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Effects of Growth Conditions on *tri4* Gene Expression in *Fusarium culmorum*

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Abstract: Expression of *tri4*, found in the *tri5* gene cluster, is essential for DON production. In this study, effects of different growth conditions on *tri4* expression, as well indirectly on DON production, were investigated in F15 isolate of *Fusarium culmorum* via qPCR (real time polymerase chain reaction). Control group was grown on potato dextrose agar (PDA) at 25°C (pH 5.6). The effects of pH 3.0 and -7.0 were examined on cultures grown at 25°C. Moreover, 0.5 mM hydrogen peroxide (H_2O_2) was concurrently added to medium. High quality ($A_{260/280}$= 1.9-2.0) and quantity (2-3µg/µL) of total RNAs were isolated from all groups. *β-tubulin* expression was used as internal control and relative quantification values were recorded. *tri4* expression was detected in all experiments except F15 grown on pH 3.0. $\bar{x}$ Cp values were calculated as 22.26±1.14-26.84±4.79. *tri4* expression levels in experiments were lower than control. Their ΔΔCT and $2^{-\Delta\Delta CT}$ values were 0-5.54 and 0-0.582, respectively. While maximum *tri4* expression was recorded in control, minimum expression was detected in the conditions consisting of pH 5.6 and at 15°C. Findings showed that different pH and temperature values and supplementation of H_2O_2 resulted in decreasing of *tri4* expression. Also, it was detected that acidic pH was a potential repressor for DON production. Findings support the importance of kit development requirement for mycotoxin detection based on gene expression analysis in the field or harvested crops.

Keywords: *Fusarium culmorum,* qPCR, *tri4* gene expression

Üreme Koşullarının *Fusarium culmorum*'da *tri4* Gen Anlatımı Üzerindeki Etkileri

Öz: *tri5* gen kümesindeki *tri4* geninin anlatımı DON üretimi için temeldir. Bu çalışmada, farklı büyüme koşullarının *tri4* anlatımı üzerindeki, dolaylı olarak da DON üretimi üzerindeki etkisi *Fusarium culmorum*'un F15 izolatında qPZR (gerçek zamanlı polimeraz zincir reaksiyonu) aracılığıyla araştırıldı. Kontrol grubu patates dekstroz agar (PDA) ortamında 25°C'de üretildi (pH 5.6). Sıcaklığın etkisi 8°C ve 15°C uygulamalarıyla test edildi. pH 3.0 ve -7.0'nin etkisi 25°C'de üretilen kültürlerde incelendi. Ek olarak, 0.5 mM hidrojen peroksit (H_2O_2) besi ortamına diğer bir faktör olarak eşzamanlı eklendi. Bütün deney gruplarından yüksek kalite ($A_{260/280}$= 1.9-2.0) ve miktarda (2-3µg/µL) total RNA izolasyonu gerçekleştirildi. *β-tubulin* geninin anlatımı içsel kontrol olarak kullanıldı. Elde edilen rölatif kantitasyon değerleri kaydedildi. *tri4* anlatımı pH 3.0 koşulundaki grup hariç tüm deney gruplarından belirlendi. $\bar{x}$ Cp değerleri 22.26±1.14-26.84±4.79 aralığında hesaplandı. Deney gruplarındaki *tri4* anlatım düzeylerinin kontrol grubuna göre daha düşük olduğu saptandı. ΔΔCT ve $2^{-\Delta\Delta CT}$ değerleri sırasıyla 0-5.54 ve 0-0.582 idi. En yüksek *tri4* anlatımı kontrol grubunda kaydedilirken, en düşük anlatım pH 5.6/15°C koşullarında belirlendi. Bulgular, farklı pH ve sıcaklık değerleri ile H_2O_2 uygulamasının *tri4* anlatımındaki azalmayla sonuçlandığını gösterdi. Ayrıca, asidik pH'nın DON üretiminin potansiyel bir baskılayıcısı olduğu belirlendi. Bulgular mikotoksinlerin tarlada ya da hasat edilmiş tahıllarda gen anlatımı analizine dayalı olarak belirlenebilmesi için kit geliştirilmesi gereksiniminin önemini desteklemektedir.

