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Identification, virulence factors characterization, pathogenicity and aggressiveness analysis of *Fusarium* spp., causing wheat head blight in Iran

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Abstract Fusarium head blight (FHB), mainly caused by *Fusarium graminearum* species complex (FGSC) and also by other species of this genus, is one of the most destructive cereal diseases with high yield losses and mycotoxin contamination worldwide. The aim of this study was to identify *Fusarium* species, characterize their virulence factors such as trichothecene genotypes and cell wall degrading enzymes (CWDEs), and also investigate virulence of the isolates obtained from wheat plants with FHB symptoms in Golestan province of Iran. Among 41 isolates tested, 24 were *F. graminearum sensu stricto* (*s.s.*), six were *F. proliferatum*, four were *F. culmorum*, three isolates belonged to each of *F. subglutinans* and *F. meridionale* species and one isolate of *F. asiaticum* was identified. Among *Fusarium* isolates, the nivalenol (NIV) genotype could be found more frequently, followed by 3-acetyl deoxynivalenol (3-ADON) and 15-acetyl deoxynivalenol (15-ADON) genotypes. Production of trichothecenes in autoclaved rice cultures was analyzed by gas chromatography (GC) and confirmed by GC–MS. The mean levels of NIV, 3-ADON and 15-ADON produced by *Fusarium* spp. were 824, 665 and 622 μg kg^{-1}, respectively. All *Fusarium* isolates were capable of producing CWDEs, mainly cellulase and xylanase. Lipase and pectinase activities appeared later and at less quantities. In overall, the isolates FH1 of *F. graminearum* and FH8 of *F. proliferatum* showed the maximum activity of CWDEs, which was correlated with high level of their virulence and aggressiveness on wheat. On the other hand, correlation was observed between the level and type of trichothecene produced by each isolate and its virulence on wheat. Virulence of trichothecene producing isolates was higher than that of non-trichothecene producing isolates. Our results suggested that CWDEs and trichothecenes, as virulence factors, have considerable roles on virulence and aggressiveness of the pathogen. This is the first report on the effect of trichothecenes and CWDEs on virulence and aggressiveness of *Fusarium* spp. associated with FHB disease in wheat growing regions of Iran.

Keywords *Fusarium* spp · Head blight · Trichothecenes · Cell wall degrading enzymes · Virulence

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Introduction

Wheat (*Triticum aestivum* L.) is one of the major cereal crops and a major source of human food worldwide. The genus *Fusarium* has a global distribution and many species in the genus are phytopathogenic fungi infecting a wide range of crop plants including cereals such as wheat, maize, oat and barley (Boutigny et al. 2011). Fusarium contamination is a major agricultural problem, which significantly reduce grain yield and quality. More importantly, many species in the genus *Fusarium* produce mycotoxins that inhibit protein synthesis and cause several health problems in humans and animals (Pestka 2010).

The FHB, also called ear blight or scab, is economically one of the most important fungal diseases of wheat (Spanic et al. 2010). In recent years, the crop losses in Iran due to FHB increased significantly (Haratian et al. 2008; Davari et al. 2013). The causal agents of this destructive disease are several *Fusarium* species, especially *F. graminearum* species complex (FGSC) and *F. culmorum* (Boutigny et al. 2014; Jennings et al. 2004; Nicholson et al. 2007). However, the spectrum of *Fusarium* spp. involved in the disease varies at a local or regional level depending on weather conditions especially during wheat anthesis (Oerke et al. 2010).

To date, 16 species have been identified and formally described within the FGSC (Van der Lee et al. 2015). During the infection process, *Fusarium* spp. are able to produce different mycotoxins such as trichothecenes, zearalenone and fumonisins. Among them, trichothecenes are considered to be the most important ones (Kimura et al. 2007). Trichothecenes are a large group of sesquiterpenoid fungal metabolites, which are demonstrated as virulence factors in wheat-Fusarium interactions (Proctor et al. 1995). Increased trichothecene accumulation is associated with higher level of fungal virulence (Gardiner et al. 2010).

Trichothecene biosynthetic gene (*Tri*) clusters have been characterized in FGSC and *F. sporotrichioides* (Proctor et al. 2009; Brown et al. 2002). In both species, the cluster consists of 12 genes that are involved in the biosynthesis, regulation or transport of trichothecenes. These genes are: *Tri 5* (encoding a terpene synthase); *Tri 4*, *Tri 11* and *Tri 13* (encoding cytochrome P450 monooxygenases); *Tri 3* and *TRI7* (encoding acetyl transferases), *Tri 8* (encoding an esterase), *Tri 6* and *Tri 10* (proposed to be regulatory genes) and *Tri 12* (encoding a transporter). They have been classified into four types (A, B, C, and D) based on structural features (Chaudhary et al. 2011). Among type B-trichothecenes, deoxynivalenol (DON), NIV, and their acetylated derivatives 3-ADON, 15-ADON, and 4-acetylnivalenol (4-ANIV, syn. fusarenone-X) are those having a significant impact on human and animal health (Pasquali and Migheli 2014).

