochraceus* species based on ITS sequences. The primer-pair denoted OCRA1/OCRA2 was designed on the basis of ITS sequence comparison of several strains of *Aspergillus* species. ITS2 region from several Brazilian strains of both species showed specific nucleotide variations (ITS and β-tubulin genes) characterizing *A. westerdijkiae* and *A. ochraceus* (Fungaro *et al.*, 2004a; Fungaro *et al.*, 2004b; Morello *et al.*, 2007). *Aspergillus ochraceus* can be rapidly and specifically detected in green coffee by PCR.(Schmidt *et al*.,2004).

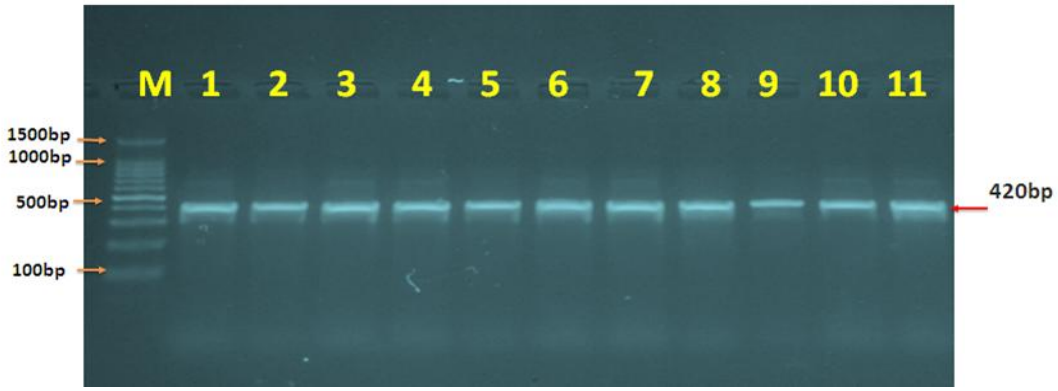


Figure 1: Agarose gel electrophoresis image that showed the PCR product analysis of 18S rRNA gene ITS2 gene from extracted DNA of *Aspergillus ochraceus* isolates. Where M: marker (1500-100bp) and the positive *Aspergillus ochraceus* (1-11) isolates were showed at (420bp) PCR product.

Quantitative Reverse Transcription Real-Time PCR in *A. niger* and *A. ochraceus*

The data which were revealed by figure 2 and table 11 for 6 isolates of *A.niger* 3 isolated from wheat and 3 from maize grains showed the presence of the gene that responsible for the production of OTA, figure 3 and table 12 for 6 isolates of *A.ohraceus* isolated from maize grains showed the presence of the gene that responsible for the production of OTA. DNA-based techniques such as real-time PCR (RT-PCR) are providing new tools for fungal detection and quantification by detecting and quantifying their DNA. RT-PCR can be performed using different chemistries, such as SYBR® Green I dye (González-Salgado *et al*.,2009).

One of the major motivations for the development of PCR based detection systems in many publications is the prospect of using this kind of analysis to estimate OTA concentrations in sample material. One might therefore anticipate that assays based on OTA biosynthetic genes might better fit that purpose as compared to systems based on genes unrelated to their biosynthesis. By using real-time PCR, a positive correlation between OTA content and DNA quantity has been indicated for P. nordicum and A. ochraceus(Farber and Geisen,2004) and more recently, in A. carbonarius (Selma *et al*.,2008). Such a correlation has been established with quantitative real-time PCR on mycotoxin biosynthesis genes (Geisen *et al*.,2004), or when using primers targeted sequences of housekeeping genes (Mulè *et al*.,2006) Currently, RT- PCR quantification of A. carbonarius in grapes is clearly the best alternative to conventional methods in order to investigate the relation between OTA producers and OTA content. With regards to food safety, Atoui *et al*. (2007) established, according to their correlation, that A. carbonarius DNA content has to be lower than 10 ng DNA g−1 grape berry to

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fulfill the maximum OTA permitted levels in the European Union (Commission regulation No. 123/2005 amending Regulation No. 67 446/2001 as regards to ochratoxin A). For this reason Real time PCR technology provides an insight into the mycotoxigenic status of food sample as well as it has the power to estimate its mycotoxin content.

