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ORIGINAL ARTICLE

Detection of nivalenol and deoxynivalenol chemotypes produced by *Fusarium graminearum* species complex isolated from barley in Iran using specific PCR assays

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Abstract

In order to identify trichothecenes chemotypes produced by *Fusarium graminearum* species complex (FGSC) isolated from barley, 68 barley samples were collected from markets in Kermanshah and Hamedan provinces, Iran. Thirty-one *Fusarium* isolates were obtained from grains and morphologically classified into three species FGSC (14), *F. equiseti* (9), and *F. proliferatum* (8). The identification of the members of FGSC was confirmed molecularly using Fg16F/Fg16R primers. *Fusarium asiaticum* isolates (4) were distinguished from other FGSC using Fg6CTPSf177/Fg16R primers. Polymerase chain reaction-based (PCR-based) detection of mycotoxin-synthesis-pathway gene was also used to determine the potential of the analysed strains to produce deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-AcDON), 3-acetyldeoxynivalenol (3-AcDON), and nivalenol (NIV). Of 14 tested isolates, 10 and 4 isolates belonged to DON and NIV chemotype, respectively. Also, the results of DON chemotype survey using specific primers MinusTri7F/R and Tri315F/R showed 1 and 9 isolates produced 3-AcDON and 15-AcDON, respectively. These results show that DON was the most common chemotype in western Iran. To our knowledge, this is the first report on 15-AcDON, 3-AcDON, and NIV isolated from barley in Iran.

Key words: barley, deoxynivalenol, FGSC, *Fusarium graminearum,* PCR, trichothecenes

Introduction

Many strategic agricultural crops such as members of the Poaceae family are subject to pathogenic fungi, particularly species from the *Fuarium* genus (Waalwijk *et al.* 2003). One well-known disease in Poaceae species such as barley, rye and wheat is *Fusarium* head blight (FHB). FHB outbreaks are common on many continents including North and South America, Europe, Asia and Australia and impose extremely high economic losses (McMullen *et al.* 1997; Obanor *et al.* 2013). *Fusarium* head blight agents invade heads and kernels of small cereal plants and contaminate them with secretion of mycotoxins (Boutigny *et al.* 2011; Postic *et al.* 2012). In many part of the world, contamination of crops with mycotoxins is a major problem in the agricultural industry. Mycotoxins are secondary metabolites produced by some species of fungi, such as *Fusarium graminearum* species complex (FGSC) members, which are responsible for hazardous effects on animals as well as humans. Mycotoxins can enter the food-chain through direct consummation of infected food either by humans or livestock (Postic *et al.* 2012).

Studies show that outbreaks of FHB have been increasing throughout the world over the past decades (Obanor *et al.* 2013). FHB has been identified worldwide and is caused by multiple agents from *Fusarium* spp. which can be very diverse in different countries. *Fusarium culmorum, F. poae,* and *F. avenaceum* are observed in many European countries while *F. asiaticum* is observed in Asia (Waalwijk *et al.* 2003). *Fusarium graminearum* and *F. asiaticum,* members of the FGSC, are the most important agents of FHB. *Fusarium graminearum* are predominant in Asia, Australia, Europe, and America (Akinsanmi *et al.* 2004; Somma

et al. 2014), whereas *F. asiaticum* is the major agent of FHB in many Asian countries such as China, Korea, Japan and Nepal (Zhang *et al.* 2007; Karugia *et al.* 2009; Lee *et al.* 2009; Sarver *et al.* 2011).

FGSC possesses extremely important species which are very widespread. At least 15 distinct lineages which are morphologically indistinguishable have been identified by molecular studies (Sarver *et al.* 2011). Some members of FGSC produce mycotoxins type B trichothecenes that can be classified into three strain-specific chemotypes, including the nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON) and 15-acetyldeoxynivalenol (15-AcDON) chemotypes. The NIV chemotype produces nivalenol, and 3-AcDON and 15-AcDON are acetylated forms of deoxynivalenol (DON) (Miller *et al.* 1991; Boutigny *et al.* 2011). For chemotype identification, there is a rapid procedure based on the amplification of specific genes encoding for proteins involved in mycotoxin biosynthesis. Genes such as: *TRI2*, *TRI3*, *TRI5*, *TRI6*, *TRI7*, *TRI13* and *TRI101* are encoded for biosynthesis of trichothecene and can be assayed by PCR to identify the ability of trichothecene biosynthesis and also chemotypes of the fungi (Chandler *et al.* 2003; Haratian *et al.* 2008; Sampietro *et al.* 2010; Yoruk *et al.* 2016).