Different trichothecenes have various toxicological properties (Van der Lee et al. 2015). DON is associated with feed refusal, vomiting and suppressed immune functions in consumers, while NIV is more toxic to humans and domestic animals compared to DON (Ryu et al. 1988). Trichothecenes also are potent phytotoxins, with DON being more phytotoxic than NIV (Desjardins 2006). Only a hydroxyl group at C-4 in NIV distinguishes it from DON. However, these chemotype differences may have important fitness consequences for the fungus, as differences in the pattern of oxygenation and acetylation can alter the bioactivity and toxicity of trichothecenes (Alexander et al. 1998).

Three strain-specific profiles of trichothecene chemotypes have been identified within the B-trichothecene lineage of Fusarium: (i) DON and 3-acetyldeoxynivalenol (3-ADON chemotype); (ii) DON and 15-acetyldeoxynivalenol (15-ADON chemotype); or (iii) NIV, its acetylated derivatives and low levels of DON (NIV chemotype) (Ward et al. 2002). On the other hand, in some sources the DON chemotype may exists which includes *Fusarium* species producing 3-ADON and 15-ADON (Pasquali and Migheli 2014; Miller et al. 1991). Substantial geographic variation in *Fusarium* spp. and trichothecene chemotype diversity have been observed (Miller 2002; Nielsen et al. 2012; Ward et al. 2008; Yli-Mattila et al. 2009). All chemotypes may be present in the same geographical location; however, only one is predominant. In cereals infected with Fusarium, the DON chemotype was found worldwide but presence of other chemotypes is restricted to geographically specific regions (Qiu and Shi 2014).

The 3-ADON chemotype is dominant in Russian Far East (Yli-Mattila and Gagkaeva 2010), Norway (Aamot et al. 2015), northern Japan (Suga et al. 2008), Canada (Ward et al. 2008) and northern Europe and has recently been spreading from Finland to the north west of Russia (Talas et al. 2011; Yli-Mattila et al. 2009). While, the 15-ADON chemotype is dominant in central and southern Europe (Yli-Mattila et al. 2013), northern China (Ji et al. 2007; Zhang et al. 2007), south Africa (Boutigny et al. 2011), Brazil (Scoz et al. 2009), Argentina (Alvarez et al. 2011; Reynoso et al. 2011), southern Russia (Yli-Mattila et al. 2009) and the mid-west of USA (Gale et al. 2007). In Asia, NIV chemotype is the most commonly found type of trichothecene (Gale et al. 2007; Zhang et al. 2007). Chemotype occurrence seems to be temperature dependent, the 15-ADON chemotype occurs in cooler regions of China, whereas the NIV chemotype occurred in warmer regions (Zhang et al. 2007). There is also evidence for shifts in trichothecene chemotypes of Fusarium. In China, DON strains are displacing NIV strains (Suga et al. 2008; Zhang et al. 2010). In North America, where for many years 15-ADON was the most prevalent chemotype found in wheat (Schmale et al.

2012), a shift from 15-ADON to 3-ADON occurred in the last decade. In Russian Far East a shift from 15-ADON to 3-ADON chemotype has been found (Yli-Mattila and Gagkaeva 2010). The 3-ADON chemotype has been found to grow more quickly and to produce more trichothecenes and conidia than the 15-ADON chemotype (Ward et al. 2008). So far, little is known about trichothecene chemotypes in different regions of Iran. Among the *Fusarium* isolates, NIV, 3-ADON and 15-ADON chemotypes were detected from different fields of Mazandaran and Golestan provinces in the northern region of Iran (Haratian et al. 2008; Malihipour et al. 2012), while 15-ADON was the only chemotype detected among the isolates collected from fields of Ardabil province in the north west of Iran (Davari et al. 2013; Malihipour et al. 2012).

The knowledge of mechanisms involved in virulence of *Fusarium* spp. on wheat is very limited till now. Fungal pathogens belonging to the genus *Fusarium* have no specialized structures for penetration into plant cell and enters the host via natural openings (Pritsch et al. 2000), or penetrates the epidermal cell walls directly with short infection-hyphae (Wanyoike et al. 2002). *Fusarium* spp. are able to penetrate and invade a host with the help of secreted CWDEs. Production of CWDEs also enables the pathogen to penetrate, grow and infect through the plant tissue (Kikot et al. 2009a). Various CWDEs such as cellulase, xylanase, pectinase and lipase could be produced by *F. graminearum* during infection of wheat spikes (Ortega et al. 2013).

