J. Crop Prot. 2013, 2 (4): 487-500

Chemotyping of *Fusarium graminearum* using *Tri13* trichothecene biosynthetic gene

Mostafa Abedi-Tizaki[1], Seyed Kazem Sabbagh[2]*, Mahta Mazaheri Naeini[3] and Saedeh Sepehrikia[4]

1. MSc Student of Plant Pathology, Department of Plant Pathology, University of Zabol, Zabol, Iran.
2. Department of Plant Protection and Institute of Plant biotechnology, University of Zabol, Zabol, Iran.
3. Faculty of Medicine, Zabol University of Medical Sciences, Zabol, Iran.
4. Laboratory expert, Mashad University of Medical Sciences, Khorasan-Razavi, Iran.

Abstract: *Fusarium graminearum* is one of the most important causes of FHB or wheat scab in different part of the world. This fungus is able to produce diffrent Trichothecene mycotoxins such as Nivalenol (NIV) and Deoxynivalenol (DON) which are harmful for both human and animals. To determine chemotypes of Trichothecene, a total of 100 isolates from different fields of Golestan province in Iran including Gorgan, Kordkuy, Bandaregaz, Gonbad, Minodasht, Kalaleh and Azadshahr were identified as *F. graminearum* using morphological features. The identity of 96 isolates was confirmed by polymerase chain reaction (PCR) assay using *F. graminearum* species-specific primers (Fg16F/Fg16R). Based on sequences of *Tri13* gene involved in the mycotoxin biosynthetic pathway, PCR assays were used to detect Nivalenol (NIV) and Deoxynivalenol (DON) chemotypes. Of the 96 tested isolates in Tri13 PCR assays, 70 classified as NIV chemotype and the remaining 26 isolates as DON producers. These results indicated that NIV chemotype was the most dominant chemotype in studied region. A greater proportion of NIV chemotype was found in Gorgan fields ($P < 0.05$, $P < 0.0001$), whereas greater proportion of DON was detected in Gonbad fields ($P < 0.05$, $P < 0.0001$). PCR assay-based chemotyping was confirmed by HPLC method. These results demonstrated that PCR assay and HPLC could be used as rapid, reliable and cost-effective methods for the detection and identification of mycotoxin-producing *Fusarium*-species and may thus help to develop strategies to avoid or reduce mycotoxin contamination of cereals.

Keywords: *Fusarium graminearum*, trichothecene, chemotypes, NIV, DON

Introduction

Fusarium Head Blight (FHB) caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] is one of the most important fungal diseases of wheat worldwide that causes serious losses in both yield and quality of grain (Parry *et al*., 1995). This fungus produces different kinds of mycotoxins, which pose a serious health threat to humans and animals (Arseniuk *et al*., 1993). It has been estimated that 25% of the world food crops is affected by mycotoxins (Charmley *et al*., 1995). The most predominant mycotoxins found in small-grain cereals are 8-ketotrichothecenes (type B trichothecenes) such as Deoxynivalenol (DON) (also known as vomitoxin) and Nivalenol (NIV) and their acetylated derivatives including 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as an oestrogenic mycotoxin, zearalenone (Mirocha *et*

Handling Editor: Dr. Mahbobeh Haratian

* **Corresponding author**, e-mail: sk.sabbagh@uoz.ac.ir
Received: 26 August 2012, Accepted: 7 September 2013

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

al., 1989; Waalwijk *et al*., 2003). DON and NIV chemotypes appear to differ in geographic distribution. The NIV chemotype has been reported in several countries of Africa, Asia, and Europe (Desjardins *et al*., 2000; Logrieco *et al*., 2003), but it has not been reported in North America (Mirocha *et al*., 1989; Abbas *et al*., 1989). DON is more associated with head blight of cereals throughout Europe and the USA than NIV and is therefore thought to be the more significant toxin (Bottalico, 1998). However, the toxicity of NIV has been shown to be greater than that of DON (Ryu *et al*., 1988). Also, these chemotypes are potent phytotoxins (Eudes *et al*., 2000), with DON being more phytotoxic than NIV (Desjardins, 2006). There is scanty knowledge about the chemotypes of *F. graminearum* isolates, there distribution and their different agricultural host in Iran (Haratian *et al*., 2008). Golestan region is located in the North and northwestern Iran with favorable conditions for *Fusarium* growth. Fusarium head blight of wheat and barley are two important diseases in Golestan Province (Golzar *et al*., 1993).

Haratian *et al*., (2008) have reported that NIV chemotype is dominant in Mazandaran province in north of Iran. Due to the toxicological differences between NIV and DON, it is important to determine the chemotypes of the fungus strains present in any given geographic region. There is therefore a need for a rapid and precise identification of trichothecene-producer species of *Fusarium* (Ji *et al*., 2007). Previously conventional methods such as HPLC or GC/MS were performed to assess mycotoxins in *Fusarium* species which they are laborious and time-consuming processes. The PCR-based assay is rapid and specific method and its high sensitivity allows detection of target DNA molecules in a complex mixture. Several species-specific primer assays have been developed to detect some of the trichothecene-producing species of *Fusarium* (Edwards *et al*., 2001). PCR assays have been used to determine chemotype based on the sequences of the genes responsible for trichothecene biosynthesis (Chandler *et al*., 2003; Jennings *et al*., 2004b; Lee *et al*., 2001; Waalwijk *et al*., 2003). Molecular characterization of trichothecene mycotoxin biosynthesis pathways has revealed the mycotoxin gene clusters and their regulations (Proctor *et al*., 1995; O'Donnell *et al*., 2000). Many trichothecene biosynthesis genes are localized in a gene cluster comprising at least 10 genes. These genes include those encoding trichodiene synthetase (*Tri5*), P450 oxygenases (*Tri4* and *Tri11*), acetyltransferase (*Tri3*), a transcription factor (*Tri6*), a toxin efflux pump (*Tri12*), and several unidentified hypothetical proteins (*Tri7*, *Tri8*, *Tri9*, and *Tri10*) (Lee *et al*., 2001). The *Tri13* and *Tri7* genes are critical for DON/NIV chemotype switching and the *Tri7* gene is responsible for acetylation of NIV to produce 4-AcNIV (Brown *et al*., 2002; Lee *et al*., 2002). Sequencing of these genes from *F. graminearum* revealed that a repeated insertion sequence of 11 nucleotides within a putative intron of *Tri7*, has disrupted the gene function in DON-producing isolates. Moreover there are three deletions within the *Tri13* gene sequence in DON producers (Brown *et al*., 2002; Lee *et al*., 2001). The objective of this study was to determine trichothecene chemotypes of *Fusarium graminearum* isolates in wheat obtained from different subregions of the main wheat production area in Iran, and to determine whether there are significant differences between geographical areas and distribution of the chemotypes.

