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Real Time Quantitative Polymerase Chain Reaction Detection of Trichothecenes Producing *Fusarium* Species in Animal Feed and Their Control By Silver and Zinc Nanoparticles.

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Abstract

Up to date, Real Time-PCR technologies successfully used in detection and quantitative estimation of fungal DNA. In the present study, fungal examination of 60 feed samples (20 of each of yellow corn, white corn and animal feeds) yielded the detection of *Fusarium species* namely *F. poae, F. equiseti* and *F. sporotrichoides*. They produced trichothecenes mycotoxins at mean levels of (68.5±5.63, 10±1.0 and 97.5±2.5 ppm), respectively. While, the feed samples of yellow corn, white corn and animal feed were contaminated with T2 toxin at Incidence rates (70%, 30%, and 75%) with mean levels of (35 ±7.4, 72±8.27 and 60±9.5 ppm) respectively. Currently, the antifungals as Zn NPs, Ag NPs and olive oil were used for controlling the growth of trichothecenes producing *Fusarium* and these activities were evaluated by molecular detection of the changes occurred in genes density and their structure before and after treatments. The RT-q PCR combines the sensitivity of conventional PCR with the generation of a specific fluorescent signal providing real-time analysis of the reaction kinetics and allows quantification of specific DNA targets. At the first step in this direction, the use of quantitative RT-PCR detected that as the doses of treatment with antifungal increased the *Tri5* gene expression efficacy, molecular weight of DNA and toxin production were decreased. Otherwise, the treatments of trichothecenes producing *Fusarium* as (*F. poae, F. equiseti* and *F. sporotrichoides*) by Zn NPs, Ag NPs and olive oil could adversely affect the efficacy of *Tri5* gene expression, the efficacy of DNA consequently, its ability for mycotoxin production as shown also in traditional PCR. More investigations are required to detect the effect of different doses of antimycotoxins on degradation of mycotoxins genes and regulation of *Tri5* gene expression and quantitative detection of different mycotoxins by quantitative RT-PCR assays.

Keyword: *Trichothecenes, Genus Fusarium, RT-q PCR, PCR, antifungal.*

Introduction

The animal health represents the major role in food security for human consumption in developing country. Fungal contaminations are a worldwide problem and were detected on a wide variety of environmental factors as feed, litters, air, soil, plants, water and animal discharges. The mycotoxigenic fungi and their mycotoxins can induce many toxic and immunomodulatory diseases in a variety of animal and poultry species.

Several species of *Fusarium*, *Aspergillus* and *Penicillium* are the most abundant moulds that produce mycotoxins, contaminate human foods and animal feeds. The major *Fusarium* mycotoxins of significant importance to human, animal and poultry health are the trichothecenes and Fumonisin (**Sweeneyand Dobson, 1998 and El Hamaky *et al*., 2001**).

The trichothecenes are secondary metabolites

produced by some *Fusarium* species as (*F. sporotrichoides*, *F. culmorum*, *F. equiseti*, *F. poae* and *F. graminearum*). The produced trichothecenes are mainly T2, deoxynivalenol, nivalenol and zearalenone (**Ueno, 1983 and Hassan *et al.*, 2010a and b)**. These *Fusarium* mycotoxins have dangerous effects on animal and human health (it has potent inhibitors of protein synthesis, cause diseases such as alimentary toxic aleukia (ATA) and even cytotoxicity, reproductive failure, and may lead to death in poultry and cattle (**Nelson and Plattner, 1993**).

In addition; during the control of diseases caused either by fungal or mycotoxins; traditional antimicrobials are insufficient to report the minimal inhibitory concentrations. Whereas; in many cases the antifungal agents used for inhibition of microbial activity don't affect the genes responsible for fungal growth as observed by DNA-PCR genome (**Scherm *et al.*, 2005, Bilodeau, 2011 and Hassan *et al.*, 2017**).

Hence, there are high significant demands to investigate new antimicrobial agents for controlling the infection caused by *Fusarium* sp. and their toxins. Recently, Nanotechnology has been used in pharmaceutical industries to find new antimicrobial agents (**Gajjar *et al.*, 2009**).

Furthermore, several studies evaluating the antifungal activity of metal nanoparticles of the least hazards to the environment in culture media particularly ZnNPs and AgNPs (**Hassan *et al.*, 2015, 2016 and 2017**). **Ansari *et al.*, (2016)** regarded that olive oil has antifungal potential which act as natural, non-irritating, non-toxic permeation enhancer. It was reported that the olive oil has shown the antifungal potential against *A. niger, C. albicans* and *R. stolonifera* (**Upadhyay *et al.*, 2011 and Upadhyay, 2014**). Therefore, the present study was undertaken to evaluate the prevalence of *Fusarium* species and trichothecenes mycotoxins in feeds; in addition using of quantitative RT-PCR for evaluation of the treatment efficacy of nanoparticles and olive oil for toxic *Fusarium*.

Materials and Methods

1. Feed samples: A total of sixty feed samples (20 each of yellow corn, white corn and animal feed) were collected from animal farms that had animals suffering from symptoms of toxicity as vomiting, diarrhea, refuse feed and loss of weight and/ or sudden death. Five hundred gram of each was collected in a clean polyethylene bag and transferred to the laboratory for further investigation.

2. Antifungal agents

2.1. Zinc and Silver Nanoparticles:
Synthesis and characterization of ZnNPs and Ag NPs were kindly done by fund of Prof. Dr. H. H. Mansour, Head of Central Laboratory of Elemental and Isotopic Analysis, Nuclear Research Centre, Atomic Energy Authority, Egypt.

2.2. Olive Oil: was purchased from ALDRIK Sigma chemical company.

2.3. *Fusarium* Mycotoxins standard solution for TLC: Mycotoxins standard of Trichothecenes (T2, DON, NIV) were purchased from ALDRIK Sigma Chemical Company, St. Louis, U.S.A)

3. Isolation and Identification of Molds in Feed Samples

The collected samples were examined microbiologically for fungi with references to *Fusarium* species as recommended by **Conner *et al.* (1992)**. After pouring of medium, the plates were left to solidify at room temperature then incubated at 25ºC for 5 -10 days. While, the identification of *Fusarium* species was done according to **Pit and Hocking (2009)**.