On the other hand, once the infection is established, mycotoxins are released and they interfere with the metabolism, physiologic processes and structural integrity of the host cell (Wagacha and Muthomi 2007). Trichothecenes are considered as virulence factors during plant infection (Pasquali and Migheli 2014). In wheat, the mycotoxin appears to be necessary for fungal passage from infected florets into the rachis from where it can further colonize the head (Jansen et al. 2005). Trichothecenes are also associated with the pathogen aggressiveness (Bai et al. 2002; Foroud and Eudes 2009). These mycotoxins are inhibitors of the protein translational apparatus (Pestka 2007) and have elicitor-like activity in stimulating plant defence and cell death (Desmond et al. 2008; Nishiuchi et al. 2006). Studies on a strain of *F. graminearum* with mutation in the *Tri5* gene encoding a DON biosynthetic enzyme revealed that *F. graminearum* strains unable to produce DON was less aggressive in both wheat and barley (Boddu et al. 2007; Langevin et al. 2004). So, it is suggested that DON and other trichothecenes are involved in virulence by enabling pathogen spread within a spike, but they are not required for initial infection (Bai et al. 2002).

In wheat and barley, trichothecene accumulation is associated with aggressiveness of the fungal pathogen (Gardiner et al. 2010). Reduced aggressiveness of NIV chemotype compared to DON chemotypes, may be due to the lower phytotoxicity of NIV on wheat (Eudes et al. 1997). These findings indicate that fitness and aggressiveness of FHB pathogens change with different chemotypes.

The main objectives of this study were to: (i) identify and determine the frequencies of *Fusarium* spp. isolated from wheat plants with FHB symptoms, (ii) investigate trichothecene chemotypes, (iii) evaluate activities of CWDEs such as cellulase, xylanase, pectinase and lipase which are involved in the infection process of FHB pathogens on host plant, and (iv) characterize virulence factors, pathogenicity and aggressiveness of *Fusarium* spp. and identify possible correlation. So, we mainly described variability of *Fusarium* spp. isolates associated with wheat head blight under various perspectives, which are directly or indirectly correlated with pathogenicity.

Materials and methods

Sample collection

Forty-one isolates of *Fusarium* spp. were collected from different wheat grain samples of various wheat cultivars with symptoms such as ear blight and bleached grains on ears in several regions of Golestan province in Iran during the 2014 growing season (Fig. 1).

Isolation and morphological identification of *Fusarium* species

For isolation of *Fusarium* spp., the grains were surface sterilized by immersion in 1 % sodium hypochlorite for 3 min, and then rinsed three times in sterile distilled water. The sterilized samples were placed in water agar as a general medium and a semi-selective medium for Fusarium, i.e., peptone- pentachloronitrobenzene agar (PPA), and incubated at 25 °C in a 12 h light/dark cycle for 10 days. The resulting Fusarium colonies were single-spored and transferred to potato dextrose agar

