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DETECTION OF TRICHOTHECENS CHEMOTYPE PRODUCED BY *FUSARIUM GRAMINEARUM* ISOLATED FROM GRAINS USING SPECIFIC PCR ASSAYS

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The present study aims at using several techniques in detecting some isolates of the Fusarium fungi and testing their ability to produce tricothecencs toxin and detection of the genes responsible for production of trichothecenes (Nivalenol) in *Fusarium graminearum* by using the PCR and Real time PCR. The eight isolates of *Fusarium* spp. are isolated from clinical and environmental sources. The clinical isolates are isolated from a cows lung, while the environmental isolates are isolated from dry grains of wheat and barley. These fungi are cultured on Sabouraud Dextrose agar (SDA). The isolates are identified depending on their morphological characteristics (cultural and microscopical). Concerning the molecular part of the study, genomic DNA in extracted from all *Fusarium graminearum* isolates. These genomic DNA samples are found to have suitable concentration and purity for Real time PCR technique. The genes sites are amplified (Tri4, Tri6, Tri10) in the targeted DNA by using Real time PCR. One the first hand, the results of macroscopic and microscopic examination of the *Fusarium graminearum* showed that colonies had a cottony appearance at the beginning of growth and then whitely turned into irradiated which was floral and had the ability to form abundant conidial structures with a large banana shape in microscopic examination . On the other hand, the average yield of DNA was in the range of (96.9-181.2) ng/µl with purity of (1.6-1.8). Three structural genes have been observed in the present study; Tri4, Tri6 and Tri10. These genes are involved in the biosynthesis of trichothecens (Nivalenol), the regulating gene that plays a major role in the production of trichothecens.

Key words: Trichothecens, *Fusarium graminearum* Tri4, Tri6 and Tri10 genes.

Introduction

The name of *Fusarium* comes from Latin fusus, meaning a spindle, is a large genus of filamentous fungi. It is a part of a group often referred to as hyphomycetes which is widely distributed in soil and associated with plants. Most species are harmless saprobes, and are relatively abundant members of the soil microbial community. Some species produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain. The main toxins produced by these *Fusarium* species are fumonisins and trichothecenes (Desjardins & Proctor, 2007). Toxins are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize. They include a range of mycotoxins, such as: the fumonisins, which affect the nervous systems of horses and may cause cancer in rodents; the trichothecenes, which are most strongly associated with chronic and fatal toxic effects in animals and humans; and zearalenone, which is not correlated to any fatal toxic effects in animals or humans. Some of the other major types of *Fusarium* toxins include: beauvercin and enniatins, butenolide, equisetin, and fusarins (Proctor, 2007).

Fusarium head blight (FHB) caused by Fusarium species is a devastating disease of wheat (*Triticum aestivum*) worldwide. Mycotoxins such as deoxynivalenol (DON) produced by *Fusarium graminearum* negatively affect plant and animal health, and cause significant reductions in grain quantity and quality (Authors *et al*., 2017).

Trichothecenes are a very large family of chemically related mycotoxins produced by various species of Fusarium, Myrothecium, Trichoderma, Trichothecium, Cephalosporium, Verticimonosporium and Stachybotrys trichothecenes belong to sesquiterpene compounds. The most important structural features causing the biological activities of trichothecenes are: the 12,13-epoxy ring, the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene nucleus and the structure and position of the side-chain. They are produced many different grains like wheat, oats or maize by various Fusarium species such as *F. graminearum, F. sporotrichioides, F. poae* and *F. equiseti* (Etzel, 2002). Some species of Fusarium can produce mycotoxins during food processing procedures that facilitate fungal growth, such as the malting of barley. In many of studies were to develop a 5′ fluorogenic (Taqman) real-time PCR assay for group-specific detection of trichothecene and fumonisin producing Fusarium spp. and to identify *Fusarium graminearum* and *Fusarium verticillioides* in field-collected barley and corn samples, Primers and probes were designed from genes involved in mycotoxin biosynthesis (TRI6 and FUM1), and for a genus-specific

internal positive control, primers and a probe were designed from Fusarium rDNA sequences (Bluhmb *et al*., 2004).