Barley is a strategic plant cultivated on more than 190,000 and 120,000 ha in just Kermanshah and Hamedan provinces, respectively. However, very few studies about FGSC invading barley and their chemotypes have been done. The aims of this study were firstly, the identification of the *Fusarium* spp. isolated from barley in Iran in 2012, 2013, and 2014 using morphological and molecular methods by species-specific PCR and secondly, determination of NIV and 3-AcDON and 15-AcDON chemotypes of the isolates.

Materials and Methods

Barley grains were purchased from agricultural markets in Kermanshah and Hamedan provinces. To isolate fusaria, barley grains were randomly hand-selected and placed on water agar amended with peptone-pentachloronitro benzene plates (Nash and Snyder 1962) and incubated at 25°C for 4 days. The single spores of *Fusarium* grown on water agar medium were transferred onto potato dextrose agar and carnation leaf agar media to prepare pure colonies. In order to identify species, macroscopic and microscopic characteristics were studied and the results were compared to the species descriptions of Leslie and Summerell (2006).

DNA was extracted from all FGSC isolates and analysed. Briefly, *Fusarium* isolates were cultured in potato dextrose broth (Sigma) medium, shaking at 150 rpm at 25±2°C for 5 days. Mycelia were harvested by filtration through Whatman paper 1 and freeze-dried for 20 h. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol.

All isolates were investigated by species-specific PCR assay to identify *F. asiatecum* from other members of FGSC. Previously published primer pairs were used for this step (Table 1). Amplification reactions were performed in a total volume of 25 µl, by mixing 1 µl of template DNA with 17.8 µl ddH_2O, 1 µl of deoxynucleotide triphosphate (dNTP) (Promega) – 1 µl of each primer, 0.2 µl of *Taq* DNA polymerase (Promega), 0.5 µl of $MgCl_2$ (Promega) and 2.5 µl of PCR 5X reaction buffer (Promega, Madison, Wl, USA). PCR amplification was done in the Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) with the following programs: an initial denaturation step at 94°C for 5 min, 35 cycles at 94°C (1 min)/56°C (1 min)/72°C

Table 1. Species-specific primers sequences and size of PCR products

Primer name	Sequence 5′-3′	Product size [bp]	Source
Fg16F	CTCCGGATATGTTGCGTCAA	400–500	Nicholson *et al.* (1998)
Fg16R	GGTAGGTATCCGACATGGCAA		
Fg6CTPSf177	GTCTCACTTCAAGCCA	162	Yang *et al.* (2008)
FgCTPSrR306	CCTTGGTCATCCATAGAG		
Tri315F	CTCGCTGAAGTTGGACGTAA	864	Jennings *et al.* (2004)
Tri315R	GTCTATGCTCTCAACGGACAAC		
MinusTri7F	TGGATGAATGACTTGAGTTGACA	483	Ward *et al.* (2002)
MinusTri7R	AAAGCCTTCATTCACAGCC		
ToxP1	GCCGTGGGGATAAAAGTCAAA	300–360	Li *et al.* (2005)
ToxP2	TGACAAGTCCGGTCGCACTAGCA		Nicholson *et al.* (1998)

(3 min), and a final extension step at 72°C for 10 min. The PCR products were visualized by 1X Tris/Borate/EDTA buffer (TBE) electrophoresis in ethidium-bromide-stained, 1% agarose gel.

Trichothecenes chemotypes of *Fusarium* spp. isolates were determined by PCR-based molecular analyses of the mycotoxin-synthesis pathway genes of NIV, DON, 15-AcDON, and 3-AcDON. ToxP1/P2 primers were designed for *Tri5* and *Tri6* gene sequences and used to identify the isolates able to produce NIV and DON. Tri315F/R and MinusTri7F/R were used to make 864 bp specific fragments for 15-AcDON and 483 bp specific fragments for 3-AcDON, respectively (Table 1).

Amplification reactions were made in a total volume of 25 µl for each of the isolates which contained 4 µl 10X buffer (Promega, Madison, WI, USA), 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTP) (Promega), 0.4 µM of each primer, 0.75 units of Taq DNA polymerase (Promega), and 50 ng of template DNA. PCR amplification was carried out in the Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) according to temperature profiles described by Lenc *et al.* (2008) and Lenart *et al.* (2013). To visualize the PCR products, 1X TBE electrophoresis in ethidium-bromide-stained and 1% agarose gel were used.