Anahtar Kelimeler: *Fusarium culmorum*, kantitatif PZR, *tri4* gen anlatımı

1. Introduction

Mycotoxins are secondary metabolites produced by fungal species. Among them, sesquiterpenoid structured trichothecenes are one of the five main mycotoxin groups generated by *Fusarium* spp. These toxins separated into four classes; A-, B-, C- and D-trichothecenes (Özer and Soran, 1991; Sudakin, 2003). More than 200 variants related to these groups were identified. Among them the class A- and B- trichothecenes are the most common toxins and they include deoxynivalenol (DON), nivalenol (NIV) and T-toxins. DON, NIV and their acetylated derivatives are belonging to class B-trichothecenes also known as phytotoxins. The phytotoxins frequently accumulate on diseased small grain cereals after *Fusarium* infection process (Desjardins and Proctor, 2007; Foroud and Eudes, 2009).

The *tri5* gene cluster is responsible for trichothecene biosynthesis. There are 12 genes located into the cluster, beside additional three genes are flanking of it (Chandler et al. 2003; Kimura et al. 2007). Total nucleotide sequence of the genes belonging to reference strains of *F. graminearum* and *F. sporotrichioides* which they produced DON and NIV mycotoxins have been currently released on Genbank database (Lee et al. 2002; Kimura et al. 2003). It is shown that high level of genetic diversity has been determined the nucleotide sequences among the references with different chemotypes of a certain species (Chandler et al. 2003). Pseudogenes, insertions and/or deletions found into a gene, and even completely deletion of a gene found in the *tri5* cluster responsible for genetic diversity. These variations provide an opportunity for discrimination of fungal chemotypes. Many strategies have been developed and used in the chemotype identification (Chandler et al. 2003; Kimura et al. 2003; Jennings et al. 2004a, b; Wang et al. 2008). Moreover, conserved and/or certain regions of the genes found in the cluster can be utilized in gene expression studies and quelling. The *tri4, tri5* and *tri6* are commonly targeted genes in these studies (McDonald et al. 2005; Scherm et al. 2011; Yörük and Albayrak, 2014; Yörük, 2014).

Necrotrophic pathogen, *F. culmorum*, causes serious diseases in small grain cereals and able to produce DON, 3-acetyldeoxynivalenol (3-ADON) and NIV endotoxins (Parry et al. 1995; Sudakin, 2003; Bai and Shaner, 2004). DON is the main toxin type of *F. culmorum* (this pathogen), whereas wheat is the major host of it (Scherm et al. 2013; Yli-Mattila et al. 2013). DON production has an important role for pathogenesis, accumulation of the vomitoxin together with other secondary metabolites results in the reduction in crop quality and quantity (Wagacha and Muthomi, 2007). Besides, the DON inhibit the protein synthesis in the other eukaryotic organisms and it maintains the stable structure even if exposed to high temperatures (Lauren and Smith, 2001; Gutleb et al. 2002). Therefore, DON detection and its quantitation on cereals and on foods have currently become an obligatory in providing the food safety. For that purpose, parameters effecting the DON production should be clearly identified. Metal ions, plant secondary metabolites, different pH and temperature conditions have been demonstrated as the key factors that influences the DON production (Pinson-Gadias et al. 2008; Ponts et al. 2009; Merhej et al. 2010). It was reported that real time polymerase chain reaction (qPCR) was the efficient method for detection of trichothecene production, indirectly. Also, qPCR is a reliable, reproducible and fast approach for DON production analysis (Merhej et al. 2010; Scherm et al. 2011, 2013). Therefore, in this study, expression levels of *tri4* gene- encodes multifunctional oxigenase which is essential in the DON production- were quantified under six different conditions *in vitro* by Sybr green I-based qPCR.