4. Production, Extraction and Detection of Trichothecenes by the Isolated *Fusarium* species from Feeds: The recovered *Fusarium* species from the present feed samples were grown on PDA (Potato Dextrose Agar) for seven days, at 25ºC. Five hundred ml flasks, each containing 100 gm of fine grounded yellow corn and 40-50 ml of distilled water were mixed and autoclaved at 121ºC for one hour. The flasks were shaken to prevent cooking of the yellow corn, inoculated with slant spores of each *fusarium* and incubated for 4 weeks at 25-28ºC. Then the flasks were transferred to 8-10ºC for additional 2 weeks (**D'Mello *et***

***al*., 1998**). After the end of incubation period, the corn was removed from flasks, dried, finely grounded and 50 gm of each was subjected to trichothecenes toxin extraction and measured by thin layer chromatography as recommended by (**Kamimura *et al*., 1981; Bottalico *et al*., 1983 and 1985**).

5. Evaluation of Antifungal Potential of Olive Oil and Zinc and Silver Nanoparticles Against *Fusarium* sp. using Agar Dilution Method (Jin *et al*., 2009):

5.1. Preparation of spore suspension of the isolates (Gupta and Kohli, 2003)

The tested isolates of *Fusarium* sp. were cultivated on SDA for 1-3 days at 28°C. At the end of incubation period, the fungal mycelia / spore mat of colonies was washed off with sterile distilled water using sterile loops, the outer most layer of growth was scraped, the mycelia were removed by filtration through a 500-um pore sieve. These spores' suspensions were counted in hemocytometer slide considering the dilution factor and the spores count was adjusted to10^5spores /ml.

5.2. Antifungal Potential of Zinc and Silver Nanoparticles against *Fusarium* sp. using Agar Dilution Method (Jin *et al*., 2009):

Different gradual concentrations (0, 100, 200, 400, 600, 800, 1000µg/ ml) of zinc and silver nanoparticles were mixed with SDA (either streaked or incorporated with 10^5 of tested *Fusarium s*p. spore suspensions) sterile petri dishes and solidified. The plates then were incubated at 25-28˚Cfor 3-5 days.

5.3. Antifungal Activity of Olive Oil on the Growth of *Fusarium* sp. by Agar Dilution-Method (Upadhyay, 2014)

SDA were prepared and autoclaved at 121˚C for 15 minutes then kept at 55˚C. Olive oil was sterilized by filtration (pore size, 0.45 µm), then mixed with SDA according to the tested concentrations (0, 100, 200, 400, 600, 800, 1000 µg/ ml). The oil-agar mediums poured into sterile petri dishes then were left to solidify. Equal amounts (0.5ml) of the fungal suspensions were inoculated and spreaded onto the agar plates. The plates were then examined after incubation at 25-28ºC for 3-5 days.

After the end of incubation period, the sensitivity of fungi to the tested drug was determined by recording growth or non-growth inhibition zone.

6. Molecular identification of mycotoxigenic *Fusarium* sp. isolates using polymerase chain reaction:

6.1. Preparation of *Fusarium* strains (Doohan, 1999):

The recovered *Fusarium* sp. isolated from feed was subjected to the detection of genes responsible for trichothecenes mycotoxin production. A total of 3 isolates including, *F. poae, F. equiseti* and *F. sporotrichoides* were cultured on Sabouraud dextrose broth medium and incubated at 25°C for 2-3 days then stored at -20°C until used for DNA extraction.

6.2. DNA extraction and PCR amplification

6.2.1. Extraction of DNA (Doohan, 1999 and Paterson, 2006)

Genomic DNA of the trichothecenes producing *Fusarium* isolates was obtained using the genomic DNA Extraction Kit (Gene JET plant Genomic DNA purification kit, cat. No. k0791 Germany) following the manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260/230 nm using SPECTRO star Nano "BMG LABTECH".

6.2.2. PCR amplification (Paterson,2006)

The used PCR primers for *Fusarium* sp. (**Bluhm *et al.,* 2002*)*** and trichothecenes producing *Fusarium* sp. (**Doohan *et al*., 1999**) were recorded in (Table 1).

Table (1). The used Primers for DNA Amplification for *Fusarium* sp. and Trichothecenes Producing Mycotoxins

Species	Primer pairs	Sequence (5′→3′)	Amplicon size (bp)
***Fusarium* sp.**	ItsF ItsR	AACTCCCAAACCCCTGTGAACATA TTTAACGGCGTGGCCGC	431 bp
Trichothecenes- producing *Fusarium* sp.	Tri5F Tri5R	AGCGACTACAGGCTTC AAACCATCCAGTTCTCCATCTG	544 bp

6.2.3. PCR amplification conditions used for Its F and Its R by *Fusarium* sp. were 5 min initial step followed by 38 cycles at 94°C for 1 min, 59°C for 1 min and 72°C for 1.5 min and a final extension step at 72°C for 5 min. While The PCR amplification protocol used for Tri5 by *Fusarium* sp. was as follows: 30 cycles at 95°C for 30 sec. (denaturation), 20 sec. at 62°C (annealing), 45 sec. at 72°C (extension). The PCR negative control reaction mixtures contained no DNA as *A. flavus*.

Amplification products were electrophoresed in agarose gels in TBE buffer with 1 ul of ethidium bromide per gel added for visualization under UV light (1.5% w/v) (Agarose, Sigma, USA), Using 100 bp DNA Ladder H3 RTU (Ready-to-Use) Cat. No. DM003-R500from Gene Direx, Inc. Company, Litwania.

6.2.4. Real-Time Quantitative polymerase chain Reaction (RT-qPCR) (Rashtchian, 1994 and Bilodeau, 2011):

The RT-qPCR mix was specific oligonucleotide primers and syber green Mix, DNA extracted according to the purification of DNA from culture Protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). The purity of DNA was assayed using the 260:280 nm ratios on a Spectrostar Nano spectrophotometer (BMG Labtech). The quantities were determined by RT- qPCR in a total reaction volume of 50 µL containing 3 µL of DNA template, 25 µL of syber Green (Trans Start Green qPCR super Mix code #AQ101-01). 20 µL PCR grade water, 1 µL of primer at a concentration of 10 pmol/µL. cycle was done in real time PCR machine (Chrom4-BIO-RAD, USA), amplification condition as described before in PCR.