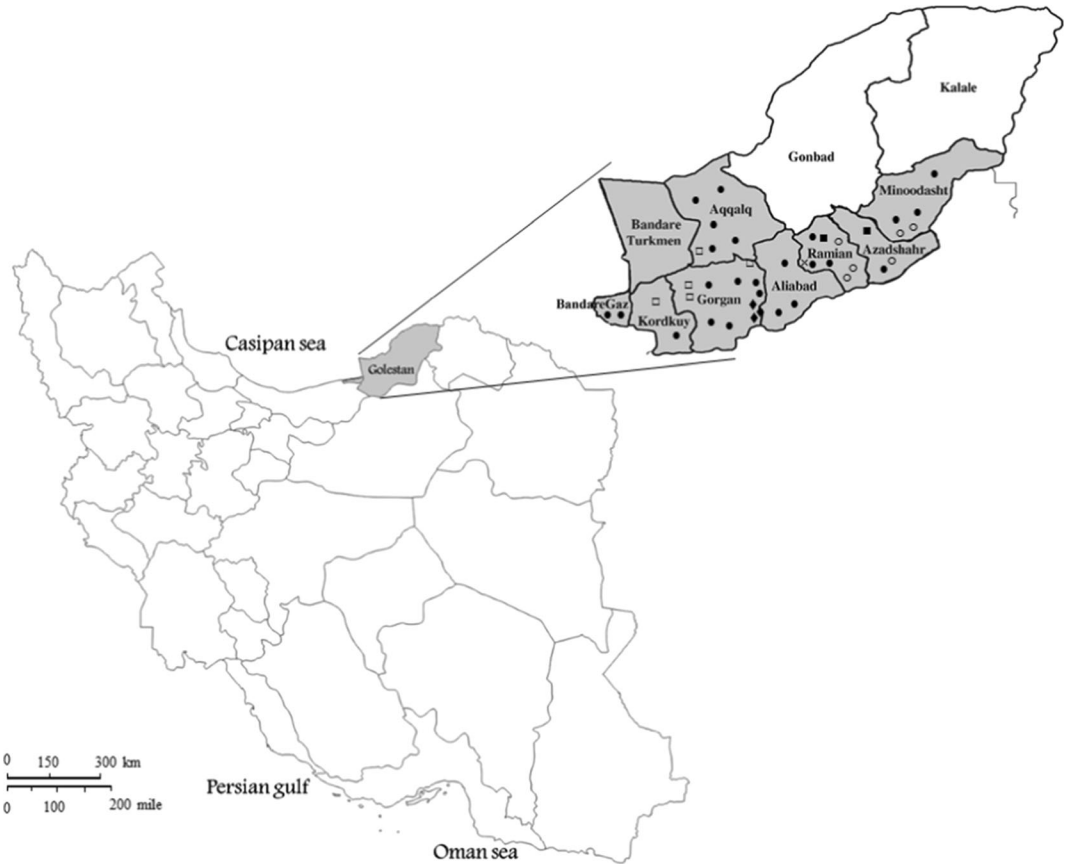

Fig. 1 Geographic locations of *Fusarium* species isolates obtained from wheat-growing regions in the Golestan province of Iran. Sampling was performed of gray areas. ● *F. graminearum*; ■ *F. culmorum*; ○ *F. proliferatum*; □ *F. meridionale*; ♦ *F. subglutinans*; × *F. asiaticum*

(PDA), carnation leaf agar (CLA) (Fisher et al. 1982) and spezieller nährstoffarmer agar (SNA) plates for morphological identification (Leslie and Summerell 2006). *Fusarium* species were identified on the basis of macroscopic characteristics such as pigmentations and growth rates on PDA plates, as well as their microscopic features including size of macroconidia, presence of microconidia and chlamydospores in cultures grown on SNA and CLA (Leslie and Summerell 2006).

Isolation of fungal genomic DNA

Mycelial plugs (0.5 cm^2) were picked up from PDA plates and transferred into bottles containing 100 ml potato dextrose broth (PDB) medium in 250 mL Erlenmeyer flasks, then incubated at 25 °C for 10 days. Mycelial mats were dried between sterile filter papers and ground to a fine powder with liquid nitrogen. Total genomic DNA was extracted with a commercially available DNA extraction kit (Genomic DNA isolation kit IV; DENA Zist Asia, Iran) according to the manufacturer's instructions. DNA concentration was quantified with a NanoDrop spectrophotometer and the quality was verified by 1 % agarose gel electrophoresis. The DNA samples were diluted using sterilized distilled water with final concentration of 50 ng μL^{-1} and stored at −20 °C until use.

Species identification and trichothecene genotype determination by PCR

To confirm the morphological identification of species, conventional PCR was performed using specific primers (Table 1) for molecular identification of seven *Fusarium* species, which may potentially infect wheat kernels in the investigated area. The PCR reaction was performed in a 25 μl volume, each reaction contained 7.5 μL of sterile water, 12.5 μL of PCR Master Mix (Pars Tous,

Table 1 Primer sequences, product sizes and annealing temperatures used for PCR identification of *Fusarium* species

Species	Primer	Sequences (5′-3′)	Product size (bp)	PCR conditions (anneal/extend)	Reference
F. graminearum	Fg16F Fg16R	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	400–500	60 °C/60s	Nicholson *et al.* (1998); Castañares *et al.* (2014)
F. culmorum	OPT18F OPT18R	GATGCCAGACCAAGACGAAG GATGCCAGACGCACTAAGAT	472	59 °C/30s	Schilling *et al.* (1996); Williams *et al.* (2002)
F. subglutinans	61-2 F 61-2R	GGCCACTCAAGCGGCGAAAG GTCAGACCAGAGCAATGGGC	445	64 °C/60s	Möller *et al.* (1999)
F. proliferatum	PRO1-F PRO1-R	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG	585	57 °C/50s	

Iran), 1 μL of 10pM each forward and reverse primers and 3 μL of template DNA. The PCR cycle consisted of an initial denaturation step at 94 °C for 2 min followed by 35 cycles of denaturation (95 °C for 35 s), annealing (times and temperatures for each primer pair listed in Table 1), extension (72 °C for 30 s) and final extension at 72 °C for 7 min. All primers used in this study were purchased from Macrogen (South Korea).

For trichothecene genotypes identification, specific primers for DON, NIV, 3-ADON and 15-ADON forms were used (Table 2). Two multiplex PCR assays were used to evaluate trichothecene genotypes in field populations of Fusarium. The DON and NIV genotypes were identified using a multiplex PCR assay to amplify portions of the *Tri5*, *Tri5-Tri6* intergenic, *Tri7* and *Tri13* genes (Doohan et al. 1999; Li et al. 2005; Waalwijk et al. 2003). The DON, 3-ADON, 15-ADON and NIV genotypes were identified using a multiplex PCR assay to amplify portions of *Tri3* and *Tri12* (Ward et al. 2002).