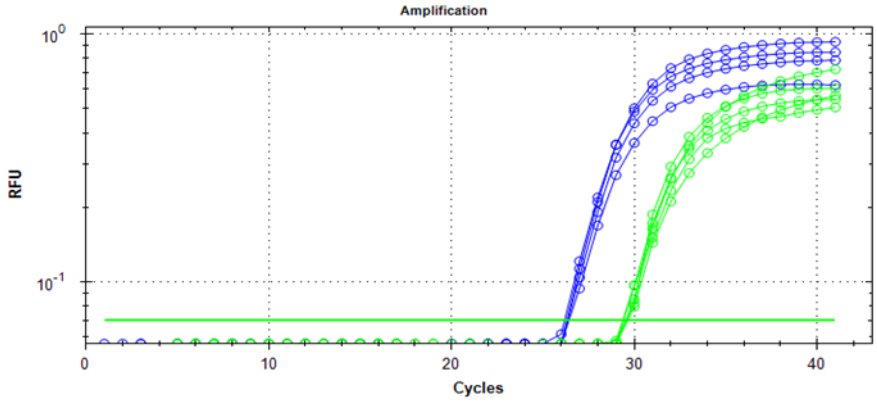


Figure 2: Real Time PCR amplification plot for target gene (ochratoxin biosynthesis protein as blue plots and housekeeping gene as Green plots

Table11: Gene expression analysis for ochratoxin A in *A.niger isolated from Wheat and*

Gene expression				
No. sample	**(Ochratoxin A)**	**CT (actin)**	**ΔCT**	**ld change (2^ΔCT)**
1-Wheat	**26.35**	**29.67**	**3.32**	**9.987**
2-wheat	**26.26**	**29.79**	**3.53**	**11.551**
3- Wheat	**26.49**	**29.32**	**2.83**	**7.111**
4-Maize	**27.23**	**29.71**	**2.48**	**5.579**
5-Maize	**26.26**	**29.43**	**3.17**	**9.000**
6-Maize	**27.49**	**29.27**	**1.78**	**3.434**

Maize

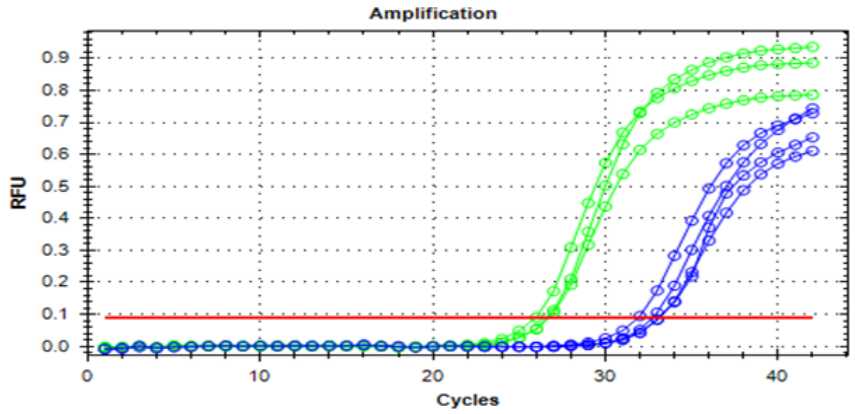


Figure 3: Real Time PCR amplification plot for target gene (ochratoxin biosynthesis protein gene) as blue plots and housekeeping gene as Green plots.

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Gene expression				
No. sample	T (Ochratoxin A)	CT (actin)	ΔCT	l change (2^ΔCT)
	28.43	**30.54**	**2.11**	**4.317**
	25.87	**30.66**	**4.79**	**27.656**
	26.55	**30.19**	**3.64**	**12.467**
	28.45	**30.58**	**2.13**	**4.377**
	27.43	**30.3**	**2.87**	**7.311**
	28.43	**30.14**	**1.71**	**3.272**

Table 12: Gene expression analysis for ochratoxin A in A.ochraceus isolated from Maize grains

Conclusion:

Gene expression results show that all the strains of the *A.niger* and *A.ochraceus* that secret ochratoxin A have the gene that responsible for the production of the toxin.

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