7. Statistical analysis

The obtained data was statistically analyzed for the mean and standard deviation of the mean significance of the results was determined by conducting a one-way analysis of F- test and least significant difference between pair groups as well as t-student test (**SPSS 14, 2006**).

3. Results and Discussion

The animal health represents the major role in food security for human which are a worldwide especially in the subtropical and tropical regions, which have huge economic significances in food production as (milk, meat and eggs). Over the past decades, fungi of *Fusarium* sp. and their toxins gained more attention due to their dangerous effect on animal health. Whereas, the identification of *Fusarium* species in food and grain is problematic and showed some diverse (**Moss, 1991**). Currently, the differentiation of *Fusarium* sp. is based on morphological characteristics of macro-conidia and micro-conidia which consume long time. Therefore, a rapid and reliable assay for the routine identification of toxigenic *Fusarium* sp. will have great benefits in the food-processing industry.

Table (2). Incidence of *Fusarium* species in yellow corn, white corn and animal feeds

Feed samples	Total moulds			*Fusarium* species		
	No. of +ve	%	MEAN of CC	No. of +ve	%	MEAN of CC
Yellow corn (20)	18	90	$3x10^4$	7	35	$1x10^2$
White corn (20)	16	80	$3.7x10^3$	6	30	$9x10^1$
Animal feeds (20)	17	85	$6x10^4$	7	35	1x10
Total (60)	51	85		20	33.3	

In the present study, fungal examination of 60 feed samples (20 of each of yellow corn, white corn and animal feeds) for detection of *Fusarium* species incidence in the samples was carried out. The results (Table, 2) all the examined samples revealed variable rates of contamination, where, 85% of all examined samples were contaminated with different mould species included 33.3% contaminated by *Fusarium* sp. The higher incidence of *Fusarium* species was recovered from yellow corn (35%) and animal feeds (35%) followed by white corn (30%), respectively.

These findings were in agreement with the results of (**Buckley *et al*., 2007) ; Hassan *et al.,* 2015, 2016, 2017 and 2018) and El-Hamaky *et al.,* (2016)**, who recovered most of these fungi from the examined feed and water samples.

Currently, the *Fusarium* species and respective mycotoxins are widely distributed through the world and cause significant economic losses in animal production due to its capability of killing cells through damaging the cellular membrane (**Abou-Elyazeid *et al.,* 2011**).

Cvetnic *et al.* (2004) found that *Fusarium* sp. were the most common fungi in maize (78.6%) in 1999 and (85%) in 2003 in Croatia.

Some moulds of *Fusarium* were detected in sixty percent of diseased sheep and their feeds and water at some desert districts in Egypt. The most predominant isolates belong to members of genus *Aspergillus* with a range of (5-100%), followed by *Fusarium* sp. with a range of (40-90%), *Penicillium* sp. with a range of (10-55%) and *Mucor* sp. with a range of (10-50%) (**Hassan *et al.,* 2010 a)**.

Table (3). Identification of members of *Fusarium* sp. isolated from yellow corn, white corn and animal feeds:

Fusarium sp.	Yellow corn(20)		White corn (20)		Animal feeds(20)		Total(60)	
	No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of + ve	%
F.poae	2	10	1	5	3	15	6	10
F. equiseti	1	5	1	5	1	5	3	5
F. graminearum	1	5	1	5	1	5	3	5
F. sporotrichoides	3	15	3	15	2	10	8	13.3
Total	7	35	6	30	7	35	20	33.3

However, the identification of *Fusarium* species yielded that the *F. equiseti* and *F. graminearum* were recovered from (5%) of yellow corn, white corn and animal feeds (Table, 3). Whereas, *F. sporotrichoides* was recovered from (15%) of examined yellow corn and white corn samples.

On the other hand, the isolation of all examined feed samples(60) was (10%) for *F. poae,* (13.3%) for *F. sporotrichoides*, (5%) for *F. equiseti* and *F. graminearum.*

While, over 33.3 % of feed samples yielded *Fusarium* sp., most isolates were *F. sporotrichoides* recovered from (13.3%) followed by *F. poae* (10%) and *F. equiseti* and *F. graminearum* (5% for each) in the present feeds (Table, 3).

Whereas, white corn samples yielded (30%) with relative lower incidence rate than that of animal feeds and yellow corn (35%). The differences in these levels of contamination may be due to exposure of the examined samples to different climatic condition either during preparation, transportation or storage. In addition, molds of *Fusarium* sp. constituted a public health hazard due to mycotoxin production such as trichothecenes and zearalenone. Most of these mycotoxins have potential carcinogen effects on liver cells due to high long term of mycotoxins intake (**FDA, 2000**).

Similar findings were obtained by **Buckley *et al.* (2007),** who detected *Fusarium* sp. only in 2.6% of animal concentrated feed. Moreover, **Khosravi *et al.* (2008)** isolated *Fusarium* in 6% of animal feed mainly; corn seed, barley and corn silage samples.

While, **Abou-Elyazeid *et al.* (2011)** recovered twenty-one *Fusarium* sp. isolated from animal feed stuff. These isolates belong to three different *Fusarium s*pecies, namely *F. verticillioides*; *F. anthophilum* and *F. proliferatum.*

Table (4). Mean levels of *Fusarium* mycotoxins produced by the isolated *Fusarium* sp. from feeds (mg/kg of feeds) (ppm).