Molecular characterization of trichothecene mycotoxin biosynthesis pathways has revealed the mycotoxin gene clusters and their regulations (Schollenberger and Drochner 2007) Abundances of the transcripts of Tri4, Tri6 and Tri10 were analyzed using the ABI 7500 real-time PCR system, The expression of three representative Tri cluster genes (Tri4, Tri6, and Tri10) and T-2 toxin production are controlled by Tri10, a regulatory gene in the cluster, Disruption of the Tri10 coding sequence dramatically reduces the transcription Tri genes and effectively blocks T-2 toxin production. Disruptions in the region upstream of the Tri10 coding sequence result in T-2 toxin hyperproduction and coordinated over expression of Tri10 and the other two Tri genes (Tag *et al*., 2001).

Materials and Methods

Isolation and Identification of *Fusarium* spp: Eight isolates of the *fusarium* fungi were isolated from different sources, seven samples were environmental (3Barley and 4wheat) and one clinical sample was obtained from the Veterinary Medicine / University of Baghdad and the Agricultural Research Center (Table 1), isolated from sample and their morphological characterization of the strains was examined based on their growth on potato dextrose agar (PDA) and(SDA).

Table 1 : *Fusarium graminearum* isolates which recovered from different source and origin

Isolate No.	Isolate origin	Source of isolate
Fus1	cows Lung	Clinical
Fus 2	Barley	Environmental
Fus 3	Wheat	Environmental
Fus 4	Barley	Environmental
Fus 5	Wheat	Environmental
Fus 6	Wheat	Environmental
Fus 7	Wheat	Environmental
Fus 8	Barley	Environmental

DNA extraction : 1-DNA was extracted 0.5 g of fungal mycelia, 2-Spores were harvested from 5 days growing cultures in PDA. 3-Mycelium / spores were transferred into a mortar, frozen in liquid nitrogen and were ground well, the genomic isolation kit was provided by Wizard Genomic DNA Purification Kit (Promega, USA).

Molecular Identification: PCR analysis Abundance of the transcripts of the genes Tri4, Tri6 and Tri10 was evaluated in 0.5 lL of each cDNA solution, by PCR. PCR reaction was carried out at 95 1C for 30 s, followed by 55 cycles of 95 1C for 30 s, 55 1C for 30 s and 72 1C for 30 s, Results were obtained from three replicate experiments. Primer pairs for amplifying Tri10 were designed as previously described (Ponts *et al*., 2007). The other tow primer pairs were designed based on the sequences of F. strain Tri4 (GenBank Accession no. EF685280), and Tri6 (GenBank Accession no. EF685281). The primers used for real-time PCR are listed in (Table 2). All cDNA samples were checked for residual genomic DNA using the primers pair that was designed in two different exons, the absence of nonspecific PCR amplification or primer-dimer formation was checked by running melting curve and agarose gel analysis of the final PCR product.

Table 2 : Names and sequences of the primers used in this study by Jiao *et al.* (2008)

Gene	Sequence forwards (50–30)	Sequence reverse (50–30)	Tm (C)
Tri4	Tri4-F TATTGTTGGCTACCCCAAGG	Tri4-R TGTCAGATGCGCCTTACAAA	58
Tri6	Tri6-F AGCGCCTTGCCCCTCTTTG	Tri6-R AGCCTTTGGTGCCGACTTCTTG	58
Tri10	Tri10-F TCTGAACAGGCGATGGTATGGA	Tri10-R CTGCGGCGAGTGAGTTTGACA	58

Results and Discussion

The isolates are cultured on SDA medium and supplied with 0.05 g/l chloramphenicol. The growth reaches to the edge of the petri dishes after a period of time estimated one week. Colonies of *Fusarium* spp. on SDA show a rapid growth compared with colonies that cultured on PDA. The diameter of the colony reaches to 9 cm within a week when grows on SDA and 7 cm when grows on PDA at 37°C . The color of this colony appeared as cottony aerial mycelium when young but soon becomes white in the front direction. Their color is floral in the opposite direction (Fig. 1). For the study of optical microscopy, it is a large banana-like form (Fig. 2). This is in consistent with what Bryan *et al*. (2013) pointed out. The other characteristics of the fungus are then complemented by the classification keys of the fusarium genera that are mentioned by John and Brett, (2006).