Materials and Methods

Sampling, fungal isolates and culture conditions

Wheat samples were collected from the diseased wheat spikes in different fields of Golestan province of Iran during 2009-2010. One hundred grains of each sample were surfaced- sterilized in an aqueous solution of 3% (w/v) chloramines T (Sigma, saint–Quentin, France) plus one drop of tween 80 for 5 min, rinsed with sterile distilled water, submerged in a 75% ethyl alcohol solution and dried over a filter paper. For the isolation of *Fusarium* species, ten grains were placed on a *Fusarium*-selective peptone pentachloronitrobenzene (PCNB) agar medium

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

(Nirenberg, 1981) and incubated for 10 days at 25 °C under fluorescent light (12 h photoperiod). *F. graminearum* identification was performed on PDA, Spezieller Nährstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA) meda according to the morphological criteria keys of Nelson *et al*., (1983).

DNA extraction from fungal cultures

Mycelia disks excised from the margin of 10-day-old PDA cultures of all isolates were inoculated into 100 ml Erlenmeyer flasks containing 20 ml of PDB liquid medium (Merck, Germany). Submerged fungi cultures were incubated on a rotary shaker at 120 rpm for 8 days at 25 °C. Mycelia were harvested by filtration through Whatman paper 1, ground to fine powder with liquid nitrogen and keep at -80°C for further DNA extraction. Total genomic DNA was extracted from dried mycelium using the CTAB method as described by Nicholson (1997).

Total DNA was quantified using a Scanodrop 200 (Analytik Jena, Germany) spectrophotometer and DNA quality was assessed by 1% agarose gel electrophoresis stained by ethidium bromide. The concentration of DNA was adjusted to 25 ng/µl for use in PCR assay.

PCR assay

PCR assays to identify and determine *F. graminearum* chemotype was performed with species-specific primers Fg16F and *Tri13* respectively (Table 1). Amplification reactions were carried out in volumes of 25 µl containing 1.5 µl of genomic DNA (25 ng), 1.5 µl of 10 × buffer PCR (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8), 1 µl of Mg Cl_2 (50mM), 0.25 µl of dNTPs (100 mM), 0.2 µl of *Taq* DNA polymerase (5 U/ml), and. 25 µl of each primer (20 mM). PCR reaction was performed in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) with the following programs: an initial denaturation step at 94° for 2 min, 40 cycles of 94 °C (30 s) / 57° (30 s)/72° (60 s), and a final extension step at 72 °C for 10 min. A negative control without DNA template was used in every set of reactions. PCR products were separated by electrophoresis on 1.2% agarose gels stained with ethidium bromide (0.5 µg/ml) in 40mM Tris–acetate and 1.0mM EDTA 1-buffer and photographed under UV light.

Determination of *F. graminearum* chemotype

Functional *Tri13* gene is required for the production of NIV, while DON-producing strains contain three deletions of 178, 61 and 37 bp in the gene, indicative of a non-functional pseudogene (Chandler *et al*., 2003). Primers Tri13F and Tri13R were designed from conserved regions within both functional and nonfunctional *Tri13* alleles, in both NIV, and DON-producing isolates. The designated primers by Waalwijk *et al*., (2003) were used to determine *F. graminearum* chemotypes (Table 1). PCR assays developed to the *Tri13* gene sequence were used to determine the chemotype. A generic *Tri13* assay used primers Tri13F and Tri13R and in PCR amplified a fragment in the range of 200-300bp from NIV producers and 400-450bp from DON-producing isolates of *F. graminearum*.

Table 1 Sequences of primers used in this study.

Primer	Sequence (5'-3')	Size (bp)	Reference
Fg16	Fg16F: CTCCGGATATGTTGCGTCAA	420-520	Nicholson *et al.,* (1998)
	Fg16R: GGTAGGTATCCGACATGGCAA		
Tri13	Tri13F: TACGTGAAACATTGTTGGC	234-415	Waalwijk *et al.,(*2003)
	Tri13R: GGTGTCCCAGGATCTGCG		

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

Sample preparation and clean-up procedure

DON and NIV production was assessed in cultures grown on autoclaved rice. Brifly, 50 g of polished rice (Tarom) and 50 ml distilled water were autoclaved in 500 ml Erlenmeyer flasks. The sterilized flasks were left for 24 h at room temperature then were reautoclaved. Eight representative isolates of *F. graminearum* (FgT16, FgaT28, FgmT15, FggT11, FggT7, FgbT6, FgbT12, and FgT9) were used for mycotoxin analysis in rice culture medium. All selected isolates were cultured on PDA medium and used for inoculation procedure. Each flask was inoculated with mycelium plugs (10 plugs/flask) from 7-day-old PDA (Merk, Germany) of each isolates and incubated for 3 weeks at 25 °C in the dark (Desjardins *et al.,* 2000). The entire contents of each flask of inoculated rice cultures was transferred to a screen bottomed tray and allowed to dry in a ventilated hood at room temperature (Langseth *et al*., 1999). The dried cultures were finely ground with a Parskhazar mill (Parskhazar, Rasht, Iran) and mixed well. Subsamples (about 100 g) were taken and stored at -20 ^{o}C until used for analysis. Fifty grams of milled sample were extracted with 2 g NaCl, 40 ml of hexane and 100 ml of acetonitrile: water (84: 16v/v) with occasional shaking in a rotary shaker at high speed for 30 min. The extract was cooled down to 4 ^{o}C and filtered through filter paper Whatman No. 4 and glass wool then was stored at 4 °C until used. The filtrates (5 ml) were passed through a solid phase extraction column clean up (SPE) (Libios, Bully, France) consisting of active carbon, alumina and glass wool (7: 5: 3) and it was slowly pressed into the tube with the rubber flange end turned down until 3 ml of the extract had passed through the column then SPE column was washed with 2 ml of acetonitrile 84% and purified extraction (2 ml) was transferred to a vial and evaporated to dryness under nitrogen stream at 60 ^{o}C.

HPLC Analysis

Five hundred microliter of water: methanol (86: 14, v/v) was used to redissolve dried residue. The solution was homogenized for 5 min in a vortex mixer then 25 µl of this extraction was injected to HPLC system (Cecil composing CE 4104, UK) by high-pressure pump (CE 4100, Cecil instrument, Cambridge, UK). Chromatographic separations were performed on a stainless steel C18 reversed-phase column (250 × 4.6 mm, Nucleodur 100-5, C18ec, 5 µm, Duren, Germany) connected to a pre-column Security Guard (20 × 4.6 mm, 5 µm particle size, Duren, Germany). The mobile phase was water: methanol (86: 14, v/v) at a flow rate of 1.5 ml/min. The mycotoxins were detected by UV (model CE 4300 programmable UV detector, Cecil instrument Ltd, Cambridge, UK) at 220 nm and quantified by Power Stream- Chromatography Software (Cecil instrument, Cambridge, UK). The mycotoxin levels were calculated by comparing the area of the chromatographic peak of the samples with those of the standard calibration curve.