2. Materials and Methods

2.1 Fungal Isolate and Culture Conditions

Monosporic *F. culmorum* F15 isolate, originated from scabby kernels of wheat planted in Sinop-Turkey, was kindly provided by Dr. Berna Tunali from Department of Plant Protection, Agricultural Faculty, Ondokuz Mayis University in Samsun, Turkey. Control group was

grown on potato dextrose agar (PDA: Biolife) at 25°C (pH 5.6). Effect of temperature was tested in two different conditions; at 8°C and 15°C. The effects of pH 3.0 and pH 7.0 were examined on cultures grown at 25°C. 0.5 mM H_2O_2 was concurrently added to medium with pH 5.6 and cultures were grown at 25°C. Seven-day-old fungal cultures belong to control group and testing sets were used in further analysis.

2.2 Total RNA Extraction and cDNA Synthesis

Total RNAs were extracted from 6 testing sets and control group. Tripure RNA isolation reagent (Roche, Switzerland) including phenol and guanidine thiocyanate was used in RNA isolation. 50-100 mg of mycelium was homogenized with liquid nitrogen by using pestle and mortar. Manufacturer's recommendations were then followed in RNA isolation protocol. Quality and quantity of RNAs were analysed both by spectrophotometer (Thermo, USA) and by agarose (1%) gel electrophoresis. Imaging was carried out by gel imagination system Gel Pro Analyzer 3.2 software. cDNA molecules were synthesized in a volume of 25 µl comprising of; 1µg total RNAs, 1x reaction buffer, 60 µM random hexamer, 60 µM oligo dT primer, 5 µM DTT, 1U of protector RNase inhibitor, 1 mM dNTPs and 1U reverse transcriptase of Roche (Switzerland). cDNA synthesis was carried out in a thermal cycler (Biorad, France). Incubation steps at 65°C for 10 min, 55°C for 30 min and 85° for 5 min were orderly applied.

2.3 Gene Expression Assays by qPCR and RT-PCR

tri4 expression of F15 isolate was determined via two different strategies: Sybr Green I based-qPCR and two step RT-PCR. Primer molecules (Table 1) were designed using primer3 software (see www.frodo.wi.mit.edu) and secondary structure formation possibilities were checked by olygoanalyzer of integrated DNA technologies (see www.idtdna.com). In qPCR experiments, Sybr Green I dye (Thermo, USA) was used as fluorescence agent. qPCRs were set at 20 µl final volume comprising 1x Sybr Green I master mix, 5 pmol of each primer and cDNA corresponding to 1µg of total RNA. qPCR assays were maintained using Roche Light Cycler II gene expression system (Roche, Switzerland).

Table 1. Primer sets used in the gene expression analysis and their amplification product sizes
Çizelge 1. *Gen anlatımı analizinde kullanılan primer setleri ve çoğaltım ürün boyutları*

Primers	Primer sequence (5'-3')	Band size (bp)	PCR type
Tri4f	ATGGATGAAAGGCTCGAGGT	139	RT-PCR
Tri4r	ACTGTCGGTGCTTTTGACG		
FusTblf	GAAGCCATTGATGTTGTTCGT	465	RT-PCR
FusTblr	TCCGACCATGAAGAAGTGAAG		
Tri4f	GCGAGAGGATACTGGTCGTC	63	qPCR
Tri4r	AAGAAGCTCCGAGAGGAGTTG		
Tblf	GGTTTCCAAATCACCCACTC	61	qPCR
Tblr	TCAACAGGGTACCCATACCG		

Cycling conditions were as follows: initial denaturation step at 95°C for 10 min, repeated 45 cycle including 95°C for 20 s, 58°C for 30s, 72°C for 10s and melting curve steps at 95°C for 5 s and 65°C for 1 min. *β-tubulin* gene was used as internal control. Standard series were generated by dilutions of four logs and slope values were recorded. Melting curve scores were also calculated for both genes. $\bar{x}$ Cp, ΔΔCT and normalization values were calculated as systematic formula developed by Livak and Schmittgen (2001). Each experiment was repeated at least two times. Analysis of variance and significance of differences were conducted on one-way ANOVA with Tukey's post-test and column tests via Graphpad Prism 5.0 software.