Fig. 1 : *Fusarium graminearum* grown on SDA at 28 °C after 7 day

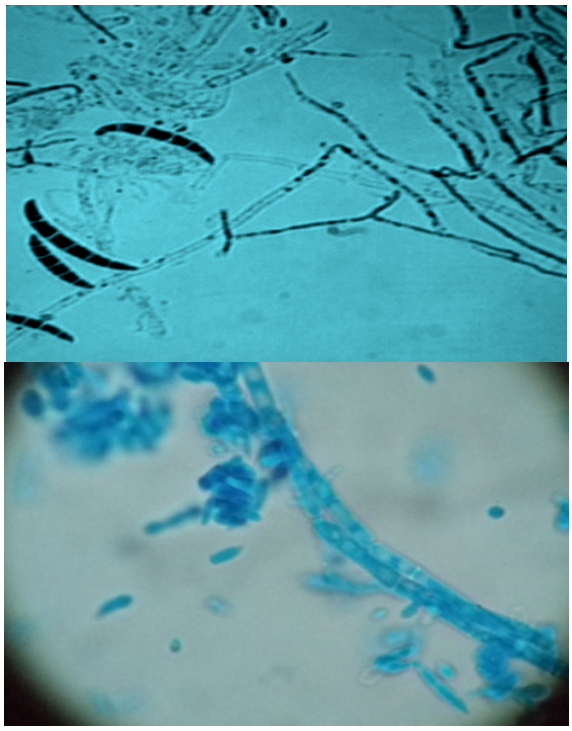

Fig. 2 : Microscopic features of *Fusarium Graminearum* stained with lactophenol cotton blue (40X)

Concentration and purity of extracted DNA from *Fusaruim graminearum* isolates

The total genomic DNA is extracted efficiently from studied isolates of *Fusaruim* spp. by using DNA extraction by Wizard Genomic DNA Purification Kit (Promega, USA). Concentration and purity of DNA are measured by using nanodrop apparatus. The yield of extracted DNA from *Fusaruim graminearum* isolates is in the range of (96.9-181.2) ng/µl with purity (1.6-1.8), (Table 3). These results corresponded with the results obtained by González-Mendoza *et al*. (2010), which pointed out the purity between (1.8-1.9) on the genetic diversity among some *Fusaruim graminearum* isolates by using Real-Time PCR. While these results are against what Kamel *et al*. (2003) found as the ratio ranged between (4-6). The process of extracting DNA from fungi is a complex process because it contains the cellular wall which composed of polysaccharide and chitin. The tissue is frozen in liquid nitrogen and then crushed using a mortar and pestle. because of the tensile strength of the chitin and other polysaccharide comprising the cell wall, this method is the fastest and most efficient way to access DNA with the *Fusaruim graminearum* The basic idea is that individual cells or masses of cells when frozen at very low temperatures (-196 C) crack easily under low-impact force (Almakarem *et al*., 2012). In the present study, DNA is precipitated by isopropanol then washed with washing buffer which contained ammonium acetate. Finally, the DNA is dissolved in TE buffer and preserved in -20°C (Moťková and Vytřasová, 2011). The total genomic DNA is shown in (Table 3).

Table 3 : DNA concentration and purity of each isolate after estimation by nanodrop technique

Sample ID	Sample Type	Con (ng/ul)	Abs 260	Abs 280	260/280
Fus.1	cDNA	134.8	0.796	0.466	1.71
Fus. 2	cDNA	102.7	0.874	0.536	1.63
Fus.3	cDNA	155.1	9.163	5.219	1.76
Fus.4	cDNA	96.9	1.938	1.115	1.74
Fus. 5	cDNA	181.2	3.624	2.098	1.73
Fus. 6	cDNA	98.3	4.226	2.415	1.75
Fus. 7	cDNA	102.3	1.386	0.768	1.8
Fus. 8	cDNA	99.6	1.173	0.641	1.83

The results of the present study showed that DNA is obtained for the purpose of use in the test of Real Time PCR and the results of DNA extracts for isolates are good. In addition, the safety and purity of DNA isolates are examined based on the bands that appeared by using the electrophoresis on agarose gel with a concentration of 1% for an hour (Figure 3).