Standard preparation and calibration curve

DON and NIV standards were purchased from Libios Company (Libios, Bully, France). DON and NIV stock solutions were prepared by dissolving the solid standard in methanol to obtain concentrations of 100 µg/ml for each toxin. Calibration curves were prepared by dissolving adequate amounts of the stock solutions in water: methanol (95: 5), previously evaporated to dryness under nitrogen stream. Solutions to perform calibration were prepared at concentrations of 4.0-0.25 µg/ml of each standard. The calibration curves showed good linearity for both mycotoxins (R2 = 0.9648 for NIV and R2 = 0.9890 for DON) (Fig. 4)

Statistical analysis

The distribution of trichothecene chemotypes (DON and NIV) was analyzed by splitting zone studied into regions of approximately equal area based on surface cultures. Chi-squared analysis was then carried out to test the proportion of DON and NIV chemotypes in each area following data analysis using SAS 9.2 software.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

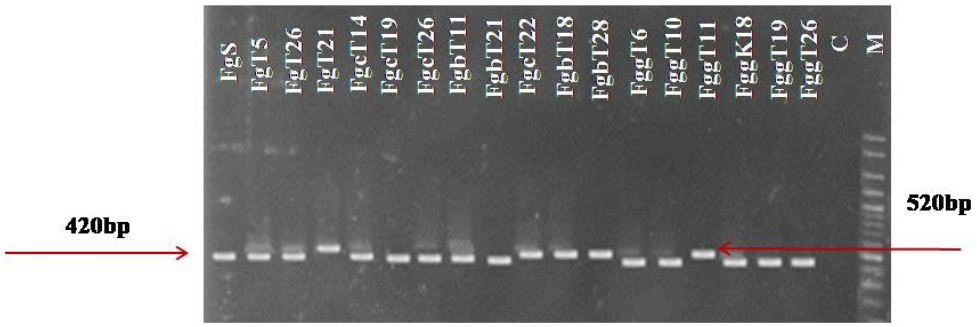


Figure 1 *F. graminearum* species-specific PCR amplification products with Fg16F/Fg16R primer set. Lane M: marker (1Kb, Qiagene); Lane C, negative control isolate Code numbers above the panel correspond to the strain codes of *F. graminearum* in Table 1.

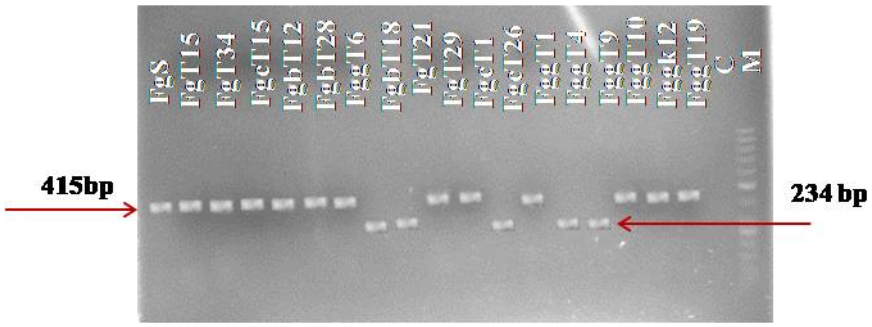


Figure 2 Amplification products from primer Tri13F/Tri13R specific to NIV and DON chemotypes from *F. graminearum* isolates. Lane M, marker (1kb); lane C, control; Isolate codes above the panel correspond to the isolate codes of *F. graminearum* in Table 1.

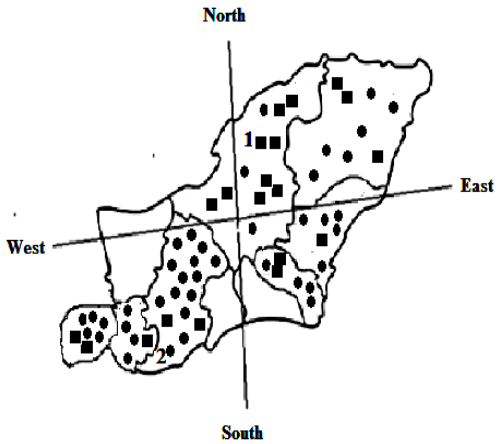


Figure 3 Map of Golestan province (1: 3500) showing distribution of chemotypes of *Fusarium graminearum* isolates. Trichothecene chemotypes from different regions are represented as follows: NIV (●) and DON (■). Number codes on the map correspond to Gonbad region (1) and Gorgan region (2).

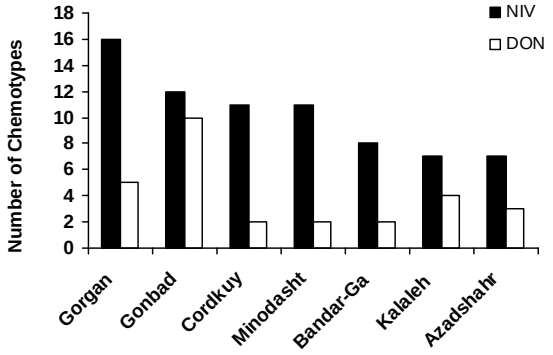


Figure 4: Distribution of chemotypes of *F. graminearum* isolates from different regions in Golestan province.

Results

F. graminearum identification

Fusarium isolates were identified by using morphological criteria and then were confirmed as *F. graminearum* by PCR analysis with *F. graminearum* specific Fg16F/Fg16R primers.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

Of one hundred isolates 96 produced an expected band of 420-520 bp, as described by Nicholson *et al.,* (1998) (Fig. 1).

Tri13 assay and determination of DON and NIV chemotypes

Two PCR assays (Tri13NIV, Tri13DON) were used for the identification of the chemotypes of *F. graminearum* isolates (Table 1). The expected product size for DON and NIV (415 and 234 bp) were amplified in PCR reaction (Fig. 2). Of 96 tested isolates using the primers designed for *Tri13* gene, 70 isolates were NIV chemotype and 26 were DON chemotype (Fig. 2) (Table 2). These results show that NIV was the most common chemotype in Golestan province (Fig. 4). The most distribution of NIV producing isolates was observed in Gorgan farms (22.2%) while DON producing isolates were dominant in Gonbad (35.7%) (Table 2) (Fig. 4). The results indicated that this pair of primers efficiently amplified a DNA fragment for all of the strains with a chemotype-specificity.

Distribution of chemotypes in different region surveyed

Analysis of the trichothecene chemotype distribution across the Golestan wheat cropping area revealed that there is a different frequency of chemotype distribution among various areas where isolates were collected (Figs. 3 and 4). Also these results indicated that there is a significant difference between wheat farms of Gorgan and Gonbad for distribution of NIV and DON chemotypes ($P < 0.05$, $P < 0.0001$) (Figs. 3 and 4).

HPLC analysis

To confirm PCR assay data and to quntify toxin, eight representative isolates were further analyzed using HPLC method. The linear regression equations for the recovery test of both DON and NIV shows R2 values > 0.96, indicating good linearity (Fig. 5).