PCR amplification of *Tri5*, *Tri5-Tri6* intergenic, *Tri7* and *Tri13* genes were performed as previously described (Doohan et al. 1999; Li et al. 2005; Waalwijk et al. 2003). The PCR amplification of *Tri12* and *Tri3* consisted of an initial step at 94 °C for 10 min, followed by two cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. The annealing temperature was stepped down every two cycles to 58, 56, 54, 53, 52 and 51 °C, then

Table 2 Primer identification, sequences and expected amplicon sizes for trichothecene mycotoxin chemotypes of *Fusarium*

Primer designation	Primer sequence	Target gene	Amplicon (bp)	Trichothecene mycotoxin chemotypes	Reference
3CON (R) 3NA (F)	TGGCAAAGACTGGTTCAC GTGCACAGAATATACGAGC	*Tri3*	 840	 NIV	Ward *et al.* (2002)
3D15A (F)	ACTGACCCAAGCTGCCATC		610	15-ADON	
3D3A (F)	CGCATTGGCTAACACATG		243	3-ADON	
TRI5 (F) TRI5 (R)	AGCGACTACAGGCTTCCCTC AAACCATCCAGTTCTCCATCT	*Tri5*	544	Trichothecene producers	Doohan *et al.* (1999)
ToxP1 ToxP2	GCCGTGGGGRTAAAAGTCAAA TGACAAGTCCGGTCGCACTAGCA	*Tri5-Tri6* intergenic	300 360	DON NIV	Li *et al.* (2005)
MinusTri7F MinusTri7R	TGGATGAATGACTTGAGTTGACA AAAGCCTTCATTCACAGCC	*Tri7*	483	DON	Doohan *et al.* (1999)
Tri13F Tri13R	TACGTGAAACATTGTTGGC GGTGTCCCAGGATCTGCG	*Tri13*	234 415	DON NIV	Waalwijk *et al.* (2003)
12CON (R) 12NF (F)	CATGAGCATGGTGATGTC TCTCCTCGTTGTATCTGG	*Tri12*	 840	 NIV	Ward *et al.* (2002)
12-15 F (F)	TACAGCGGTCGCAACTTC		670	15-ADON	
12-3 F (F)	CTTTGGCAAGCCCGTGCA		410	3-ADON	

50 °C for 21 cycles, with a final extension at 72 °C for 10 min (Schmale et al. 2011).

Mycotoxin analyses in laboratory cultures

Mycotoxin production of the *Fusarium* isolates in laboratory cultures was investigated according to Alvarez et al. (2009). Briefly, 25 g of rice (*Oryza sativa* L.) grains was soaked in 100 ml of sterile distilled water for 6 h. Water was drained and the soaked rice was autoclaved twice. Five milliliters inoculum suspension of 1×10^5 conidia mL^{-1} from each isolate was added to each flask and incubated at 26 ± 1 °C in darkness for 3 weeks. The rice-fungi mixtures were ground in a mortar and then dispensed in an Erlenmeyer flask with 75 ml of acetonitrile: methanol: water (80:5:15, *v/v*). Approximately 10 ml of the sample extract was gravity filtered and 5 ml aliquot of the purified extract was transferred into a vial. The solvent was evaporated at 55 °C for 1 h. The analysis was carried out using a fused silica capillary DB-5 column (30 m × 0.25 mm i.d.; film thickness 0.25 μm) for detection and quantification of NIV, DON, 3-ADON and 15-ADON. Nitrogen was both the carrier and auxiliary gas. The injection volume was 1 μl and total running-time was 30 min for each sample. Detection limits were 20 μg kg^{-1} for DON, 50 μg kg^{-1} for NIV, 3-ADON and 15-ADON. Mycotoxin production was confirmed using an Agilent 7890A gas chromatograph with mass spectroscopy, which was performed under electron energy conditions of 70 eV as described by Alvarez *et al*. (2009).

Enzymatic analyses

According to previous studies on the activity of CWDEs *in vitro*, most enzyme activities are observed within 10 days after inoculation (Kikot et al. 2009; Ortega et al. 2013). Based on these observations, pectinase and cellulase activity were evaluated in this study within 10 days. The test for each enzyme had three replicates for each isolate and the experiment was repeated two times.

Pectinase assay

Cultures were performed in 500 mL Erlenmeyers with 250 mL culture medium as previously described (MacMillan and Voughin 1964). Pectinase activity was determined based on the amount of reducing sugar (D-galacturonic acid) released in culture supernatant. The amount of D-galacturonic acid was determined by dinitrosalicylic acid colorimetric method of Colowich (1995) and absorbance was measured at 540 nm. The unit of enzyme activity was defined as the amount of enzyme that released 1 μ mol of galacturonic acid per minute according to the standard curve. The standard curve was drawn based on the absorbance in different concentrations (μg ml^{-1}) of D-galacturonic acid.