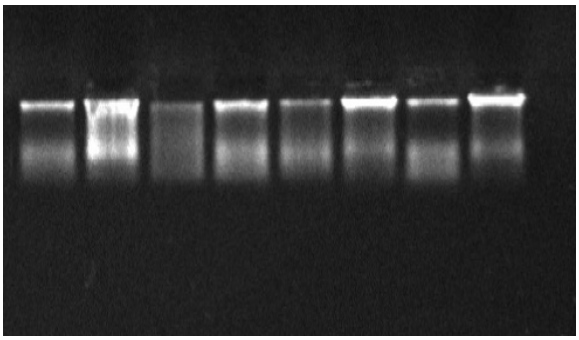

Fig. 3 : Agarose gel electrophoresis of total genomic DNA isolated that are extracted by a commercial kit (Wizard Genomic DNA Purification). Fragments are fractionated by electrophoresis on 1% agarose gel (1hr, 5v/cm, 1xTris borate buffer) and visualized under U.V light after staining with ethidium bromide. PCR Analysis

A rapid procedure based PCR of specific genes encoding for proteins involving in mycotoxins biosynthesis is developed which help to identify the chemotypes of fungal species through a rapid way. Molecular studies of *Fusarium graminearum* revealed that a gene cluster is responsible for Trichothececns production. Their presence in a species can be investigated using PCR to identify ability of a particular

maycotoxin biosynthesis and also chemotypes of the fungi (Yoruk *et al.*, 2016). Since wheat and barley grains are widely used in our country, it is necessary to identify the fungi that cause pollution as well as determination of chemotypes of the Fusarium isolates using the PCR-based molecular analyses.

Molecular characterization of trichothecene mycotoxin biosynthesis pathways has revealed the mycotoxin gene clusters and their regulations (Schollenberger and Drochner, 2007). Abundances of the transcripts of Tri4, Tri6 and Tri10 are analyzed using the ABI 7500 real-time PCR system. PCR reaction is carried out at 95 1C for 30 s, followed by 40 cycles of 94 1C for 10 s, 58 1C for 15 s and 72 1C for 35 s.

The expression of three representative Tri cluster genes (Tri4, Tri6 and Tri10), and T-2 toxin production are controlled by Tri10, a regulatory gene in the cluster. Disruption of the Tri10 coding sequence dramatically reduces the transcription Tri genes and effectively blocks T-2 toxin production. Disruptions in the region upstream of the Tri10 coding sequence result in T-2 toxin hyperproduction and coordinated over expression of Tri10 and the other two Tri genes (Tag *et al*., 2001)

Tri10 is hypothesized to control all other Tri genes, both inside and outside the Tri gene cluster, in part by positively regulating Tri6, Tri6 encodes previously characterized Cys2His2 zinc finger DNA-binding protein that functions as a pathway-specific transcription factor and positively regulates the other Tri genes (15, 25). Both Tri10 and Tri6 also control transcript levels for the gene encoding farnesyl pyrophosphate synthetase (FPPS). Since FPPS catalyzes the last step in the isoprenoid biosynthetic pathway. The primary metabolic pathway lead to trichothecene biosynthesis. We further hypothesized that Tri10 and/ or Tri6 regulate the expression of additional isoprenoid biosynthetic genes (Hohn *et al*., 1999). The present study shows that 50 % of isolates (4) are positive for the Real Time PCR test while the remaining 50% gives negative results for the same test. This shows that the isolates that give negative results are unable to produce trichothecene toxin.

Detection of *Tri4, Tri6, Tri10* by PCR in Samples study:

All samples are subjected to PCR testing as shown in (Fig 4 – 5 – 6)

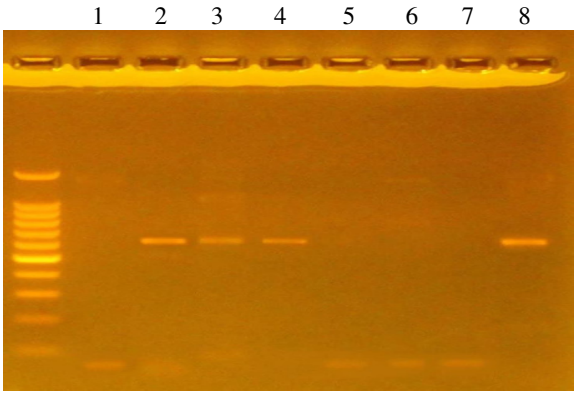

Fig. 4 : PCR product gel electrophoresis of *Tri 6* gene of of cytochrome P450 in agarose 2% and voltage 70V for 75 min . Lan1 ladder ,lan 1-8 PCR product indicate the gene with size 618bp.

