

Detection of 3-Acetyldeoxynivalenol, 15-Acetyldeoxynivalenol and Nivalenol-Chemotypes of *Fusarium graminearum* from Iran using specific PCR assays

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Abstract

Fusarium graminearum is a serious causing agent of Fusarium Head Blight (FHB) or scab of wheat. It produces different types of trichothecene mycotoxins that are detrimental to both humans and domestic animals. These fungi produce various types of trichothecene mycotoxins, principally including nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON) and 15-acetyldeoxynivalenol (15-AcDON). In this research, in order to determine the trichothecene chemotypes, a total of 100 isolates of *F. graminearum* were collected from different fields in Golestan province (Iran). The identity of species were determined using morphological criteria and then were confirmed using species-specific Fg16 primers. A genomic Polymerase Chain Reaction (PCR) was developed to predict the chemotypes based on the sequence of the *Tri3* gene. Out of the 100 tested *F. graminearum* isolates, 72 detected a 549 bp fragment from NIV chemotypes, 10 yielded a 583 bp fragment from 3-AcDON chemotypes and 18 yielded an 863 bp fragment from 15-AcDON producing isolates. According to our findings, NIV chemotype was predominant in the studied areas.

Keywords: *Fusarium graminearum*; Trichothecene; NIV (Nivanelol); DON (Deoxynivalenol) 3- AcDON (3-Acetyldeoxynivalenol); 15-AcDON (15-acetyldeoxynivalenol); *Tri3*.

Introduction

Fusarium graminearum Schwabe [teleomorph: *Gibberella zeae* (Schwein) Petch] is a fungal pathogen of various crops and is the main causal agent of "Fusarium Head Blight" (FHB) of wheat (Cook, 1981; Kommedahl and Windels, 1981). FHB is known to reduce the yield of grain products. The fungal agent can also contaminate the grains with toxic secondary metabolites (mycotoxins) which cause a health risk to both humans and animals (Marasas et al., 1984; Mc Mullen et al., 1997). The fungus produce various types of trichothecens such as deoxynivalenol (DON chemotype), Nivalenol (NIV chemotype) and their several acetylated derivatives, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) as well as an estrogenic mycotoxin, Zearalenone (Mirocha et al., 1989; Seo et al., 1996). The presence of DON and NIV chemotypes and occurrences of their acetylated derivatives have been reported by different authors (Placinta et al., 1999). Specifically, the NIV chemotype has been reported in several countries of Africa (Mirocha et al., 1989), Asia and Europe (Desjardins et al., 2000; Jennings et al., 2004a) and America (Puri and Zhong, 2010) but it has not yet been reported in North America (Abbas et al., 1989). In cereals infected with *Fusarium*, the DON chemotype was found worldwide but presence of the NIV chemotype is restricted to geographically specific regions (Desjardins et al., 2000). The 15-AcDON chemotype is predominant in North America while the 3-AcDON chemotype is predominant in some areas of Asia including China, Australia and New Zealand (Li et al., 2005; Mirocha et al., 1989). Current molecular studies have shown that the 3-ADON chemotype is replacing 15-ADON from eastern to western Canada (Ward et al., 2008)

where isolates belonging to the 3-ADON chemotype accumulate more trichothecenes than those belonging to the 15-ADON chemotype (Alvarez et al., 2009). Miller et al. (1991) indicated that 15-AcDON production was prevalent in USA and 3-AcDON production in Asia. Epidemics of FHB occur frequently in northern Iran (Golzar et al., 1998; Safaie et al., 2005). DON and NIV chemotypes differ only at the C-4 position; NIV has a hydroxyl group at this position whereas DON does not (Hye-Seon et al., 2003). Each *F. graminearum* isolate is either a DON or an NIV producer (Ichinoe et al., 1983; Seo et al., 1996). Although the molecular genetics of trichothecene production by *Fusarium* species have been studied intensively (Hohn et al., 1998), the biosynthetic and genetic bases for chemotype determination are not well understood. Molecular structures of trichothecene mycotoxin biosynthesis pathways has been demonstrated the mycotoxin gene clusters and their regulations (Gutleb et al., 2002; Hye-Seon et al., 2003; Lee et al., 2001; O'Donnell et al., 2000). Chemical analyses and determination of the mycotoxin chemotype of toxigenic *F. graminearum* is a laborious and time-consuming process (Jian-Hua et al., 2008). Recently, PCR-based assays have been used to determine the mycotoxine chemotype based on the sequences of the *Tri7* and *Tri3* genes sequence of *F. graminearum* (Hye-Seon et al., 2003; Seifert and Le'vesque, 2004; Wang et al., 2008; Yörük and Albayrak, 2012). *Tri3* is located between *Tri7* and *Tri4* and highly conserved for two chemotypes (Brown et al., 2002; Lee et al., 2001; Lee et al., 2002). The aim of the research was to characterize the exact chemotypes of *F. graminearum*, the main agent to cause head blight on winter wheat in northern Iran during 2010-2011 in order to