Cellulase assay

Cultures were performed in 500 mL Erlenmeyers with 250 mL culture medium as described by Abdel-Razik (1970). After inoculation, incubation was carried out under shaking (150 rpm) at 27 °C and darkness for 10 days. Cellulase activity was investigated using the method of Wood and Bhat (1988). The absorbance was measured at 550 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity was defined as the amount of enzyme that catalyzed 1.0 μ mol of glucose per minute during the hydrolysis reaction.

Xylanase assay

Cultures were performed in 500 mL Erlenmeyers with 250 mL culture medium as described by Miller (1959). Xylanase activity was investigated using the method of Bailey et al. (1992). Absorbance was read at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of xylanase activity was defined as the amount of enzyme that liberates 1.0 μ mol of reducing sugars equivalent to xylose per minute under the assay conditions described.

Lipase assay

Cultures were performed in 500 mL Erlenmeyers with 250 mL culture medium as described by Ortega et al. (2013). Lipase activity was investigated using the method of Ortega et al. (2013). Lipase hydrolytic activity was measured spectrophotometrically at 440 nm with p-nitrophenyl palmitate (p-NPP, 1 mM in acetone) as substrate at 37 °C in 50 mM Tris–HCl buffer (pH 7.0). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of p-NPP per minute under the above mentioned reaction conditions.

Plant materials

Spring wheat cultivar (cv.) Falat, which is susceptible to FHB (Soltanloo et al. 2011) and obtained from Agricultural Research Center of Khorassan Razavi province in Iran, was used for pathogenicity testes. The seeds were surface sterilized with 1 % sodium hypochlorite for 1 min, rinsed three times with sterile distilled water and incubated for 5 days on a wet sterile filter paper in Petri dishes at 25 °C. Germinated seeds were each sown in the 15 cm-diameter plastic pots filled with potting soil, which had been autoclaved at 121 °C for a minimum of 30 min at 100 kPa (15 psi) on 2 successive days and grown in greenhouse (30 ± 4 °C; 16/8 h light/dark photoperiod). The soil used in this experiment, was a combination of clay, sand and farmyard manure with the ratio of 2:1:1 (*v*/*v*/*v*).

Inoculum preparation

Fungal inocula were produced in Mung Bean Broth (MBB) medium using the method described by Zhang et al. (2013). Conidial suspensions were diluted to a final concentration of 1×10^5 conidia mL^{-1} containing 0.05 % (*v*/*v*) Tween 20.

Virulence analysis on seedlings

Virulence capability of all *Fusarium* isolates on seedlings was investigated using the method described by Gargouri-Kammoun et al. (2009). At the two- to three-leaf stage (Zadoks' growth stage (ZGS) 12 to 13), a volume of 250 μl of a spore suspension (1×10^5 conidia mL^{-1}) was placed at the stem base and leaf primordial of each plant. Plants used as controls were inoculated in a similar manner with sterile distilled water. Plants were incubated in the dark at constant 100 % relative humidity and 22 °C for 24 h, and twenty one days after inoculation, each plant was carefully pulled out and washed. Disease severity was graded into five classes based on the proportion of stem discoloration (0 = no discoloration; 1 = 1 to 25 %; 2 = 26 to 50 %; 3 = 51 to 75 %; 4 = more than 75 %; 5 = dead plant) as described by Fernandez and Chen (2005) and the disease index (DI) was calculated as described previously (Taheri and Tarighi 2010). Each test had ten replicates arranged in a completely randomized design, and the experiment was repeated two times.

Virulence analysis on wheat spikes

Virulence of all isolates on wheat spikes was evaluated using the method described by Yoshida et al. (2007). At the flowering stage (ZGS 64 to 65), 10 mL of spore suspension (1×10^5 conidia mL^{-1}) amended with 0.05 % Tween 20 was sprayed onto the spikes of each plant. The inoculated plants were incubated overnight in greenhouse at 18-25 °C, with 100 % humidity. Then, the plants were placed in a plastic bag for 3 days to maintain high relative humidity. Control plants were only treated with sterile distilled water. Inoculated wheat heads were evaluated after 10 days and the FHB disease severity was estimated. Disease severity was measured as the percentage of infected spikelet(s) within the spike using a 0 to 5 scale (0 = no disease, 1 = to 20 %, 2 = to 40 %, 3 = to 60 %, 4 = to 80 % and 5 = more than 80 % disease severity) (Wan et al. 1997) and the FHB index was calculated as described previously (Amarasinghe et al. 2013). Each test had ten replicates arranged in a completely randomized design, and the experiment was repeated two times.