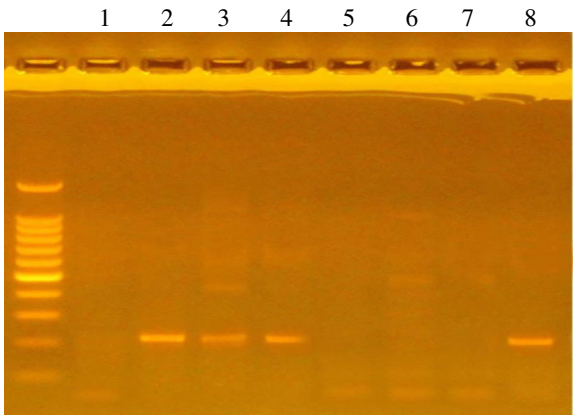

Fig. 5 : PCR product gel electrophoresis of *Tri 4* gene ofcytochrome P450 in agarose 2% and voltage 70V for 75 min . Lan1 ladder ,lan 1-8 PCR product indicate the gene with size 201bp.

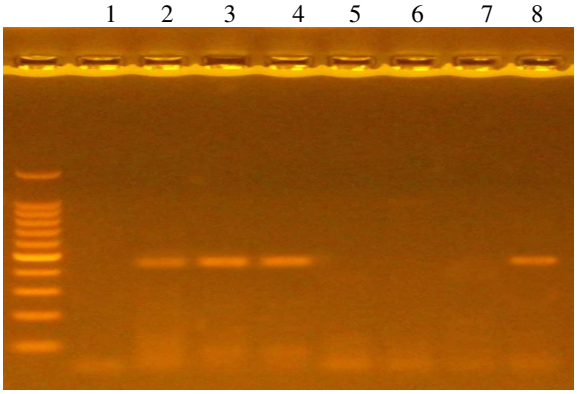

Fig. 6: PCR product gel electrophoresis of *Tri 10* gene of cytochrome P450 in agarose 2% and voltage 70V for 75 min . Lan1 ladder ,lan 1-8 PCR product indicate the gene with size 481bp.

The Role of *Tri 4* In Synthesis of Trichothecenesnivalenol (NIV) in Real Time PCR:

Amplification of Tri4 gene targeted by PCR and electrophoresis by agarose gel electrophoresis shows that out of 8 isolates of *Fusarium* spp., 4 (50%) isolates of them are positive for Tri4 gene. While 4 (50%) isolates are negative, Table (4) (Figure 7).

Table 4 : Real time Cq*Tri 4* in trichothecenesnivalenol (NIV)

Well	Col.	Sample Name	Cq	Efficiency	Efficiency R^2	Result
1		1	-	-	-	Exc.
2		2	34.17	0.68	0.99469	
3		3	34.68	0.66	0.99286	
4		4	35.38	0.55	0.99336	
5		5	-	-	-	Exc.
6		6	-	-	-	Exc.
7		7	-	-	-	Exc.
8		8	34.58	0.78	0.99485	

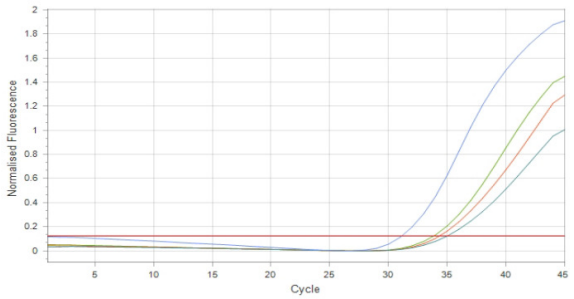

Fig. 7 : Real time Cq*Tri 4* in trichothecenesnivalenol (NIV)

This is due to knowledge of important genes, and the central region of the trichothecene gene cluster contains two pathway and two regulatory genes (four Tri genes) that are necessary for forming the trichothecene skeleton. One of these genes, Tri4, encodes a multifunctional cytochrome P450 monooxygenase. The expression of this gene is highly induced under trichothecene-inducing conditions. Thus, we can conclude that the toxin of the promoter in the gene cluster is important for native transcriptional regulation of Tri4 in trichothecene biosynthesis, it is considered the basic rule in the composition of the toxin of the trichothecence toxin by the fusarium fungi .This is true to what is found by Takeshi *et al.* (2013). It is therefore possible to reach the inability of the Tri4 promoter to cause heterologous gene expression from a non-native locus that may suggest the necessity of additional elements located in the coding region of Tri4 for transcriptional activation of its own gene. Alternatively, the physical linkage of Tri genes or chromatin remodeling at the central region of the gene cluster may have a regulatory role in the epigenetic activation of Tri4 and some other Tri genes Palmer and Keller (2010).