FgT16, FgmT15, FggT7, FgbT12 and FgT9 isolates were predicted to produce NIV by PCR analysis produced NIV in culture and FgaT28, FggT11 and FgbT6 isolates were predicted to produce DON by PCR analysis produced DON in culture. In the north of Iran, there are reports of DON and NIV trichothecenes on grains of cereals. It is noteworthy that Karami-Osboo *et al*., (2010) detected DON chemotype in contaminated corn from the Golestan and Moqan Areas by HPLC.

Discussion

All trichothecene producing *Fusarium* species are destructive pathogens that can attack a wide range of plant species. The accurate identification of toxin producer *Fusarium* species is very important because each of them possesses a specific toxigenic profile and it is important to know the potential toxigenic risk of the contaminated plant or food products (Mulé *et al.*, 2004). Species of *Fusarium* have high heterogeny in morphological characters. S classification of species within genus is very difficult (Llorens *et al.,* 2006). The identification of *Fusarium* species based on morphological criteria is complex and laborious, specially for the non-specialist (Bluhm *et al.,* 2002; Windels, 1992). Therefore, molecular approaches could provide a rapid and reliable means for complete and the routine identification of *Fusarium spp*. confirming morphological diagnostics. So, for complete identification of *Fusarium* species additional molecular analysis such as species-specific PCR assays must be conducted. Species-specific primers have been developed and used for PCR detection and screening of *Fusarium* species (Spanic *et al.,* 2010). *F. graminearum* is one of the most important species to produce estrogenic toxin in wheat. Different species of *Fusarium* on wheat in the north of Iran have been reported (Zamani-Zadeh and Khoursandi, 1995b) but no molecular confirmation overview has been published on the toxin producer *Fusarium* species in our studied region. In this work, the isolates belonging to this species were identified based on morphological characters and then were confirmed by using species-specific primers.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

Table 2 Chemotype and PCR assay results of *F. graminearum* strains examined in this study.

PCR assay results				
Tri13 primer				
Culture number	**cultivar**	**Sample site**	**NIV**	**DON**
FgT1	Tajan	Gorgan	+	-
FgT3	Tajan	Gorgan	+	-
FgT4	Tajan	Gorgan	+	-
FgT5	Tajan	Gorgan	+	-
FgT7	Tajan	Gorgan	+	-
FgT8	Tajan	Gorgan	+	-
FgT9	Tajan	Gorgan	+	-
FgT10	Tajan	Gorgan	-	+
FgT13	Tajan	Gorgan	+	-
FgT14	Tajan	Gorgan	+	-
FgT15	Tajan	Gorgan	+	-
FgT16	Tajan	Gorgan	+	-
FgT17	Tajan	Gorgan	-	+
FgT18	Tajan	Gorgan	-	+
FgT21	Tajan	Gorgan	-	+
FgT24	Tajan	Gorgan	+	-
FgT26	Tajan	Gorgan	+	-
FgT29	Tajan	Gorgan	+	-
FgT30	Tajan	Gorgan	+	-
FgT31	Tajan	Gorgan	-	+
FgT34	Tajan	Gorgan	+	-
FgcT1	Tajan	Cordkuy	+	-
FgcT2	Tajan	Cordkuy	+	-
FgcT6	Tajan	Cordkuy	+	-
FgcT7	Tajan	Cordkuy	-	+
FgcT10	Tajan	Cordkuy	+	-
FgcT11	Tajan	Cordkuy	+	-
FgcT14	Tajan	Cordkuy	+	-
FgcT15	Tajan	Cordkuy	+	-
FgcT19	Tajan	Cordkuy	+	-
FgcT22	Tajan	Cordkuy	+	-
FgcT26	Tajan	Cordkuy	-	+
FgcT27	Tajan	Cordkuy	+	-
FgcT33	Tajan	Cordkuy	+	-
FgbT1	Tajan	Bandar gaz	+	-
FgbT6	Tajan	Bandar gaz	-	+
FgbT11	Tajan	Bandar gaz	+	-
FgbT12	Tajan	Bandar gaz	+	-
FgbT13	Tajan	Bandar gaz	+	-
FgbT18	Tajan	Bandar gaz	+	-
FgbT21	Tajan	Bandar gaz	-	+
FgbT23	Tajan	Bandar gaz	+	-
FgbT24	Tajan	Bandar gaz	+	-
FgbT28	Tajan	Bandar gaz	+	-
FggT1	Tajan	Gonbad	+	-
FggT2	Tajan	Gonbad	-	+
FggT4	Tajan	Gonbad	-	+
FggT6	Tajan	Gonbad	+	-
FggT7	Tajan	Gonbad	+	-

Table 2 Continued

PCR assay results				
Tri13 primer				
Culture number	**cultivar**	**Sample site**	**NIV**	**DON**
FggT9	Tajan	Gonbad	-	+
FggT10	Tajan	Gonbad	+	-
FggT11	Tajan	Gonbad	-	+
FggK12	Koohdasht	Gonbad	+	-
FggK14	Koohdasht	Gonbad	+	-
FggT27	Tajan	Gonbad	-	+
FggT29	Tajan	Gonbad	-	+
FggT30	Tajan	Gonbad	+	-
FggT31	Tajan	Gonbad	-	+
FggT33	Tajan	Gonbad	-	+
FggT34	Tajan	Gonbad	+	-
FgmT1	Tajan	Minoodasht	+	-
FgmT3	Tajan	Minoodasht	+	-
FgmT4	Tajan	Minoodasht	+	-
FgmT7	Tajan	Minoodasht	+	-
FgmT8	Tajan	Minoodasht	+	-
FgmT11	Tajan	Minoodasht	-	+
FgmT12	Tajan	Minoodasht	+	-
FgmT15	Tajan	Minoodasht	+	-
FgmT16	Tajan	Minoodasht	+	-
FgmT20	Tajan	Minoodasht	-	+
FggK16	Koohdasht	Gonbad	-	+
FggK18	Koohdasht	Gonbad	+	-
FggT19	Tajan	Gonbad	+	-
FggT21	Tajan	Gonbad	-	+
FggT24	Tajan	Gonbad	+	-
FggT26	Tajan	Gonbad	+	-
FgmT22	Tajan	Minoodasht	+	-
FgmT23	Tajan	Minoodasht	+	-
FgmT26	Tajan	Minoodasht	+	-
FgkT28	Tajan	Kalale	+	-
FgkT1	Tajan	Kalale	-	+
FgkT2	Tajan	Kalale	+	-
FgkT6	Tajan	Kalale	+	-
FgkT7	Tajan	Kalale	-	+
FgkT8	Tajan	Kalale	-	+
FgkT11	Tajan	Kalale	+	-
FgkT12	Tajan	Kalale	+	-
FgkT13	Tajan	Kalale	+	-
FgkZ15	Zagros	Kalale	+	-
FgkZ16	Zagros	Kalale	-	+
FgaZ18	Zagros	Azadshar	+	-
FgaZ20	Zagros	Azadshar	+	-
FgaZ21	Zagros	Azadshar	-	+
FgaZ22	Zagros	Azadshar	+	-
FgaT24	Tajan	Azadshar	-	+
FgaT25	Tajan	Azadshar	+	-
FgaT28	Tajan	Azadshar	-	+
FgaT29	Tajan	Azadshar	+	-
FgaT30	Tajan	Azadshar	+	-
FgaT32	Tajan	Azadshar	+	-