Table 1. Primers sequences used in this study.

Primer	Sequence(5'-3')	Size(bp)	Reference
Fg16F/Fg16R	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	400-500	Nicholson et al. (1998)
Tri3NIVF/Tri3NIVR	GGACGTGASTACTCTTGGCAA CCCAGRCCTCTAAGAARGGB	549	Jennings et al. (2004a)
Tri303F/Tri303R	GATGGCCGCAAGTGGA GCCGGACTGCCCTATTG	583	Jennings et al. (2004a)
Tri315F/Tri315R	CTCGCTGAAGTTGGACGTAA GTCTATGCTCTCAACGGACAAC	863	Jennings et al. (2004a)

S= C or G, R= A or G

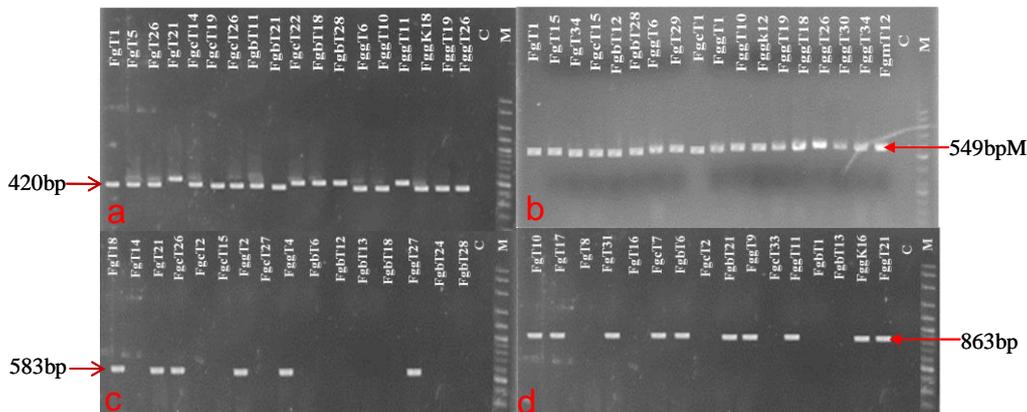


Fig1. (a) PCR products ranging of 420-520 bp by using Fg16F/Fg16R specific primer pair in all *Fusarium* isolates. (b) Amplification products from Tri3NIVF/Tri3NIVR primers, specific to NIV chemotype, (c) *Tri303F/Tri303R* specific to 3-ADON chemotype and (d) *Tri315F/Tri315R* primers specific to 15-ADON chemotype. Lane M: marker (100bp); Lane C, negative control (omitting DNA template); Codes numbers above the panel correspond to the strain codes of *F. graminearum* in complementary data.

understand structure population and distribution of this species.

Results

Fusarium graminearum identification

A total of one hundred isolates of *Fusarium* species obtained from seven different areas belonging to Golestan province in north of Iran were identified as a *F. graminearum* species based on morphological criteria. All isolates were confirmed as *F. graminearum* by a single pair species-specific primer (Fg16F/Fg16R). All samples produced a common band ranging from 420-520 bp pair (bp), as described by Nicholson et al. (1998) (Fig1. a). These data and the morphological examination confirmed that all studied isolates were belonged to *F. graminearum* clade (Table 1).