In this study, it is found that a gene located in the central region of the trichothecene gene cluster is subject to position-dependent regulation and that the promoter does not function when placed at the end of the gene cluster.

The Role of *Tri 6* in Synthesis of Trichothecenesnivalenol (NIV):

The results of the present study show positive results for gens Tri6 in isolates (Tri 2, Tri3, Tri4, Tri8) when examined by Real Time PCR and in percentages ranging from (37.95-50.91). This gene is considered encode key factors for trichothecene biosynthesis pathway .This is identical to what it was found by Feng *et al.* (2008) as shown in the following Table (5) Figure (8).

Table 5 : Real time Cq*Tri 6* in trichothecenesnivalenol (NIV)

Well	Col.	Sample No.	Cq	Efficiency	Efficiency R^2	Res.
1		1	-	-	-	Exc.
2		2	37.95	0.46	0.99865	
3		3	38.47	0.51	0.99744	
4		4	41.19	0.48	0.99597	
5		5	-	-	-	Exc.
6		6	-	-	-	Exc.
7		7	-	-	-	Exc.
8		8	50.91	0.20	0.99768	

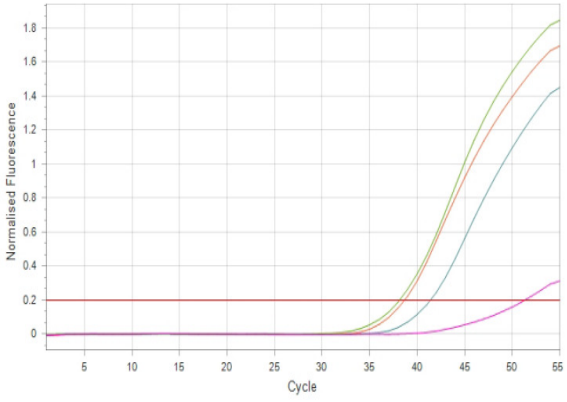

Fig. 8 : Result Real time Cq *Tri 6* in trichothecenesnivalenol (NIV)

This is illustrated by some fungal genes for trichothecene biosynthesis (*Tri* genes) that are known to be under control of transcription factors encoded by *Tri6*. *Tri6* deletion mutants are constructed in order to discover additional genes regulated by these factors in plant. Both mutants are greatly reduced in pathogenicity and toxin production. These phnotypes are largely restored by genetic complementation with the wild-type gene. It can be found through (Seong *et al.,* 2009) regulated genes that will be targeted for functional analysis to discover additional factors involved in toxin biosynthesis, toxin resistance and pathogenesis.

A gene *Tri6* is considered an organizational gene as it encodes a previously characterized Cys_2His_2 zinc finger DNA-binding protein that functions as a pathway-specific transcription factor and positively regulates the other *Tri* genes and *Tri6* also control transcript levels for the gene encoding farnesyl pyrophosphate synthetase (*FPPS*) (Tag *et al.*, 2001).

The results show the absence of this gene in a number of isolates supporting the hypothesis that the lack of T-2 toxin production in The Real Time BCR test yielded positive results for this gene in four isolates. While the results were negative in four other isolates and in percentages (32.27-32.86). These strains is due to a lack of transcripts for later pathway-specific genes. Moreover, the loose versus tight division in *Tri* gene expression mediated by *Tri6* may define a previously unrecognized important regulatory control point for trichothecene biosynthesis .This is identical to hypothesis (Peplow *et al.,* 2003) who confirmed the absence of toxin in some isolates for the same reason.

The Role of *Tri 10* in Synthesis of Trichothecenesnivalenol (NIV):

A third gene that has been revealed under study is an important gene in the process of detecting the presence of toxin or detecting its work in inhibiting the production of fungal toxins in general .The importance of this gene (Katarzyna *et al.*, 2018) has been emphasized when it is observed its role in inhibition of mycotoxin production by flavonoids occurred at the transcriptional level. (Table 6) (Figure 9).

Tao *et al.* (2018), noted that the role of this gene inexpression of trichothecene biosynthesis (*Tri*) genes and DON production are mainly regulated by the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway and two pathway-specific transcription factors. In 2017, other gene studies have demonstrated the role of this gene in inhibiting the production of trichothecens toxin by several acids .This was found by Tomasz *et al.* (2017) who confirmed the role of the gene in responsible for trichothecene biosynthesis (Tri10) which proved that the inhibition of mycotoxin production by sinapic acid occurred at the transcriptional level. *Fusariam* responds to sinapic acid by stimulation of ergosterolbiosynthesis.