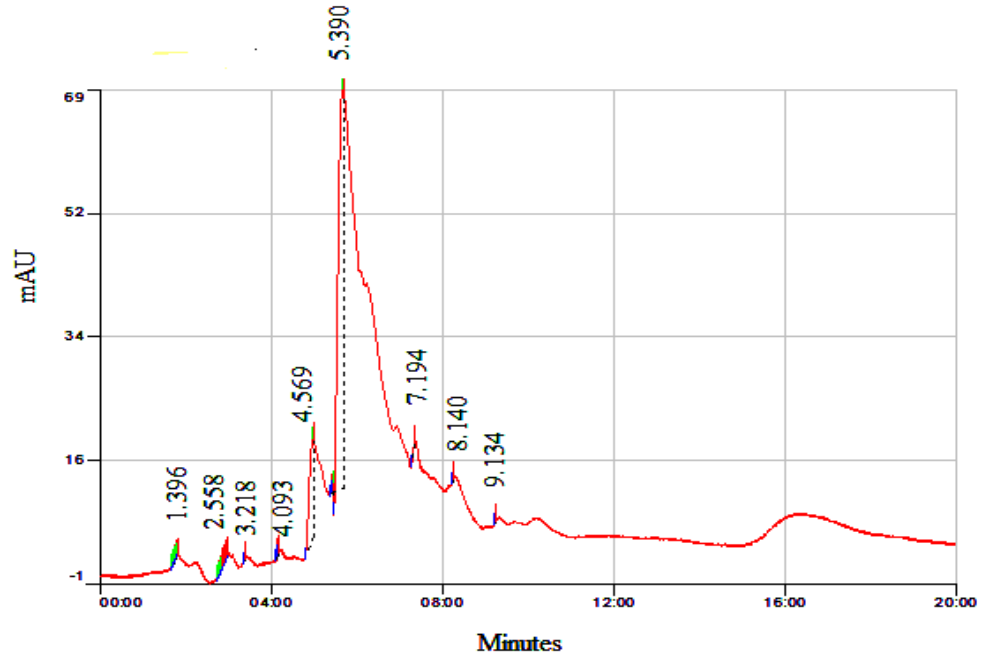
+ corresponding fragment amplified; No corresponding fragment amplified.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

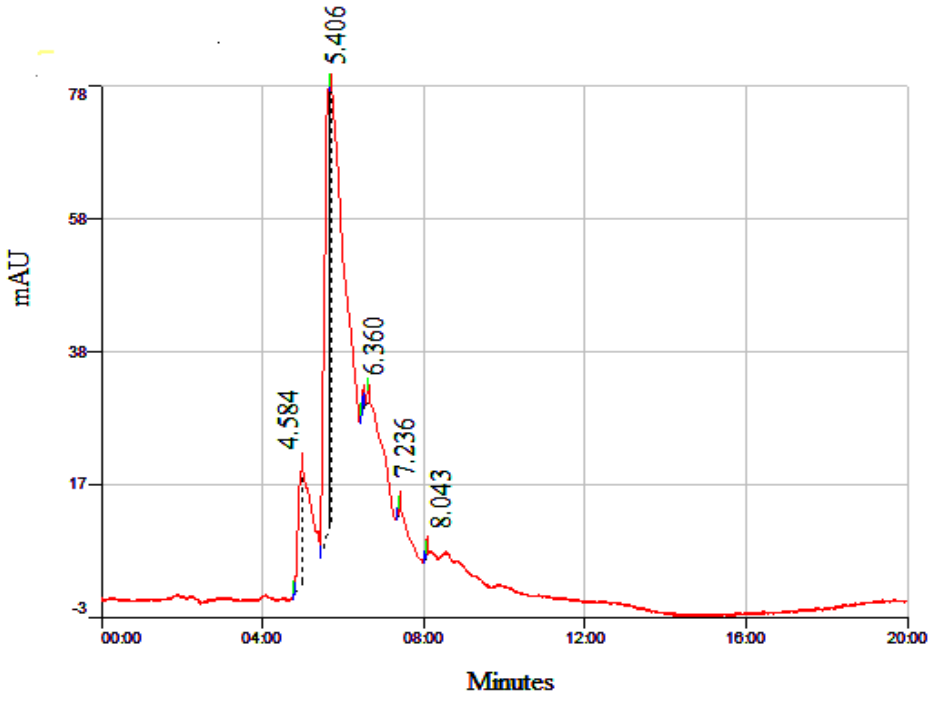
There are few reports based on geographical distribution of *F. graminearum* chemotypes in different regions and hosts in Iran. Haratian *et al*., (2008) reported the different chemotypes of *F. graminearum* species in Mazandaran region where NIV was known as dominant chemotype. Our observation is completely congruent with the previous chemical and molecular assays carried out in neighboring province. Other studies in different parts of the world such as Africa, Asia and Europe have confirmed the presence of both NIV and DON chemotypes, but only DON type has been detected in North America (Miedaner *et al*., 2000). NIV and DON chemotypes have been identified in Europe and South America at the same time so that DON chemotype was dominant type in these regions while in Asian regions such as Korea and Japan NIV have had the greatest distribution (Carter *et al*., 2002; Lee *et al*., 2002). Ji *et al*., (2007) have found that both DON and NIV chemotypes were present at the same time in deferent regions of China although, DON producer population had a greater level in comparison with NIV. Our data showed that the distribution of DON and NIV toxin is not equal in different parts of province studied. Analysis of trichothecene chemotypes in Argentina showed that 15-AcDON chemotype has had the most distribution compared with 3-AcDON chemotype (Alvarez *et al*., 2009). These results are of concern because the recent studies made on *F. graminearum* populations of Canada and America has revealed that the populations with 3-AcDON chemotype were more aggressive than 15-AcDON population in susceptible cultivars of spring wheat (Puri and Zhong, 2010). The *Tri13* gene in the genome of NIV-producer species encodes 3-acetyltrichothecene C-4 hydroxylase that plays an essential role for the addition of the C-4 oxygen to calonectrin (Kimura *et al*., 2007). Lee *et al*., (2002) have showed that the *Tri13* gene is required for the conversion of DON to NIV and that the *Tri7* gene product modifies NIV by acetylation to produce 4-ANIV. They also postulated that some DON-producing isolates might possess a non-functional *Tri13* gene while retaining a functional *Tri7* gene. It has been suggested that, following the loss of function of *Tri13*, *Tri7* will have no function (Lee *et al*., 2002; Brown *et al*., 2002). In the absence of selection pressure to remain functional, *Tri7* may accumulate mutations and become non-functional itself. Evidence in support of the degeneration of *Tri7* following loss of function of *Tri13* have been obtained among isolates of *F. graminearum* from China (Desjardins, 2009). In this study, we demonstrated that a PCR assay with detection of trichothecene producing genes such as *Tri13*, provides a rapid and reliable method for identification of chemotypes of *F. graminearum*.