Chemotype determination

Chemotyping of collected isolates was done by PCR assays using a set of specific primer of *Tri3* gene sequence including: *Tri3NIV*, *Tri303* and *Tri315* (Table 1). The results obtained from PCR reaction with *Tri3NIV* showed the presence of the NIV chemotype in our isolates. Primers *Tri3NIV* produced a fragment of 549 bp length in seventy two isolates in the result of *Tri3* gene sequence amplification (Fig1. b). Of 100 studied isolates subjected to developed PCR assays based on *Tri3* primer, 72 isolates were detected as NIV chemotypes (See complementary data). This result indicates that the majority of isolates have a potential to produce of NIV chemotype. Two sets of primers, *Tri315F/Tri315R* and *Tri303F/Tri303R*, were used to determine the 15-ADON mycotoxin and 3-ADON sub-

chemotype, respectively. According to *Tri3* gene amplification, *Tri303F/Tri303R* primers yielded DNA fragments of 583 bp (Fig1. c). Among the tested isolates with *Tri303* primer, ten isolates were recognized as 3-AcDON producers. PCR amplifications results with *Tri315* showed that 18 isolates were able to produce the 15-AcDON chemotype (See complementary data).. An amplification product with this primer was detected yielding a 863 bp amplicon (Fig1. d). Species-specific PCR primers amplified a DNA fragment of the expected size in studied isolates for which the primer was originally designed. Each isolate produced a PCR product with only one of these primer sets but not the other two. These observations indicated the specification of the primers used for the final confirmation of *Fusarium* chemotypes.

Distribution of chemotype in studied area

Among sampling regions, Gorgan farms showed high distribution of NIV producer isolates (Table 2). This chemotype was detected in all sampling regions with the exception of Bandargaz (Table 2). Our results showed a presence of three chemotypes (NIV, 3-AcDON and 15-AcDON) in *F. graminearum* isolates. 15-AcDON sub-chemotype was observed in the majority of the sampled wheat farms and the highest frequency was found in the fields of Gonabad region (%38/8).

Discussion

In this study, *Fusarium* isolated from infected wheat kernel, showing scab symptoms were identified using morphological characters and then were confirmed with a set of species-specific primers. Species determination and genetic

Table 2. Distribution of trichothecen chemotypes of *F. graminearum* from region different in Golestan province.

Region survey	<i>F. graminearum</i> chemotype		
	DON acetylated derivatives		
	3-AcDON	15-AcDON	NIV
Gorgan	2	3	16
Kordku	1	1	11
Bandargaz	0	2	8
Gonbad	3	7	12
Minoodasht	1	1	11
Kalaleh	2	2	7
Azadshar	1	2	7
Total	10	18	72

chemotyping of *F. graminearum* were used to understand the spatial distribution of these toxins in wheat from the Gorgan province. It has been suggested that knowledge of *Fusarium* chemotype's distribution could be implemented in forecasting schemes for disease development and mycotoxin contamination on a geographical region (Jennings et al., 2004b; Scoz et al., 2009). Owing to highlighting the variability of toxin production among strains of *F. graminearum* (Desjardins, 2008), we developed three sets of primers to target the *Tri3* gene (*Tri3NIV*, *Tri303*, *Tri315*) following PCR assays to determine the chemotype of each *F. graminearum* isolate. This work was the first study of chemotype distribution performed on Golestan province using a large population, with the molecular identification of *F. graminearum*. The trichothecene chemotype distribution analysis among the wheat cultivated in the region of this province revealed that the NIV chemotype was the most dominant type. In addition, two other types; 3-ADON and 15-ADON were always found throughout the sampling area. This chemotype (NIV) is more toxic than DON and is therefore a serious threat for both human and animals (Ryu et al., 1988). So it is necessary to reduce the toxin in this region by using a resistant variety and act to eliminate the toxin from infected farms. Information about secondary metabolites produced by this fungus as toxins and effective factors to inhibit toxin production, using a molecular approach could help to decrease and control the presence of the toxin in crops and food. At this time, little is known about trichothecene chemotypes in different regions of Iran. Haratian et al. (2008) reported that the NIV chemotype was dominant in Mazandaran province in northern Iran and those results were in accordance with this research. Amplification product size from *Tri3* primer was similar to other related researches (Ji et al., 2007). Ji et al. (2007) also found that the most frequently encountered chemotype detected with *Tri315* was the 15-AcDON chemotype while Jennings et al. (2004b) discovered that the 3-AcDON chemotype was detected by the *Tri303* primer. Regarding detection of chemotypes with *Tri3* primer in all isolates, it can be deduced that the *Tri3* gene is most probably contributed in production of Deoxynivalenol acetyl derivatives in *F. graminearum* isolates. *Tri3* gene encoding a 15-O-acetyltransferase enzyme which has an important role in C-15 acetylation, involved in trichothecene biosynthesis (Reynoso et al., 2011). In Europe and South America, the DON chemotype was dominant comparing NIV (Carter et al., 2002). Analysis of different chemotypes in wheat farms of Argentina showed that 15-AcDON chemotype had higher frequency than 3-AcDON (Alvarez et al., 2009). This variation could be due to an effect of geology in a specific region. On the other hand, our finding from Iran showed less diversity comparing nearby regions. It worth to note that, based on recent studies on *F. graminearum* in Canada and