Table 6 : Real time Cq*Tri 10* in trichothecenesnivalenol (NIV)

Well	Co	Sample No.	Cq	Efficiency	Efficiency R^2	Result
1		1	-	-	-	Exc.
2		2	32.86	0.96	0.99561	
3		3	32.27	0.94	0.99614	
4		4	33.33	0.78	0.99553	
5		5	-	-	-	Exc.
6		6	-	-	-	Exc.
7		7	-	-	-	Exc.
8		8	37.69	0.47	0.98711	

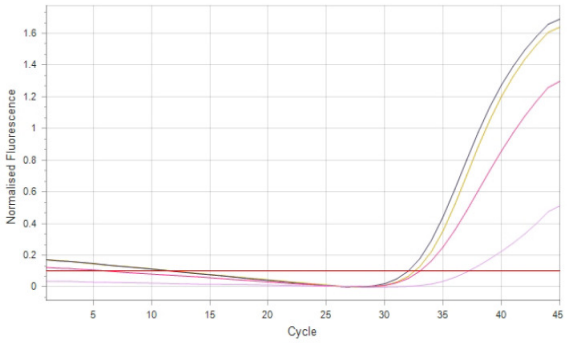

Fig. 9 : Real time Cq*Tri 10* in trichothecenesnivalenol (NIV)

In other studies, genes that inhibit the production of fungal toxins have been detected by examining the Real Time PCR which confirmed the role of ferulicacid. A basic acid found in the wheat to inhibit the production of trichothecenes toxin and decrease in the level of *Tri* gene expression that is measured, suggesting that inhibition of toxin synthesis by ferulic acid could be regulated at the transcriptional level . This explains the role of the Ferulicacidin modulating trichothecene biosynthesis by *Fusarium* in some wheat varieties (Boutigny *et al.*, 2009).

Many studies have been found to detect the gene and its role through immunological tests. Rui *et al.* (2015) noted the role in the regulation of Tri gene expression and showed that AreA transcription factor mediates the regulation of deoxynivalenol (DON) synthesis by ammonium and cyclic adenosine monophosphate interacted with Tri10 in co-immunoprecipitation assays through the transcription

factor that may interact with Tri10 and control the expression and up-regulation of MEP genes.

The results of the Real Time BCR test show the role of the three genes (Tri4, Tri6, Tri10) in detecting the production of fungal toxins, especially the trichothecens toxin type B, as well as their role in sometimes inhibition of these genes as organizational genes as well as their importance in vital pathways, noting that there are (153) genes produced by the same poison and are likely to have a significant role in cellular transport. So these genes are selected because they are genes target for functional analysis to discover additional factors involved in toxin biosynthesis, toxin resistance and pathogenesis (Seong *et. al.*, 2009).

The results of the present study can be summed up by the results of the Real Time BCR test in the following table (7) for the number of isolates. Sources are (clinical or environmental) as well as their different origins (lung cow, barley, wheat).

Table 7 : Frequency of Single Genes in *Fusarium graminearum* Isolates Collected from the Clinical and Environmental Source.

Isolate No.	Isolate origin	Source of isolate	Trichothecene genes		
			Tri4	*Tri6*	*Tri10*
Fus.1	cows Lung	Clinical	-	-	-
Fus.2	Barley	Environmental	+	+	+
Fus.3	Wheat	Environmental	+	+	+
Fus.4	Barley	Environmental	+	+	+
Fus.5	Wheat	Environmental	-	-	-
Fus.6	Wheat	Environmental	-	-	-
Fus.7	Wheat	Environmental	-	-	-
Fus.8	Barley	Environmental	+	+	+

Conclusions

- Samples of wheat and barley have become contaminated with fungi, including *Fusarium graminearum* which is a danger to the health of humans and animals.
- The highest percentage of trichothecensnivalenol (NIV) toxin was found in barley grain compared with wheat grain**.**
- Effectiveness of PCR and Real time PCR technique to detect Trichothececns toxin extracted from fusarium fungi samples.
- Genes (*Tri4, Tri6, Tri10*) are the genes responsible for the production of trichothecenenivalenol (NIV).

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