Detached-leaf assay

The wheat plants were grown in greenhouse with 12 h photoperiod, RH of 75 %, and a day: night temperature of 18 °C: 12 °C. After 14 days, 4 cm segments from the mid-section of the first leaf were harvested, and placed adaxial surface up on the surface of 0.5 % water agar as described by Browne and Cooke (2004). Leaf segments were inoculated at the center of the adaxial surface with 5 μl inoculum suspension of 1×10^5 conidia mL^{-1} containing 0.05 % (*v*/*v*) Tween 20. Sterile distilled water was applied on the control leaves. Petri dishes were incubated at 25 °C with a 12 h: 12 h light: dark cycle. After 5 days, the length of necrotic lesions was measured. The test included four replicates for each isolate and the experiment was repeated two times.

Assessment of aggressiveness

Aggressiveness of each isolate of *Fusarium* spp. on seedlings, wheat spikes, and detached leaves were carried out using the methods described by Malihipour et al. (2012) and Pariaud et al. (2009). Analysis of aggressiveness was done based on determining hours post inoculation (hpi) for disease symptom appearance.

Aggressiveness of all isolates was checked after every 12 h.

Statistical analysis

All experiments were set up in a completely randomized design. The data were analyzed by one-way analysis of variance (ANOVA) and comparison of means was carried out using the Duncan's Multiple Range Test at the level of $P \leq 0.05$. Statistical analysis was performed with statistical package for the social sciences (SPSS; version 22) software.

Results

Morphological identification of *Fusarium* isolates

Morphological observations showed that width of macroconidia ranged from 4.0 to 7.0 μm for FGSC (Fig. 2a), 4.0 to 4.6 μm for *F. graminearum* (Fig. 2b), 4.0 to 4.5 μm for *F. asiaticum* (Fig. 2c), 3.8 to 4.3 μm for *F. meridionale* (Fig. 2d), 5.0 to 6.0 μm for *F. culmorum* (Fig. 2e). Based on morphological characters of conidia, chlamydospores and conidiophores, 41 *Fusarium* isolates were identified which belonged to four species (Table 3, Fig. 2). The most common species identified were FGSC (68.3 %) and *F. proliferatum* (14.6 %). Less frequently isolated species included *F. culmorum* (9.8 %) and *F. subglutinans* (7.3 %).

Molecular identification of *Fusarium* isolates using species-specific PCR assay

The list of PCR primers used to identify *Fusarium* species is presented in Table 1. The Fg16F/Fg16R primers are not completely specific to *F. graminearum sensu stricto*, but they gave products of different size (400–500 bp), as described by Nicholson et al. (1998). *F. graminearum s.s.* gave a product of about 400 bp, while *F. asiaticum* gave a PCR product of about 550 bp and *F. meridionale* gave a product of about 500 bp, as described by Castañares et al. (2014). Molecular analysis using Fg16F/Fg16R primers revealed that from 28