***Fusarium* sp. (No. of isolates)**	**Levels of Trichothecenes (mg/kg of feeds)(ppm)**												**Total Mean of Trichothecenes.**
	T_2			**DON**			**DAS**			**NIV**			
	No. of +VE	**%**	**MEAN levels ± SE**	**No. of +VE**	**%**	**MEAN levels ± SE**	**No. of +VE**	**%**	**MEAN levels ± SE**	**No. of +VE**	**%**	**MEAN levels ± SE**	**MEAN levels ± SE**
***F. poae* (6)**	4	66.6	25± 2.30	-	-	-	3	50	18.5± 3.33	2	33.3	25± 00	68.5± 5.63
***F. equiseti* (3)**	2	66.6	10± 1.0	-	-	-	-	-	-	-	-	-	10.0± 1.0
***F. graminearum*(3)**	-	-	-	-	-	-	-	-	-	-	-	-	-
***F. sporotrichoides* (8)**	5	62.5	32.5± 2.5	-	-	-	1	12.5	35± 00	1	12.5	30± 00	97.5± 2.5

*T_2, Deoxynivalenol (DON), diacetoxy-scirpenol (DAS) and nivalenol (NIV)

Table (5) Mean levels of *Fusarium* mycotoxins in feeds (mg/kg of feeds) (ppm**)**

Feeds (No. of samples)	Levels of Trichothecenes (mg/kg of feeds)(ppm)												Total Mean of Trichothe-cenes. in tested feed
	T_2			DON			DAS			NIV			
	No. of +VE	%	MEAN levels ±SE	No. of +VE	%	MEAN levels ±SE	No. of +VE	%	MEAN levels ±SE	No. of +VE	%	MEAN levels ±SE	MEAN levels ±SE
Yellow corn (20)	14	70	35 ±7.4	6	30	16.66 ±2.1	6	30	2.2± 3.5	10	50	22.2± 00	76.0± 12.9
White corn (20)	6	30	72±8. 27	8	40	43.75 ±1.30	1	5	2.5± 00	2	10	5.0± 1.50	123.25± 9.57
Animal feeds (20)	15	75	60±9. 5	11	55	2.88 ±5.4	9	45	80.76± 00	-	-	-	143.14± 14.9

*T_2, Deoxynivalenol (DON), diacetoxy-scirpenol (DAS) and nivalenol (NIV)
*permissible limits of *Fusarium* toxins in animal feeds according to **(European Commission, 2013)** were: (0.25-0.5 ppm) for $T_2$and (2-8 ppm) for Deoxynivalenol (DON).

Here, as illustrated in table (4), screening of the recovered *Fusarium* species from feeds for production of trichothecenes mycotoxins was undertaken. The isolates of *F. poae, F. equiseti* and *F. sporotrichoides* produced mean levels (68.5± 5.63, 10.0±1.0 and 97.5±2.5 ppm) of Trichothecenes mycotoxins, respectively. Regarding, members of Trichothecenes; T_2 mycotoxins; produced by *F. equiseti*, *F. poae* and *F. sporotrichoides* at mean levels of (25±2.30, 10±1.0 and 32.5±2.5ppm) respectively and T_2 toxin not produced at all in case of *F. graminearum.* Whereas, DAS and NIV were detected only during screening of *F. poae* and *F. sporotrichoides* at mean levels of (18.5±3.33 ppm DAS and 25±00 ppm NIV) and (35±00 ppm DAS and 30±00 ppm NIV), respectively.
Whereas, **Hassan *et al.* (2010 a)** detected the *Fusarium* toxins (Fumonisin B_1) in feed samples. The largest amount was estimated in crushed yellow corn (60%) namely FB_1, T_2 and zearalenone with the mean levels of (48.4±1.0; 3.0±0.1 and 0.84±0.03 ppm), respectively.

Currently, most of the examined feed samples were contaminated with Trichothecenes Fusarium toxins, particularly, T_2 toxin. Where, the samples of yellow corn, white corn and animal feed were contaminated with T_2 toxin at the rates of incidence (70, 30 and 75%) with the mean levels of (35 ±7.4, 72±8.27 and 60±9.5), respectively (Table 5).

Whereas, other Trichothecenes *Fusarium* toxins were also detected as DON, DAS and NIV in avariable rates of incidence. Regarding DON, the rates of incidence and mean levels were (16.6 ±2.1, 43.75±1.3 and 2.88±5.4ppm) in feed samples of (yellow corn, white corn and animal feeds), respectively (Table 5).
On the other hand, the permissible limits of *Fusarium* toxins in feeds according to **(European Commission, 2013)** were: (0.25-0.5 ppm) for T_2 and (2-8 ppm) for DON. Hence, all detected levels in the present feed sample were significantly over the permissible international limits and the dangerous health hazards are expected from their consumption.
All the previous literatures of fungal diseases caused by *Fusarium* sp. infection and their toxins recorded that the pollution by these fungi affected upon the growth rate. They potentiated several health problems in human and animals including aneamia, stunted growth, hemorrhagic dermatitis, pulmonary edema, carcinogenic, tremorgenic, immune suppressive and hormonal effects (**Mogda *et al.*, 2002, Hassan *et al.*, 2010 a and Abou-Elyazeid *et al.* (2011).**
In addition, the human exposure to mycotoxins has been occurred by direct ingestion of contaminated cereals and grains, meat, milk, and eggs originating from mycotoxin-exposed animals (**Wafia and Hassan, 2000 and Hassan, 2017 and 2018**).

Recently, the frequent increase in antibiotic-resistance by fungal strains is a serious public health problem and renewed interest in the use of metals nanoparticles as antifungal agent. Some metals were found to inhibit microbial growth as nanoparticles of Ag and Zn which showed strong antibacterial and antifungal activity (**Reddy, 2007 and Hassan *et al.*, 2016**). They have gained more attention due to their special properties with fewer hazards to the environment (**Violeta *et al.*, 2011**) inhibiting the growth of toxigenic fungi consequently, their ability to produce toxins (**Mouhamed *et al.*, 2015**.)