RT-PCR assays were used in order to verify the qPCR amplification. cDNA molecules were also used in RT-PCR. PCR were conducted on 25 µl volume including; cDNA amount corresponding to 1µg of total RNA, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM each dNTPs, 5 pmol of primers and 1U of *Taq* DNA polymerase (Promega, USA). PCR cycling conditions were performed at 94°C for 5 min for pre-denaturation, 35 cycles at 94°C for 45s, 58°C for 45s, 72°C for 45s and at 72°C for 5 min for final extension. PCR bands were analysed via 1.5% agarose gel electrophoresis and gel imagination system Gel Pro Analyzer 3.2 software. Experiments were repeated at least two times.

3. Results and Discussion

Control group and five testing sets- exposed to H_2O_2, different pH and temperature conditions- were effectively grown on PDA medium in 7 days (Figure 1) and more than 100 mg of mycelium was obtained from each of them. High quality ($\Delta_{260/280}$ = 1.9-2.0) and quantity (2-3 µg/µl) of total RNAs were isolated from 7-day-old fresh mycelia (Figure 2). The RNAs were diluted to 200 ng/µl and then they were used in cDNA synthesis. Quantification of *tri4* gene expression was carried out with amplification based approaches. *β-tubulin* gene expression was selected as internal control in qPCR.

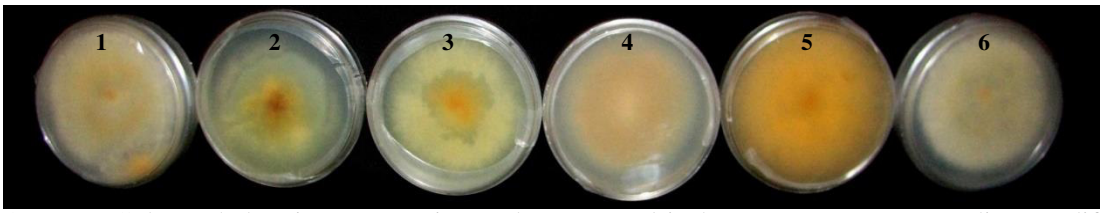

Figure 1. Cultures belonging to Fusarium culmorum F15 isolate grown on PDA medium at different conditions: (**1**) pH 5.6/25°C (control group), (**2**) pH 3.0/25°C, (**3**) pH 7.0/25°C, (**4**) pH 5.6/8°C, (**5**) pH 5.6/15°C and (**6**) PDA+0.5 mM H_2O_2 (pH 5.6/25°C)

Şekil 1. *Farklı koşullardaki PDA besi ortamında üretilen Fusarium culmorum F15 izolatına ait kültürler: (**1**) pH 5.6/25°C (kontrol grubu), (**2**) pH 3.0/25°C, (**3**) pH 7.0/25°C, (**4**) pH 5.6/8°C, (**5**) pH 5.6/15°C ve (**6**) PDA+0.5 mM H_2O_2 (pH 5.6/25°C)*

Mean cross point values ($\bar{x}$ Cp) together with their standard errors belongs to *tri4* and *β-tubulin* genes were recorded in six samples (Table 2). While the $\bar{x}$ Cp values for *tri4* gene were ranged from 0.0±0.0 to 26.84±4.79, the $\bar{x}$ Cp's for internal gene was found as 20.79±0.25-31.09±0.05. Target gene (*tri4)* expression was repressed under acidic pH condition. At the same time, acidic pH caused to down-regulation of internal gene (*β-tubulin*). 2^{10} fold decreases were detected among housekeeping gene expression of experiments.