According to these results, both NIV and DON chemotypes were detected in different regions and that NIV was dominant chemotype in Golestan province. It is noteworthy that NIV chemotype is more toxic for animals and human while DON type is mostly phytotoxic. The difference between distributions of these two chemotypes is likely due to the distribution of host, cultivars soil type, cultural practices or temperature (Jennings *et al*., 2004a). Nevertheless, it seems that more studies to determine the role of each of these factors in distribution of chemotypes is needed. In this study, chemotype distribution analysis in the wheat farms showed that Gorgan and Gonbad subregions have the highest contaminations of NIV and DON so to reduce toxin production in grain depots, adequate control measures and knowledge of amount of different trichotecin types in farms is necessary. Thus, mycotoxin monitoring using specific primers pair can replace costly and time consuming chemical methods to detect trichothecene in food and feed safety controls.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]



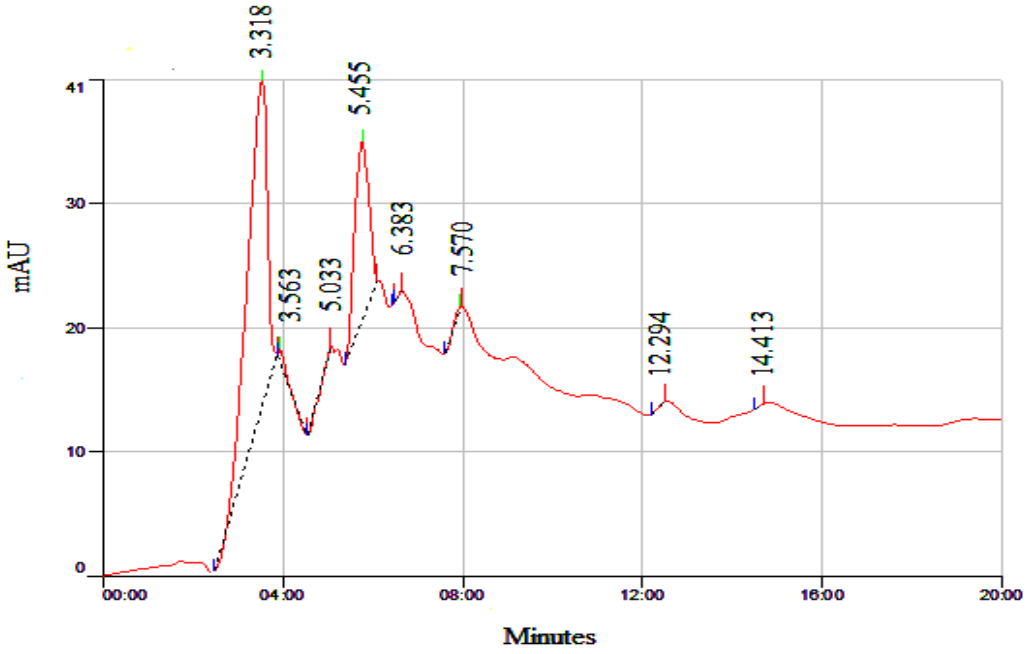
a



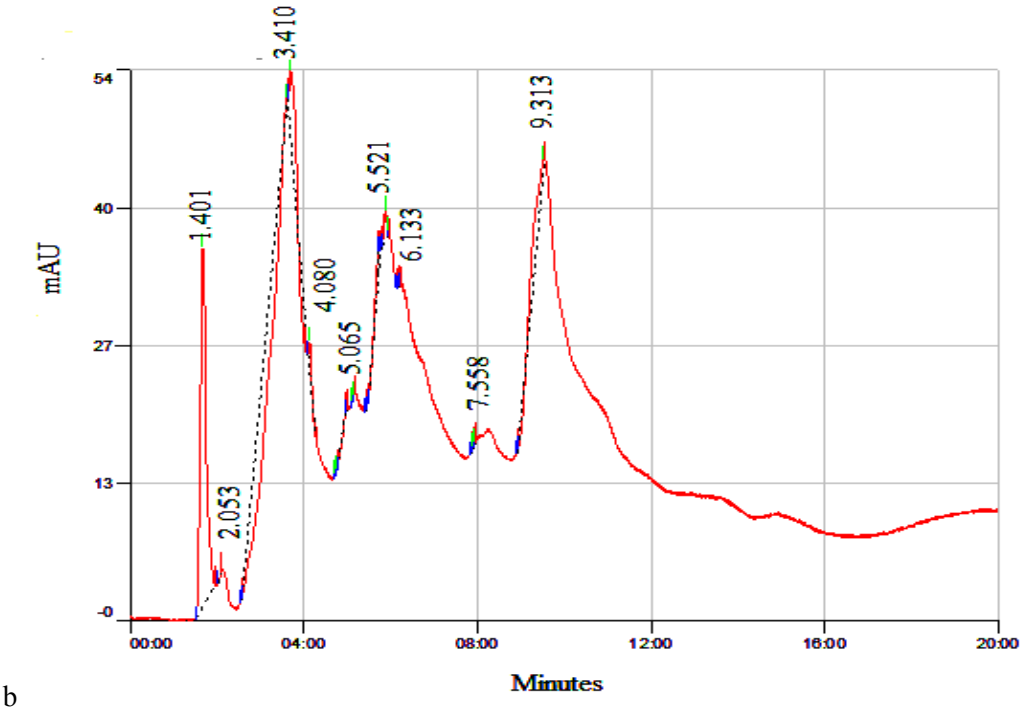
b

Figure 5 HPLC chromatogram of Deoxynivalenol (DON) (a) and Nivalenol (NIV) (b) standards, retention times 5.39 and 5.40 minutes respectively.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]



a



b

Figure 6 HPLC chromatogram of FggT11 (a) and FgmT15 (b) *Fusarium graminearum*, retention times 5.45 and 5.52 minutes, respectively.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

Acknowledgements

We would like to thank Dr. Shojaee and Mr. Karami-Osboo for technical cooperation and providing reference used as HPLC. Also, acknowledge financial support of Institute of plant biotechnology (Biocenter), University of Zabol.

Reference

Abbas, H. K., Mirocha, C. J., Kommedahl, T., Vesonder, R. F., Golinski, P. 1989. Production of trichothecene and non-trichothecene mycotoxins by *Fusarium* species isolated from maize in Minnesota. Mycopathology, 108: 55-58.