Table (6). Minimal inhibitory concentration (MIC) of Zn NPS on toxigenic *Fusarium* species isolated from feed samples:

Member of *Fusarium*	Colony count of *Fusarium sp.* at gradual concentrations of Zn NPS (µg/ml)						
	0	100	200	400	600	800	1000
F. equiseti	4.0×10^5	1.0×10^3	5.0×10	0.3×10	00	00	00
F. poae	2.0×10^5	1.0×10	0.3×10	00	00	00	00
F. sporotrichoides	7.0×10^5	2.0×10^4	2.0×10^2	1.0×10	00	00	00

In the present study, the MIC of Zn NPs for *F. equiseti, F. sporotrichoides* and *F. poae* were (600, 600 and 400 µg /ml). These variations suggested to be associated with toxigenicity of *Fusarium* species, where *F. equiseti* and *F. sporotrichoides* produced mean levels of trichothecenes (10±1.0 and 32.5±2.5 ppm) and *F. poae* (22.8±2.16 ppm) and hence, required higher MIC of Zn NPs treatments (600, 600, 400 µg /ml), respectively (Table 4,6).

Several studies showed that the antimicrobial activity of Zn NPs is influenced by the concentration and the size of their particles (**Violeta *et al.*, 2011**). While**, Hassan *et al.* (2013)** detected that the growth of aflatoxigenic moulds and aflatoxins production were inhibited by adding 8 µg/ml of ZnO NPs; while that of ochratoxin A and Fumonisin B1 producing moulds and mycotoxins production were inhibited by adding 10 µg/ml of ZnO NPs to the tested medium. The antimicrobial potentials of ZnO NPs may be due to the formation of hydrogen bond between hydroxyl group of cellulose molecules of fungi with oxygen atom of ZnO NPs; leading to inhibition of the microbial growth. In addition, the release of Zn^{2+} may occur which causes damages to the cell membrane and inter acts with intraocular contents (**Moraru *et al.*, 2003**).

Table (7). (MIC) of Ag NPS on toxigenic *Fusarium* species isolated from feed samples

Fusarium sp.	Colony count of *Fusarium* sp. at gradual concentrations of Ag NPS (µg/ml)						
	0	100	200	400	600	800	1000
F. equiseti	4.0×10^5	4.0×10^3	1.0×10	1.0×10	00	00	00
F. poae	2.0×10^5	2.0×10^2	1.0×10	00	00	00	00
F. sporotrichoides	7.0×10^5	1.0×10^3	3.0×10^2	1.0×10	1.0×10	00	00

Regarding, Ag NPs, the MIC for *F. equiseti and F. sporotrichoides* were (600 and 800 µg/ ml) and (400 µg/ml) for *F. poae,* respectively. These variation may be associated with the toxigenicity of *Fusarium* sp. member, as the toxigenicity increased, the required MIC was also increased (Table 4,7).

The silver nanoparticles exhibit higher toxicity to microorganisms without any effect on animal cells (**Kim *et al*., 2009**). While, it has been observed that AgNPs have high antimicrobial potential even when synthesized by different methods (**Alt *et al*., 2004; Kim and Kim, 2006 and Thomas *et al*., 2007**).

Furthermore, **Hassan *et al.* (2016**) illustrated that the antimicrobial potential of AgNPs against *C. albicans, A. flavus, S. aureus* and *Salmonella* sp. was concentration dependent, when the concentrations of AgNPs increased up to (300 µg /ml), the optical density of the treated spore suspension were decreased till reached 100% transmittance and clear medium. The antifungal activity of AgNPs is attributed to its effects on the fungal mycelia (**Kim *et al*., 2009**). While, **Hassan *et al.* (2016)** observed the interaction between AgNPs and the membrane structure of fungal cells by detection of significant changes to their membranes, which are recognized by the formation of pits on their surfaces, and finally cell death.

Table (8). MIC of olive oil on toxigenic *Fusarium* species isolated from the feed samples

***Fusarium* sp.**	**Colony count of *Fusarium* sp. at gradual concentrations of olive oil (µg/ml)**						
	0	**100**	**200**	**400**	**600**	**800**	**1000**
F. equiseti	4.0×10^5	3.0×10^4	2.5× 10	0.5× 10	0.3× 10	00	00
F. poae	2.0×10^5	1.0×10	0.5×10	0.1× 10	00	00	00
F. sporotrichoides	7.0×10^5	1.0×10^4	2.0×10^1	0.5×10^1	0.1× 10	00	00

Up to date, the natural oils extracted from herbal plants are widely used as antimicrobial potential. In the present study, MIC of olive oil against both *F. equiseti* and *F. sporotrichoides* species was (800 µg/ml) and (600 µg/ml) for *F. poae* (Table, 8).

Herein, MIC of all tested antifungal was detected to be associated with the toxigenicity of the treated *Fusarium* species. As when the concentration of the produced toxins increased, the required MIC of antifungal was also increased.

Regarding, several studies detected the significant antifungal potential of olive oil (**Battinelli *et al*., 2006, Pereira *et al*., 2007 and Sudjana *et al*., 2009**).These actions are related to their consistency containing phenyl ring; less lipophilicity and less skin penetration potential as compared to other antifungal agents (**Medina *et al*., 2011**). While, **Ansari *et al*. (2016)** regarded that olive oil has antifungal potential in addition to acting as natural, non-irritating, non-toxic permeation enhancer. Other studies regarded the antifungal potential of olive oil against *A. niger, C. albicans* and *Rh. stolonifer* **(Upadhyay *et al*., 2011 and Upadhyay, 2014)**. On the other hand, **Neveen (2006)** evaluated the antifungal potential of ozonized olive oil against most pathogenic fungi as *A. fumigatus, C. albicans, E. floccosum,* and *T. rubrum*. She added that the MIC ranged from (0.53 to 2.0 mg/ ml) *C. albicans* and *T. rubrum* and decrease in *M. canis* (0.03 g/100 ml) under oleozone effect. The mode of antimicrobial action of olive oils is due to their effect in increasing the permeability of microbial cells leading to leakage of fluid material in microbial cells and hence, death of cells (**Lamber *et al.,* 2001 and Walsh *et al.,* 2003**). Therefore, essential oils particularly, olive oil have antimicrobial potentials against wide range of pathogens (**Upadhyay *et al.,* 2010**).