Results

Sixty-eight barley samples purchased from markets in Kermanshah and Hamedan provinces were analyzed for the occurrence of FGSC and trichothecenes chemotypes. Twenty-one samples were found positive for the *Fusarium* species. A total of 31 isolates were obtained. The isolates were classified into three species FGSC (14), *F. equiseti* (9), and *F. proliferatum* (8) based on morphological characters (Table 2).

Members of FGSC were distinguished molecularly using Fg16F/Fg16R primers. The primers Fg16F/Fg16R produced fragments of 450 bp in 14 isolates. Based on morphological features they were identified as FGSC. Therefore, our morphological studies were confirmed by molecular outputs. Fg6CTPSf177/FgCTPSrR306 primers produced fragments of 162 bp only in 4 isolates which belonged to *F. asiaticum* (Fig. 1).

Four PCR assays (DON, 15-AcDON, 3-AcDON, and NIV) were used for identification of chemotypes of the FGSC isolates. ToxP1/P2 primers that were designed based on *Tri5* and *Tri6* gene sequences were used to identify DON and NIV producer isolates. ToxP1/P2 primers produced fragments of 300 and 360 bp in 10 and 4 isolates, respectively (Fig. 2). Specific primers Tri315F/R and MinusTri7F/R which are designed based on *Tri3* and *Tri7* gene sequences, respectively, were used to identify 15-AcDON and 3-AcDON chemotypes. Tri315F/R and MinusTri7F/R primers produced fragments of 864 bp and 483 bp in 9 and 1 isolates, respectively (Fig. 3).

PCR results showed that 15-AcDON-producing strains existed in all cities in western Iran, except Bisotun, and only one 3-AcDON-producing strain was observed in Kamyaran. NIV-producing strains were observed in the cities of Sarpole-Zahab, Kamyaran, Asad Abad, and Bisotun (Table 2).

Table 2. Place of sample collection, *Fusarium* spp., frequencies of potentially toxigenic (deoxynivalenol – DON and nivalenol – NIV) strains isolated from barley in Iran

Place of sample collection	No. of barley samples (No. of infected samples with *Fusarium* spp.)	*Fusarium* spp. identified	3-AcDON--producing strain	15-AcDON--producing strains	NIV-producing strains
Sarpol-e Zohab	7 (2)	*F. eq, F. gr, F. as, F. pr*	–	*F. gr* (1)	*F. as* (1)
Kamiaran	7 (3)	*F. eq, F. gr, F. pr*	*F. gr* (1)	*F. gr* (1)	*F. gr* (1)
Ravansar	7 (3)	*F. eq, F. gr, F. pr*	–	*F. gr* (1)	–
Sahneh	7 (2)	*F. gr, F. pr*	–	*F. gr* (1)	–
Gilan-e Gharb	7 (1)	*F. eq, F. gr, F. as*	–	*F. as* (1)	–
Kermanshah	7 (2)	*F. gr, F. eq*	–	*F. gr* (1)	–
Bisotun	7 (2)	*F. eq, F. gr, F. pr*	–	–	*F. gr* (1)
Hamedan	7 (2)	*F. eq, F. as, F. pr*	–	*F. as* (1)	–
Ghorveh	6 (2)	*F. eq, F. gr, F. as, F. pr*	–	*F. as* (1)	–
Asad Abad	6 (2)	*F. eq, F. gr, F. pr*	–	*F. gr* (1)	*F. gr* (1)