Alvarez, C. L., Azcarate, M. P., Pinto, V. F. 2009. Toxigenic potential of *Fusarium graminearum* sensu stricto isolates from wheat in Argentina. International Journal of Food Microbiology, 135: 131-135.

Arseniuk, E., Goral, T., Czembour, H. J. 1993. Reaction of triticale, wheat and rye accessions to graminaceous *Fusarium spp*. infection at the seedling stage and adult plants growth stages. Euphytica, 70: 175-183.

Bluhm B. H., Flaherty J. E., Cousin M. A., Woloshuk C. P. 2002. Multiplex polymerase chain reaction assay for the differential detection of trichothecene-and fumonisin-producing species of Fusarium in cornmeal. Journal of Food Protection 65:1955-1961.

Bottalico, A. 1998. *Fusarium* diseases of cereals: species complex and related mycotoxin profiles, in Europe. Journal of Plant Pathology, 80: 85-103.

Brown, D. W., McCormick, S. P., Alexander, N. J., Proctor, R. H., Desjardins, A. E. 2002. Inactivation of a cytochrome p-450 is a determinant of trichothecene diversity in *Fusarium* species. Fungal Genetics and Biology, 36: 224-233.

Chandler, E. A., Simpson, D. R., Thomsett, M. A., Nicholson, P. 2003. Development of PCR assays to *Tri7* and *Tri13* trichothecene biosynthetic genes and characterisation of chemotypes of *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium cerealis*. Physiological and Molecular Plant Pathology, 62: 355-367.

Charmley L. L., Trenholm H. L., Prelusky D. A., Rosenberg A. 1995. Economic losses and decontamination. Nat. Toxins 3:199-203.

Carter, J. P., Rezanoor, H. N., Holden, D., Desjardins, A. E., Plattner, R. D., Nicholson, P. 2002. Variation in pathogenicity associated with the genetic diversity of *Fusarium graminearum.* European Journal of Plant Pathology, 108: 573-583.

Desjardins A., Manandhar H., Plattner R., Manandhar G., Poling S., Maragos C. 2000. Fusarium species from Nepalese rice and production of mycotoxins and gibberellic acid by selected species. Applied and Environmental Microbiology 66:1020-1025.

Desjardins, A. E. 2006. *Fusarium* mycotoxins: chemistry, genetics, and biology. APS Press, St. Paul, MN.

Desjardins, A. E. 2009. From yellow rain to green wheat: 25 years of trichothecene biosynthesis research. Journal of Agricultural and Food Chemistry, 57: 4478-4484.

Desjardins, A. E., Manadhar, H. K., Plattner, R. D., Maragos, C. M., Shrestha, K., McCormick, S. P. 2000. Occurrence of *Fusarium* species and mycotoxins in Nepalese maize and wheat and the effect of traditional processing methods on mycotoxin levels. Journal of Agricultural and Food Chemistry, 48: 1377-1383.

Edwards, G., Pirgozliew, S. R., Hare, M. C., Jenkinson, P. 2001. Quantification of trichithecene producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. Applied and Environmental Microbiology, 67:1575-80.

Eudes, F., Comeau, A., Rioux, S., Collin, J. 2000. Phytotoxicité de huit mycotoxines associées à la fusariose de l'épi chez le blé. Canadian Journal of Plant Pathology, 22: 286-92.

Golzar, H. 1993. Distribution of Fusarium head blight in Gorgan and Gonbad areas and response of commercial wheat cultivars to

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

disease. Proceedings of 11th Iranian Plant Protection Congress, Rasht, Iran, p 36.

Haratian, M., Sharifnabi, B., Alizadeh, A., Safaie, N. 2008. PCR analysis of the *Tri13* gene to determine the genetic potential of *Fusarium graminearum* isolates from Iran to produce Nivalenol and Deoxynivalenol. Mycopathologia, 166: 109-116.

Jennings, P., Coates, M. E., Turner, J. A., Chandler, E. A., Nicholson, P. 2004a. Determination of deoxynivalenol and nivalenol chemotypes of *Fusarium culmorum* isolates from England and Wales by PCR assays. Plant Pathology, 53: 182-190.

Jennings, P., Coates, M. E., Walsh, K., Turner, J. A., Nicholson, P. 2004b. Determination of deoxynivalenol-and nivalenol-producing chemotypes of *Fusarium graminearum* isolated from wheat crops in England and Wales. Plant Pathology, 53: 643-652.

Ji, L., Cao, K. L., Ji, H. u., Wang, T. S. 2007. Determination of Deoxynivalenol and Nivalenol Chemotypes of *Fusarium graminearum* Isolates from China by PCR Assay. Journal of Phytopathology, 155: 505-512.

Karami-Osboo, R., Mirabolfathy, M., Aliakbari, F. 2010. Natural Deoxynivalenol Contamination of Corn Produced in Golestan and Moqan Areas in Iran. Journal of Agriculture, Science and Technology, 12: 233-239.

Kimura, M., Tokai, T., Takahashi-Ando, N. 2007. Molecular an genetic studies of *Fusarium* trichothecene biosynthesis; pathways, genes and evolution. Bioscience, Biotechnology, and Biochemistry, 71: 2102-2123.

Langseth, W., Bernhoft, A., Rundberget, T. B., Kosiak, T., Gareis, M. 1999. Mycotoxin production and cytotoxicity of Fusarium strains from Norwegian cereals. Mycopathologia 144: 103-113.

Lee, T., Han, Y. K., Kim, K. H., Yun, S. H., Lee, Y. W. 2002. *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Applied and Environmental Microbiology, 68: 2148-2154.

Lee, T., Oh, D., Kim, H., Lee, J., Kim, Y. Yun, S. and Lee Y. 2001. Identification of deoxynivalenoland nivalenol-producing chemotypes of *Gibberella zeae* by using PCR. Applied and Environmental Microbiology, 67: 2966-2972.

Llorens A., Hinojo M. J., Mateo R., Gonza´lez-Jaeén M., Valle-Algarra F. M., Logrieco A., Jime´nez M. 2006. Characterization of Fusarium spp. isolates by PCR-RFLP analysis of the intergenic spacer region of the rRNA gene (rDNA). International Journal of Food Microbiology 106 297-306.

Logrieco, A., Bottalico, A., Mule, C., Moretti, A., Perrone, G. 2003. Epidemiology of toxigenic fungi and their associated mycotoxins for some meditrranean crops. European Journal of Plant Pathology, 109: 645-667.

Miedaner, T., Reinbrecht, C., Schilling, A. G. 2000. Association among aggressiveness, fungal colonization, and mycotoxin production of 26 isolates of *Fusarium graminearum* in winter rye head blight. Journal of Plant Diseases and Protection, 107: 124-134.