Recently, the molecular biology techniques used PCR to detect the genomes of toxigenic fungi as *F. graminearum* and *F. verticilliodes*

(**Sarlin *et al.*, 2006 and Abouel-Yazid *et al.*, 2011)**. Hence, the sensitive and specific nested PCR assay as well as the rapid and quantitative Light Cycler PCR assay might be useful for the diagnosis and monitoring of fungal infections. **Hassan *et al.* (2017)** investigated the bio-control of some aflatoxigenic strains and resulted that the genes responsible for AFTs production, lost their role in AFTs biosynthesis pathway and inactivation of these genes may occur. While, for other aflatoxigenic isolates; after bio-control by *B. subtillus* and *C. albicans*; no genes were detected by PCR and no levels of AFTs detected by chemical chromatography indicating a successful bio-control.

In the present study, the antifungals as Zn NPs, Ag NPs and olive oil were used for controlling the growth of trichothecenes producing *Fusarium* and these activities were evaluated by molecular detection for the changes in genes density and structure, before and after treatments. A purified PCR-DNA product of the Amplified Internal Transcribed Spacer gene Forward and Reverse (Its F and Its R) using primers of *F. poae*, *F. equiseti* and *F. sporotrichoides* (Table, 1) at 431-bp (photo1-3).The DNA bands of each used isolates were similar to the general characters of a standard reference *Fusarium* species (see Lane 2 of fig. 1-3). Whereas, treating of the isolates by low (400 µg/ml) and high (600 µg/ml) doses of Zn NPs eliminated the signals of DNA bands by traditional PCR (Lane 4&5 in Fig. 1-3).

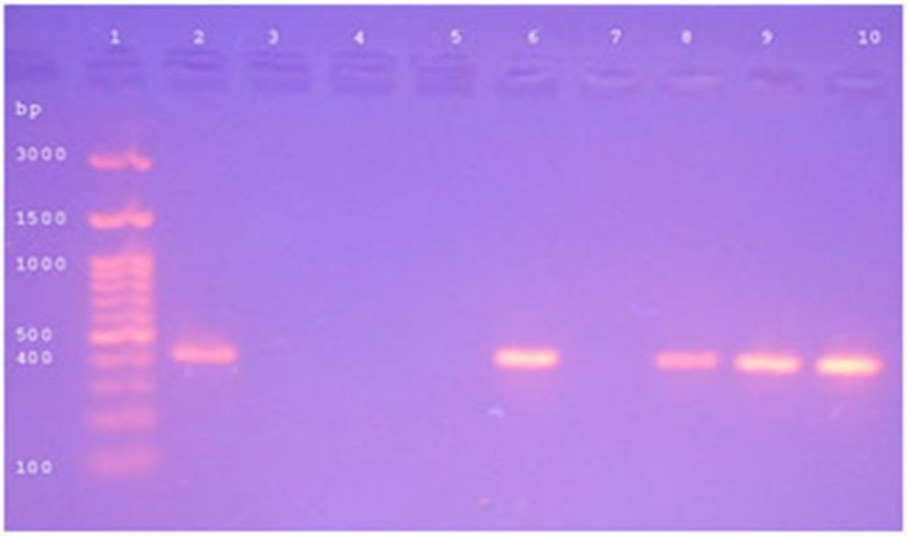

Figure (1). Electrophoretic pattern of Trichothecenes producing *F. poae*
Lane 1: 100 bp ladder, Lane 2: control positive, Lane 3: control negative (*A.flavus*), Lane 4*: Fusarium* after treatment with Zn NPs low dose, Lane 5 *Fusarium* after treatment with Zn NPs high dose,
Lane 6 : *Fusarium* after treatment with Ag NPs low dose, Lane 7: *Fusarium* after treatment with Ag NPs high dose, Lane 8 *Fusarium* after treatment with olive oil low dose , Lane 9: *Fusarium* after treatment with olive oil high dose, Lane 10: control positive.
N.B. Low doses for: Zn NPS (400µg/ml), Ag NPs (600µg/ml), olive oil (600µg/ml) - High doses for: Zn NPS (600µg/ml), Ag NPs (800µg/ml), olive oil (1000µg/ml)

Figure (2): Electrophoretic pattern of Trichothecenes producing *F. equiseti*
Lane 1: 100 bp ladder, Lane 2: control positive, Lane 3: control negative (*A.flavus*), Lane 4*: Fusarium* after treatment with Zn NPs low dose, Lane 5 *Fusarium* after treatment with Zn NPs high dose, Lane 6: *Fusarium* after treatment with Ag NPs low dose, Lane 7: *Fusarium* after treatment with Ag NPs high dose, Lane 8 *Fusarium* after treatment with olive oil low dose, Lane 9: *Fusarium* after treatment with olive oil high dose, Lane 10: control positive.
N.B. Low doses for: Zn NPS (400µg/ml), Ag NPs (600µg/ml) , olive oil (600µg/ml) - High doses for : Zn NPS (600µg/ml) , Ag NPs (800µg/ml) , olive oil (1000 µg/ml)