United states, the isolates with 3-AcDON chemotype were more aggressive than those with 15-AcDON and also the 3-AcDON chemotype isolate, since they produced more DON (Puri and Zhong, 2010; Von der Ohe et al., 2010). Distribution of these chemotypes is variable across the globe. In some regions of Asia and China, Australia, New Zealand and northern Europe, the 3-AcDON chemotype is the most dominant whilst the 15-AcDON chemotype is more dominant in North America, central Europe and southern Russia (Quarta et al., 2006).

Materials and methods

Fungal isolates and identification

Kernel samples of winter durum wheat with the characteristic scab symptom were collected from seven different regions of Golestan province during 2010-2011. . The most common wheat varieties were Tajan, Zagros and Koohdasht. Surface-sterilized rachises were placed on a *Fusarium*-selective peptone pentachloronitrobenzene (PCNB) agar (Nirenberg, 1976). Single-spore isolates were cultured on both potato dextrose agar (PDA) and carnation leaf-piece agar (CLA) plates and incubated at 25 C in a 12-h light/dark cycle for 10 days. Isolates were identified as *F. graminearum* according to the morphological criteria on PDA and the cylindrical macroconidia on CLA medium (Burgess et al., 1994; Nelson et al., 1983).

DNA extraction

DNA was extracted from the mycelia of 4 to 5 day-old cultures grown in potato dextrose broth (PDB). The mycelia were freeze-dried and ground to a fine powder and total genomic DNA was extracted using the CTAB method as described by Nicholson et al. (1998).

PCR amplification

Three sets of primers specific designed based on the trichothecene *Tri3* gene to the individual mycotoxin-producer chemotypes and were used to perform PCR detection assays (Table 1). These primers for the *Tri3* gene were developed based on Jennings et al. (2004a). PCR reactions were carried out in 25 µl volumes containing 50 ng of genomic DNA, 1.5 µl 10× buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 Mm KCl, pH 8), 2 mM MgCl₂, 0.2 mM dNTPs, 0.75U DNA *Taq* polymerase (Roche Co., Germany) 2 mM MgCl₂ (Roche, Germany), and 0.4 µM each primer. A negative control, containing all reagents but with no DNA, was used in every set of reactions. PCRs were carried out in an Eppendorph thermocycler and the cycling conditions consisted of

denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30s, 57°C for 30 s and 72°C for 60 s with a final extension step of 72°C for 5 min. The annealing modified temperatures were 60, 53 and 53°C for *Tri3NIVF/Tri3NIVR*, *Tri315F/Tri315R* and *Tri303F/Tri303R*, respectively. The samples were then cooled to 4°C until recovery. PCR products were separated by electrophoresis through 1.2% agarose gels. The gels were stained with ethidium bromide and photographed under UV light.

Species-specific PCR assay

Molecular identification of the collected isolates was further confirmed using specific primer Fg16F and Fg16R (Nicholson et al., 1998) which produces a product of 420-520 bp DNA fragment specific to *F. graminearum* (Table 1).

Conclusion

In this work, a set of primers based on the *Tri3* gene sequences of *F. graminearum* clade was designed that detected a chemotype-specific DNA fragment with different sizes from 3-AcDON-, 15-AcDON and NIV-producers of *F. graminearum* clade strains. Here, we can conclude that the PCR assays could be a beneficial approach for rapid and sensitive detection and identification of *Fusarium* species, the major producer of trichothecene. This tool can be readily used to boost the expediency of disease control and prevention practice to secure healthy food and food products.

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