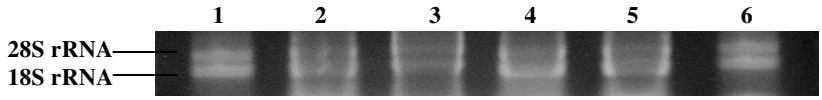

Figure 2. Agarose gel electrophoresis profiles of total RNAs isolated from F15 grown on PDA medium at different conditions: (**1**) pH 5.6/25°C (control group), (**2**) pH 3.0/25°C, (**3**) pH 7.0/25°C, (**4**) pH 5.6/8°C, (**5**) pH 5.6/15°C PDA and (**6**) PDA+0.5 mM H_2O_2 (pH 5.6/25°C)

Şekil 2. *Farklı koşullardaki PDA besi ortamında üretilen F15'den izole edilen total RNA'ların agaroz jel elektroforezi görünümü: (1) pH 5.6/25°C (kontrol grubu), (2) pH 3.0/25°C, (3) pH 7.0/25°C, (4) pH 5.6/8°C, (5) pH 5.6/15°C ve (6) PDA+0.5 mM H_2O_2 (pH 5.6/25°C)*

ΔΔCT and $2^{-\Delta\Delta CT}$ values belonging to experiments of acidic pH were accepted as "0" which is different from ΔΔCT values of control group. Thus, normalization of acidic pH group was excluded from gene expression analysis. After normalization, ΔΔCT and $2^{-\Delta\Delta CT}$ values

which were correspond to relative gene expression levels in five samples were calculated ranged from 0 to 5.54 and 0 to 0.582, respectively (Table 2). Also both of these values for control group were found as 1. Tukey's multiple comparisons test showed that findings were statistically significant ($p<0.0001$).

Table 2. $\bar{x}$ Cp, ΔΔCT and fold change values in qPCR assays
Çizelge 2. *qPZR denemelerindeki* $\bar{x}$ *Cp, ΔΔCT ve oransal değişim değerleri*

Sample	$\bar{x}$ Cp		ΔΔCT	$2^{-\Delta\Delta CT}$
	tri4	***β-tubulin***		
PDA (pH 5.6 / 25°C)	22.55±0.9	23.58±0.18	1	1
PDA (pH 3.0 / 25°C)	0.0±0.0	31.09±0.05	0	0
PDA (pH 7.0 / 25°C)	22.26±1.14	22.51±0.03	0.78	0.582
PDA (pH 5.6 / 8°C)	23.25±0.32	20.79±0.25	3.49	0.089
PDA (pH 5.6 / 15°C)	26.84±4.79	22.33±0.23	5.54	0.021
PDA+0.2 mM H_2O_2	22.39±0.1	21.08±1.45	2.34	0.197

Findings are statistically significant ($p<0.0001$).

Fold changes in *tri4* gene expression were between 0.021 and 0.582 excluding the level belonging to grown on medium with acidic pH ($2^{-\Delta\Delta CT}$ value is "0"). It seems clearly that all parameters used in this study lead to down-regulation of the *tri4* gene expression (Figure 3). Also, gene expression analysis was completed with melting curve analysis. Slope values were changed between -3.63 and -3.4 for target and internal genes. Melting curve scores were ranged from 0.83 to 1. These scores showed that qPCR analysis was efficiently carried out.

At the same time, qPCR assays were confirmed via RT-PCR analysis. Similarly, *tri4* gene expression was not amplified from cDNA of F15 isolate grown on acidic medium (Figure 4). The *tri4* and also *β-tubulin* gene expressions were verified from testing sets and control group. The139 bp and 465 bp long fragments-corresponding to *tri4* (Figure 4) and *β-tubulin* genes, respectively- were amplified from *F. culmorum* isolate (data not shown).

It was reported in different studies that media content (such as Mg^{2+} addition) and manipulation of growth condition (such as acidic pH) could lead to decrease in trichothecene production (Sudakin et al. 2003; Pinson-Gadais et al. 2008; Boutigny et al. 2009; Merhej et al. 2010).