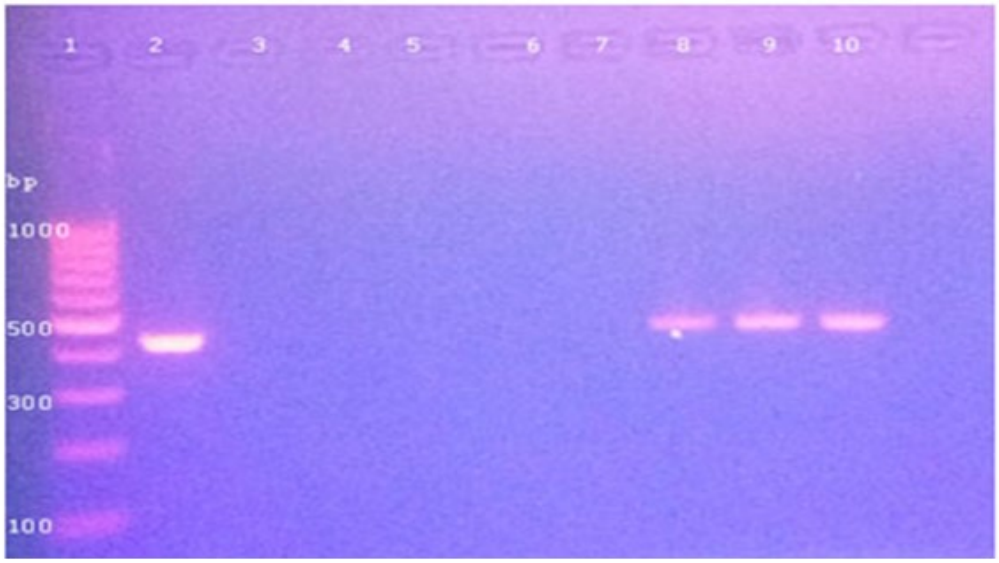

Figure (3): Electrophoretic pattern of Trichothecenes producing *F. sporotrichoides*
Lane 1: 100 bp ladder, Lane 2: control positive, Lane 3: control negative (*A.flavus*),Lane 4*: Fusarium* after treatment with Zn NPs low dose, Lane 5 *Fusarium* after treatment with Zn NPs high dose, Lane 6:*Fusarium* after treatment with Ag NPs low dose, Lane 7: *Fusarium* after treatment with Ag NPs high dose, Lane 8: *Fusarium* after treatment with olive oil low dose, Lane 9: *Fusarium* after treatment with olive oil high dose, Lane 10: control positive.
N.B. Low doses for: Zn NPS (400µg/ml), Ag NPs (600µg/ml), olive oil (600µg/ml) - High doses for: Zn NPS (600µg/ml) , Ag NPs (800g/ml) , olive oil (1000µg/ml)

On the other hand, treatment of the isolated *Fusarium* sp. with a low dose of Ag NPs (600 µg/ml) didn't affect the expression signal of DNA bands of treated *F. poae* and *F.* equiseti (Lane 6 in Fig. 1-2). But, the treatments of *F. poae* by high doses of Ag NPs (800 µg/ml) prevent the formation of DNA-PCR band (Lane 7 in Fig. 1). Similarly, treating the tested *F. equiseti* by high doses of Ag NPs (800 µg/ml) didn't affect the DNA bands. (Lane 7 in Fig. 2).But, similar dose of Ag NPs removed the signals of DNA fragments of *F. sporotrichoides* (Lane 6, 7in fig. 3).

Currently, using of low and high doses of treatment for all tested *Fusarium* sp. by olive oil didn't affect the expression of DNA bands (Lane 8, 9 in all fig. 1-3).

From the present results of DNA-PCR, it is become apparent that despite inhibiting the fungal growth by all low and high doses of the used antifungals by plate agar diffusion tests, the expression of DNA-PCR bands was completely disappeared by the tested antifungal as Zn NPS (Lane 4&5 in Fig. 1-3). While, no affection for signals of DNA-PCR bands even complete inhibition occurred by plate agar diffusion test (Tables 6, 7, 8 and Lane 8, 9 in all fig. 1-3).

Therefore, all the treated isolates of *Fusarium* sp. lost their ability for growth as detected by agar diffusion method. However, the DNA-PCR from these treated isolates showed some differences, where the corresponding DNA genes detected by PCR but the other isolates showed no growth and their viability in diffusion agar tests were completely inhibited. While, in other cases the treated strains genes were eliminated after treatment. Zinc nanoparticles were the only antifungal which showed *Fusarium* growth inhibition and disappearance of fungal DNA when examined by PCR.

On the other hand, the Real-time qPCR technologies are successfully used in detection and quantitative estimation of fungal DNA and their toxins. In addition to the conventional PCR, help in generation of a specific fluorescent signal in real-time analysis and quantification of DNA targets (**Schena *et al.*, 2004**).

Currently, Real-Time Quantitative PCR(RT-qPCR)*Tri5* system was applied on DNA extracted from the tested *F. equiseti, F. poae* and *F. sporotrichoides* at (544-bp) to detect their expression efficacy and molecular weight before and after treatments with antifungals) Zn NPs, Ag NPs & olive oil) (Tables 9, 10, 11).

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 AFB1 production. They added that, the molecular detection should give critical information on the activation or inactivation of specific genes involved in toxin biosynthesis.

Table (9). Real Time quantitative PCR (RT-qPCR) for detection of *Tri5* gene expression of the treated trichothecenes producing *F.poae* by Zn NPs, Ag NPs and olive oil.

***Fusarium* sp. DNA**	***F. poae***					
	Eff.%	**Mole**	**C.T**	**Eff.%**	**Mole**	**C.T**
	Low doses			**High doses**		
***Fusarium* sp. DNA without treatments (standard)**	97.5%	105.5	15.21	97.5%	105.5	15.21
***Fusarium* sp. DNA treated with Zn NPs**	89.2%	0.0024	27.41	83.9%	0.0015	22.88
***Fusarium* sp. DNA treated with Ag NPs**	86.5%	8.739	18.01	65.9%	0.0122	25.14
***Fusarium* sp. DNA treated with olive oil**	83.0%	0.2054	22.22	80.7%	0.1816	22.08

C.T.: Cycle Threshold – Mole: Mole, weight of DNA (µg/ml) -Eff.%: Efficacy % of *Tri5* expression Low doses of: Zn NPS (400µg/ml), Ag NPs (600µg/ml), olive oil (600µg/ml) - High doses of: Zn NPS (600µg/ml), Ag NPs (800µg/ml), olive oil (1000µg/ml)

Table (10). Real Time quantitative PCR (RT-qPCR) for detectionof *Tri5* gene expression of the treated trichothecenes producing *F. equiseti* by Zn NPs , Ag NPs and olive oil

Samples of *Fusarium* sp. DNA treated with :	***F. equiseti***					
	Eff.%	**Mole**	**C.T**	**Eff.%**	**Mole**	**C.T**
	Low doses			**High doses**		
***Fusarium* sp. DNA without treatments (standard)**	95.6%	103.41	18.1	95. 6%	103.41	18.1
***Fusarium* sp. DNA treated with Zn NPs**	85.83%	0.017	22.24	83.45%	0.007	25.72
***Fusarium* p. DNA treated with Ag NPs**	90.87%	0.529	22.41	85.26%	0.1516	21.05
***Fusarium* sp. DNA treated with olive oil**	91.77%	41.41	16.33	86.65%	38.93	13.90

C.T. : Cycle Threshold – Mole : Mole, weight of DNA (µg/ml) -Eff.%: Efficacy %of *Tri5* expression Low doses of: Zn NPS (400µg/ml), Ag NPs (600µg/ml), olive oil (600µg/ml) - High doses of: Zn NPS (600µg/ml) , Ag NPs (800µg/ml) , olive oil (1000µg/ml)

Table (11). Real Time quantitative PCR (RT-qPCR) for detection of *Tri5* gene expression of the treated trichothecenes producing *F. sporotrichoides* by Zn NPs, Ag NPs and olive oil.