F. eq = F. equiseti, F. gr = F. graminearum, F. as = F. asiaticum, F. pr = F. proliferatum

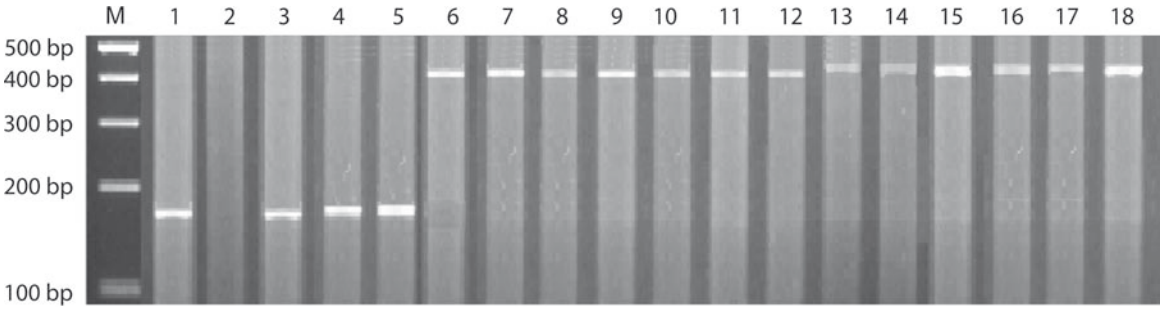

Fig. 1. PCR products obtained with specific primer pairs Fg16F/Fg16R (band, 450 bp) and Fg6CTPSf177/FgCTPSrR306 (band, 162 bp) from 14 isolates of FGSC. Lane M: GeneRuler DNA Ladder Mix, 100–10,000 bp Ladder. (1 = FGSCB1, 2 = *Fusarium proliferatum,* 3 = FGSCB2, 4 = FGSCB3, 5 = FGSCB4) amplified with primers Fg6CTPSf177 and FgCTPSrR306. (6 = FGSCB1, 7 = FGSCB2, 8 = FGSCB3, 9 = FGSCB4,10 = FGSCB5, 11 = FGSCB6, 12 = FGSCB7, 13 = FGSCB8, 14 = FGSCB9, 15 = FGSCB10, 16 = FGSCB11, 17 = FGSCB12, 18 = FGSCB13) amplified with primers pairs Fg16F/Fg16R

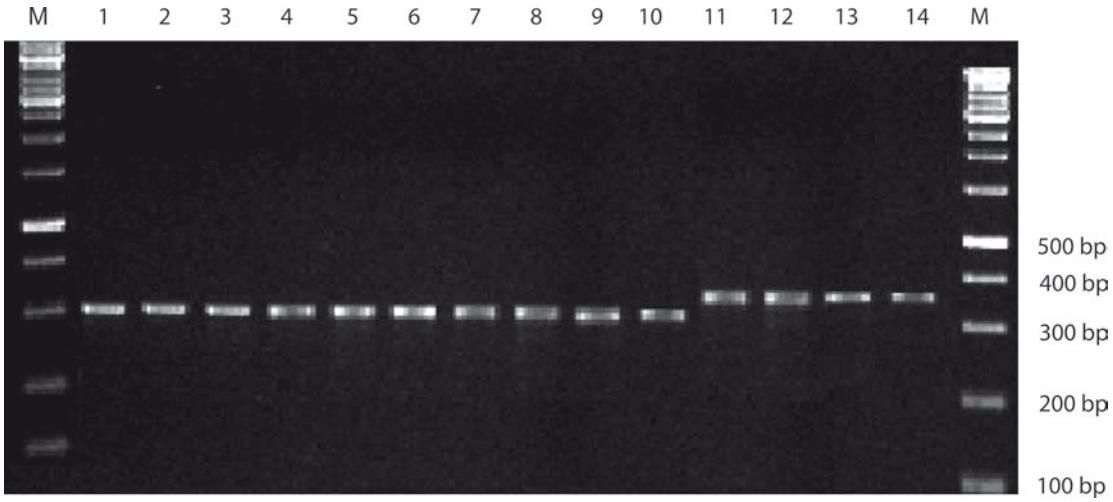

Fig. 2. PCR detection of deoxynivalenol (DON) (band, 300 bp) and nivalenol (NIV) (band, 360 bp) obtained with specific primer pairs of ToxP1/P2. Lane M: GeneRuler DNA Ladder Mix, 100–10,000 bp Ladder. Lanes 1 = FGSCB2, 2 = FGSCB3, 3 = FGSCB4, 4 = FGSCB51, 5 = FGSCB6, 6 = FGSCB8, 7 = FGSCB9, 8 = FGSCB10, 9 = FGSCB11, and 10 = FGSCB12 were positively identified as DON genotypes, and lanes 11 = FGSCB1, 12 = FGSCB7, 13 = FGSCB13, and 14 = FGSC14 were positively identified as NIV genotypes