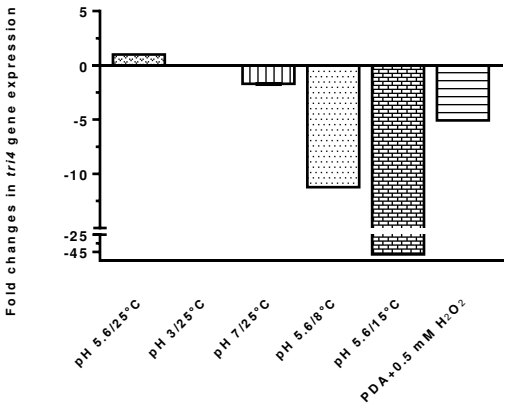

Figure 3. Fold changes in *tri4* gene expression of F15 isolate grown on PDA medium at different conditions.
Şekil 3. *Farklı koşullardaki PDA besi ortamında üretilen F15 izolatının tri4 gen anlatımındaki oransal değişimler*

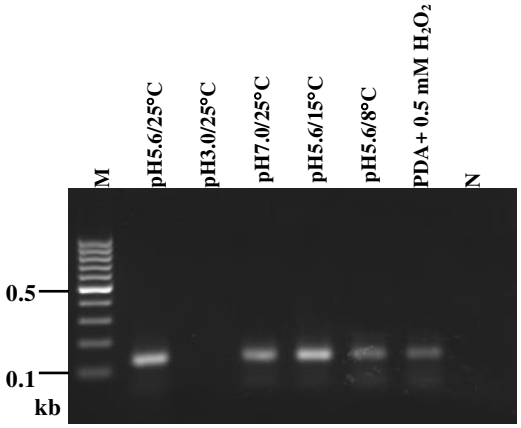

Figure 4. 139 bp long RT-PCR products belonging to *tri4* gene amplified from F15 isolate grown on different conditions. N: Negative control, M: 100 bp DNA ladder

Şekil 4. *Farklı koşullarda üretilen F15 izolatından çoğaltılan tri4 genine ait 139 bç boyutlu RT-PZR ürünleri. N: Negatif kontrol, M: 100 bç DNA standardı*

While the H_2O_2 was selected as different media content, different temperature and pH values were applied as growth conditions, in this study. Down-regulation of trichothecene production was monitored by the decreasing in the *tri4* gene expression level. The gene includes 4 exons and total CDS sequences of several *Fusarium* species including *F. culmorum* are currently present at Genbank. The *tri4* expression is essential in the DON biosynthesis. The gene encodes multifunctional monooxygenase, responsible for the conversion of trichodiene to isotrichotriol. The formation of isotrichotriol was catalysed by four independent reaction steps. The oxidation steps are essential for formation the trichothecene structure (Kimura et al. 2007). Therefore, down-regulation and/or totally inhibition of the *tri4* expression by different conditions were accepted as the indicator of decreasing the DON production. When it is compared to chromatographic or biochemical tests, gene expression analysis is inexpensive, rapid and reliable approach for determination and relative quantitation of mycotoxins produced by fungi. Merhej et al. (2010) showed that acidic pH and Mg^{2+} addition lead to down-regulation in *tri5, tri6* and *tri12* genes whose expression are also essential in DON production. Distinctly, trichothecene production was correlated with the decreasing in *tri4* expression, in this study. Girgin et al. (2001) reported that optimum pH value for DON production was the 5.6. Similarly, maximum gene expression was detected at pH 5.6 (with the combination of room temperature), in this study. However Ponts et al. (2009) found that H_2O_2 addition to fungal culture media resulted in 50 fold increase in DON production on the contrary of findings obtained from this study. Our findings revealed that *F. culmorum* isolate could produce DON without plant-microbe interactions. Detection of mycotoxin production via indirectly qPCR could be easily adapted to *in planta* studies. Requirement of kit development have to be discussed for detection of mycotoxin existence based on gene expression analysis in the field or harvested crops.

Acknowledgements

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