***Fusarium* sp. DNA**	***F. sporotrichoides***					
	Eff.%	**Mole**	**C.T**	**Eff.%**	**Mole**	**C.T**
	Low doses			**High doses**		
***Fusarium* sp. DNA without treatments (standard)**	96.3%	104.10	17.4	96.3%	104.10	17.4
***Fusarium* sp. DNA treated with Zn NPs**	82.08%	0.00026	29.31	75.4%	0.00015	28.76
***Fusarium* sp. DNA treated with Ag NPs**	81.86%	0.03386	24.04	73.98%	0.0008095	22.82
***Fusarium* sp. DNA treated with olive oil**	84.49%	1.144	20.22	63.33%	0.1038055	28.09

C.T.: Cycle Threshold – Mole: Mole, weight of DNA (µg/ml) -Eff.%: Efficacy %of *Tri5* expression
Low doses of: Zn NPS(400µg/ml), Ag NPs(600µg/ml), olive oil(600µg/ml) - High doses of: Zn NPS (600µg/ml), Ag NPs(800µg/ml), olive oil (1000µg/ml)

Recently, RT-PCR has allowed rapid, sensitive, specific and high detection and quantification of mycotoxins. However, direct DNA detection of fungi is often difficult because of the PCR inhibitors, low inoculum density and inadequate sampling techniques **(Bilodeau, 2011)**.

As the first step in this direction, the present results of RT-qPCR detected that as the treatment doses of antifungal increased, the *Tri5* gene expression efficacy, molecular weight of DNA and toxin production were decreased (Table, 9, 10, 11). Where, in case of treatments of *F. poae, F. equiseti* and *F. sporotrichoides* with Zn NP sat low and high doses, the *Tri5* gene expression efficacy were decreased from (97.5, 95.6 and 96.3%standard non-treated isolates) to (89.2 and 83.93%) (85.8and 83.4%) and (82.1 and 75.4%), respectively. While, the treatment with Ag NPs at high and low doses resulted in a significant decrease in the *Tri5* gene expression efficacy of DNA (for *F. poae, F. equiseti* and *F. sporotrichoides)* from (97.5, 95.6 and 96.3% for the standard non-treated isolates) to (86.54 and 65.97%) (90.87and 85.26%) and (81.86 and 73.98%), respectively. Whereas, the antifungal effect of olive oil on *F. poae, F. equiseti* and *F. sporotrichoides* also caused decrease in *Tri5* gene expression efficacy of DNA from (97.5, 95.6 and 96.3% for the standard non-treated isolates) to (83.71 and 80.05%), (91.77 and 86.65%) and (84.49 and 63.33%), respectively (Tables, 9, 10, 11).

Otherwise, the treatments of trichothecenes producing *Fusarium a*s (*F. poae, F. equiseti* and *F. sporotrichoides)* by Zn NPs,Ag NPs and olive oil could adversely affect the efficacy of *Tri5* gene expression and hence, its ability for mycotoxin production as shown in traditional PCR (Table, 4, 5, Fig. 1-3). Herein, the decrease in *Tri5* gene expression is accompanied with inverse increase in their cycle threshold (Table, 9, 10, 11).

It is interesting to report here that, there are high significant amounts of trichothecenes mycotoxins production by the non-treated *Fusarium* sp. (*F. poae, F. equiseti* and *F. sporotrichoides)* (Table 4) also, their*Tri5* expressions efficacy was relatively high (Tables 9, 10, 11). Similar findings were obtained by several studies (**Hohn and Beremand, 1989, Hutcheon and Jordan, 1992, Hohn *et al*., 1993 and Glass and Donaldson, 1995**) showed that,

both reduced *Fusarium* biomassin wheat.
In addition**, Doohan *et al.* (1999)** detected an increase in *Tri5* expression following treatment of *Fusarium culmorum* with fungicides, and an inverse relationship between *Tri5* expression and biomass. They added that, there was a direct power relationship between *Tri5* expression and DON produced by *Fusarium culmorum*.
RT- qPCR technologies open increasing opportunities to detect and study phyto-pathogenic and antagonistictoxigenic fungi. They combine the sensitivity of the conventional PCR with the generation of a specific fluorescent signal providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets and detection of the efficacy of their control.

Conclusion

Fusarium species produce trichothecenes that cause some degree of acute toxicity when consumed in high amounts and are potential carcinogens. Therefore, a frequent testing program of the animal feeds and other environmental factors for fungal and mycotoxin contaminations must be undertaken. Hence, the regulation of mycotoxins biosynthetic gene expression of toxigenic moulds became a critical demand as it is a successful method for controlling of these fungi; preventing their activity and inhibiting their ability for mycotoxins production. The genetic and genomic resources will significantly enhance our understanding of the mechanisms of mycotoxins production and pathogenicity of the fungus for human and animals. Otherwise, treatment of trichothecenes producing *Fusarium* sp. as *F. poae, F. equiseti*and *F. Sporotrichoides* by Zn NPs,Ag NPs and olive oil could significantly decreased the efficacy of *Tri5* gene expression and hence, decreased their ability for mycotoxin production. Herein, the decrease in *Tri5* gene expression accompanied with inverse increase in their cycle threshold. More investigations are required to detect the effect of different doses of antimycotoxins on degradation of mycotoxins genes and their effect on the regulation of *Tri5* gene expression. In addition, further studies are needed for quantitative detection of different mycotoxins of mycotoxigenic-producing fungal species by quantitative RT-PCR assays.

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