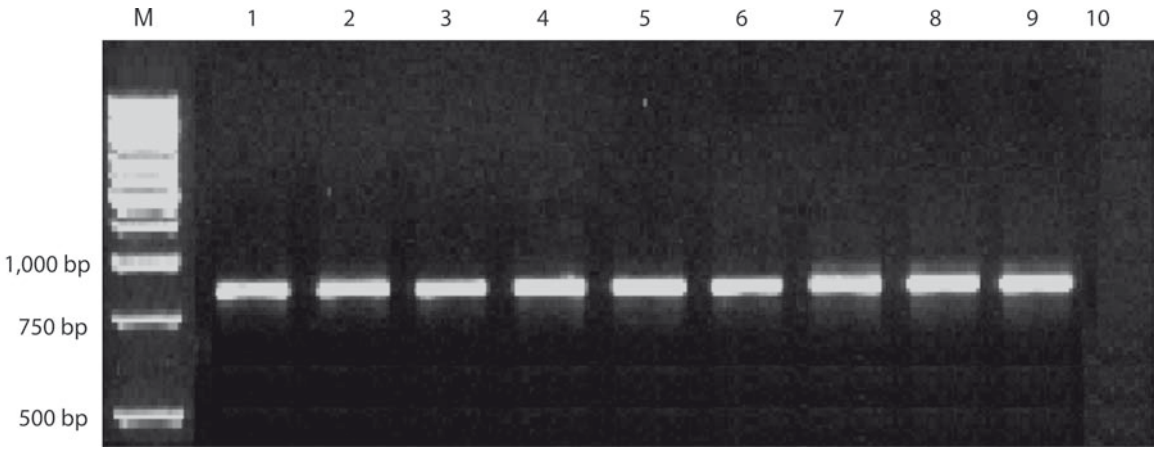

Fig. 3. PCR detection of 15-AcDON (band, 864 bp) obtained with specific primer pairs of Tri315F/R. Lane M: GeneRuler 1 kb DNA Ladder. Lanes 1 = FGSCB2, 2 = FGSCB3, 3 = FGSCB4, 4 = FGSCB51, 5 = FGSCB6, 6 = FGSCB8, 7 = FGSCB9, 8 = FGSCB10, and 9 = FGSCB11 were positively identified as 15-AcDON genotypes

Discussion

Barley is classified as a strategic crop which is cultivated in most parts of the world and is a typical host for FHB (Yang *et al.* 2008). Previous studies have shown that species causing barley FHB can vary in different part of the world (Kim *et al.* 1993; Goswami and Kistler 2004). However, there have been only limited attempts to classify fungal isolates associated with barley in Iran. Our morphological studies identified 31 isolates in the barley samples from western Iran which belong to three species including FGSC, *F. equiseti*, and *F. proliferatum*. FGSC has at least 15 morphologically unidentifiable species, including *F. graminearum sensu strico* and *F. asiaticum* which are the casual agents of FHB global epidemics (Sarver *et al.* 2011). Both species of *F. graminearum* and *F. asiaticum* have been reported from wheat in northern and northwestern areas of Iran (Davari *et al.* 2012). In the present study, for the first time, both of these species were isolated from barley cultivated in Iran and identified through molecular analyses based species-specific PCR. We molecularly detected that *F. asiaticum* constitute about 30% (4 out of 14 isolates) of the isolates belonging to FGSC. Previous studies showed that *F. asiaticum* was commonly isolated in warmer areas (above 22°C) and the majority of *F. graminearum sensu strico* was obtained from cooler areas (22°C or lower) (Qu *et al.* 2008; Backhouse 2014). In our study, *F. graminearum sensu strico* was obtained from almost all regions in western Iran and *F. asiaticum* was mainly isolated from the warmer regions of the same area.

FGSC members produce thrichothecene mycotoxins which can be determined using PCR based analysis. Previous studies identified chemotypes of FGSC members by considering genes related to biosynthesis of the compounds (Jennings *et al.* 2004; Quarta *et al.* 2006; Sampietro *et al.* 2012; Zhang *et al.* 2012). There are few reports based on geographical distribution of FGSC chemotypes and their host ranges in different regions of Iran. Haratian *et al.* (2008) examined NIV and DON chemotypes of isolates collected from different fields in Mazandaran province, in northern Iran. In the present study, we used specific primers to identify the NIV and DON trichothecenes chemotypes among the molecularly confirmed FGSC isolates. Our results revealed that there were more DON chemotypes than NIV chemotypes. Moreover, investigation of the acetylated form of DON showed that 9 and 1 isolates were classified as 15-AcDON and 3-AcDON chemotypes, respectively. To our knowledge, these results are presented for the first time for barley cultivated in Iran.

In conclusion, our findings showed that species-specific primers can be used for rapid detection of NIV and DON trichothecenes chemotypes in infected tissues and 15-AcDON was the most abundant chemotype in western Iran.

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