Mirocha, C. J., Abbas, H. K., Windels, C. E., Xie, W. 1989. Variation in deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone production by *Fusarium graminearum* isolates. Applied and Environmental Microbiology, 55: 1315-1316.

Mulé G., Susca A., Stea G., Moretti A. 2004. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides, F. proliferatum* and *F. subglutinans.* European Journal of Plant Pathology 110:495-502.

Nelson, P. E., Toussoun, T. A., Marasa, W. F. O. 1983. *Fusarium* Species*:* An Illustrated Manual for Identification. The Pennsylvania State University Press, Pennsylvania, USA, 193 pp.

Nicholson, P., Rezanoor, H. N., Simpson, D. R., Joyce, D. 1997. Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* using a PCR assay. Plant Pathology, 46: 842-856.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

Nicholson, P., Simpson, D. R., Weston, G., Rezanoor, H.N., Lees, A.K., Parry, D.W., Joyce, D. 1998.Detection and quantification of *Fusarium culmorum* and *Fusarium graminearum* in cereals using PCR assays. Physiological and Molecular Plant Pathology, 53: 17-37.

Nirenberg H. I. 1981. A simplified method for identifying Fusarium spp. occurring on wheat. Canadian Journal of Botany 59:1599-1609.

O'Donnell, K., Kistler, H. C., Tacke, B. K., Casper, H. H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineage of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences, 97: 7905-7910.

Parry, D. W., Jenkinson, P., MacLeod, L. 1995. Fusarium ear blight (scab) in small grain cereals-a review. Plant Pathology, 44: 207-238.

Proctor, R. H., Hohn, T. M., McCormick, S. P., Desjardins, A. E. 1995. *Tri6* encodes an unusual zinc finger protein invoved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. Applied and Environmental Microbiology, 61: 1923-1930.

Puri, K. D., Zhong, S. 2010. The 3-ADON population of *Fusarium graminearum* found in North Dakota is more aggressive and produces a higher level and DON than the prevalent 15-ADON population in spring wheat. Phytopathology, 100: 1007-1014.

Ryu, J., Ohtsubo, K., Izumiyama, N., Nakamura, K., Tanaka, T., Yamamura, H., Ueno, Y. 1988. The acute and chronic toxicities of nivalenol in mice. Fundamental and Applied Toxicology, 11: 38-47.

Spanic V., Lemmens M., Drezner G. 2010. Morphological and molecular identification of Fusarium species associated with head blight on wheat in East Croatia. Eur J Plant Pathol 128: 511-516.

Windels C. E. 1992. Fusarium. In: Singleton, L. L., Mihail, J. D., Rush, C. M. (Eds.), Methods for Researchon Soilborne Phytopathogenic Fungi.American Phytopathological Society Press, St. Paul, MN,: 115-128.

Waalwijk, C., Kastelein, P., Vries, I.,Kerenyi, Z., van der Lee, T., Hesselink, T., Kohl, J., Kema, G.2003. Major changes in *Fusarium spp*. in wheat in the Netherlands. European Journal of Plant Pathology, 109: 743-754.

[DOR: 20.1001.1.22519041.2013.2.4.6.0]

تعیین تیپ شیمیایی جدایه‌های *Fusarium graminearum* با استفاده از ژن *Tri13* تولیدکننده تریکوتسین

مصطفی عابدی تیزکی[1]، سیدکاظم صباغ[*2]، مهتا مظاهری نایینی[3] و سعیده سپهری‌کیا[4]

۱- دانشجوی کارشناسی ارشد بیماری‌شناسی گیاهی، گروه گیاه‌پزشکی دانشگاه زابل، ایران.
۲- گروه گیاه‌پزشکی و پژوهشکده زیست‌فناوری گیاهی دانشگاه زابل، زابل، ایران.
۳- دانشکده پزشکی، دانشگاه علوم پزشکی زابل، زابل، ایران.
۴- کارشناس آزمایشگاه دانشگاه علوم پزشکی مشهد، مشهد، ایران.
* پست الکترونیکی نویسنده مسئول مکاتبه: sk.sabbagh@uoz.ac.ir
دریافت: ۵ شهریور ۱۳۹۱؛ پذیرش: ۱۶ شهریور ۱۳۹۲

چکیده: *Fusarium graminearum*، یکی از عوامل مهم فوزاریوز سنبله یا اسکب گندم در مناطق مختلف جهان به‌شمار می‌رود. این قارچ توانایی تولید مایکوتوکسین‌های تریکوتسین خطرناکی از جمله نیوالنول (NIV) و دی اکسی نیوالنول(DON) را دارد که برای انسان و دام مضر هستند. به‌منظور تعیین تیپ‌های شیمیایی تریکوتسین، تعداد ۱۰۰ جدایه *F. graminearum* از مناطق مختلف استان گلستان از جمله گرگان، کردکوی، بندرگز، گنبدکاووس، مینودشت، کلاله و آزادشهر با استفاده از خصوصیات مورفولوژیکی شناسایی شدند، سپس ۹۶ جدایه با آغازگرهای اختصاصی گونه *F. graminearum* (Fg16F/Fg16R) از طریق واکنش زنجیره‌ای پلی‌مراز (PCR) مورد تأیید قرار گرفتند. براساس توالی ژن *Tri13* که در بیوسنتز مایکوتوکسین تریکوتسین نقش دارد، تیپ‌های شیمیایی نیوالنول (NIV) و دی اکسی نیوالنول (DON) با روش PCR ردیابی شدند. از ۹۶ جدایه بررسی شده با آغازگرهای اختصاصی ژن *Tri13*، دو تیپ شیمیایی NIV و DON ردیابی شد که ۷۰ جدایه به‌عنوان تولیدکننده تیپ شیمیایی NIV و ۲۶ جدایه به‌عنوان تولیدکننده تیپ شیمیایی DON شناخته شدند. این نتایج نشان داد که که تیپ شیمیایی NIV ، تیپ غالب در مناطق مورد بررسی می‌باشد. تیپ شیمیایی NIV بیشترین پراکنش را در مزارع گرگان داشت ($P < 0.05, P < 0.0001$) درحالی‌که تیپ شیمیایی DON در مزارع گنبدکاووس غالب بود ($P < 0.05, P < 0.0001$). درنهایت تیپ‌های شیمیایی ردیابی شده با واکنش PCR نیز با آنالیز نمونه‌ها با استفاده از HPLC مورد تأیید قرار گرفتند. این نتایج ثابت می‌کند که PCR و HPLC، روش‌های سریع، مطمئن و به‌صرفه برای ردیابی و شناسایی گونه‌های فوزاریوم تولیدکننده توکسین می‌باشند که به توسعه روش‌هایی برای جلوگیری و کاهش آلودگی‌های مایکوتوکسینی در غلات کمک می‌کند.

واژگان کلیدی: *Fusarium graminearum*، تریکوتسین، تیپ شیمیایی،NIV، DON

[DOR: 20.1001.1.22519041.2013.2.4.6.0]