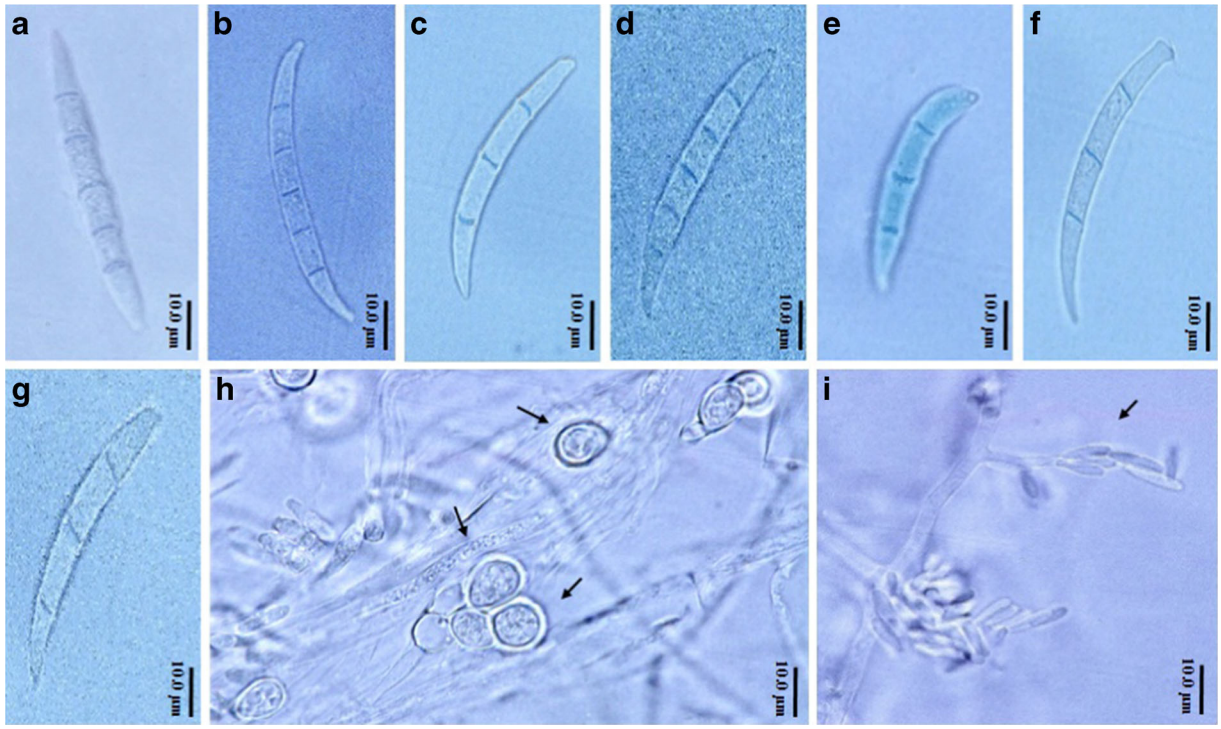

Fig. 2 Morphological characters of *Fusarium* species. macroconidium of FGSC (**a**), macroconidium of *F. graminearum* (**b**), macroconidium of *F. asiaticum* (**c**), macroconidium of *F. meridionale* (**d**), macroconidium of *F. culmorum* (**e**), macroconidium of *F. proliferatum* (**f**), macroconidium of *F. subglutinans* (**g**), chlamydospores of FGSC (**h**), conidiophores of *F. proliferatum* (**i**)

Table 3 Morphological characters of *Fusarium* isolates from different cultivars of wheat (*Triticum aestivum* L.)

Name of the Species	Pigmentation on PDA	Growth rate	Chlamydospore	Sporodochium	Microconidia	Macroconidia			
						Number of septa	Apical cell	Basal cell	size (μm)
FGSC	vary from white to pale pink to red	R	+	Pale orange	–	5–7	tapered	Fs	24–72 × 4–7
F. culmorum	red	R	+	Orange to brown	–	3–4	rounded and blunt	Nfs	37–55 × 4–7
F. subglutinans	initially white but becomes violet	S	–.	Tan to orange	+	3	curved	Pfs	54–85 × 4–7
F. proliferatum	purple-violet	S	–	Tan to pale orange	+	3–5	curved	Pf	54–85× 4–7

FGSC: *Fusarium graminearum* species complex, R: Rapid, S: Slow, +: Presence, −: Absence, Fs: Foot shape, Nfs: Notched and without a distinct foot shape, Pfs: Relatively poorly developed, Pf: Poorly developed

isolates morphologically identified as FGSC, 24 isolates belonged to *F. graminearum*, three isolates were *F. meridionale* and one isolates was *F. asiaticum*. All six isolates morphologically identified as *F. proliferatum*, were confirmed using PRO1-F/PRO1-R primers. All four isolates morphologically identified as *F. culmorum* were confirmed using OPT18-F470/OPT18-R470 primers. Also, morphological identification of three isolates belonging to *F. subglutinans* was confirmed using OPT18-61-2 F/61-2R primers specific for this species.

Trichothecene genotype detection by PCR

The gene *Tri5* encodes Trichodiene synthase, which catalyses the first step in trichothecene biosynthesis. In this study, this gene was detected using the primer set TRI5(F)/TRI5(R), which produces a unique PCR product in size of 544 bp for strains that contain *Tri5* gene (Doohan *et al*. 1999; Covarelli *et al*. 2014). In overall, results obtained from PCR of the *tri5* gene showed amplification of this gene for 78.1 % of trichothecene producing and 21.9 % of non-trichothecene producing isolates.

Also, primers for amplification of five genomic regions (*Tri3*, *Tri5-Tri6* intergenic, *Tri7*, *Tri12* and *Tri13*) involved in trichothecene biosynthesis were used (Table 2). The results obtained from PCR reaction with *Tri3*, *Tri5-Tri6* intergenic, *Tri12* and *Tri13* showed the presence of NIV genotype in our isolates. Primers used in regions of *Tri3*, *Tri5-Tri6* intergenic, *Tri12* and *Tri13* produced a fragment of 840, 360, 415 and 840 bp length, respectively. The *Tri7* primer pair which was specific for detecting DON produced a fragment of 483 bp length. Primers *Tri3* and *Tri12* for detecting 3-ADON produced fragments of 243 and 410 bp length, respectively, and for detecting 15-ADON produced fragments of 610 and 670 bp length, respectively. The results showed that 25 isolates had the NIV genotype, five classified in 3-ADON genotype, two had 15-ADON genotype, and nine were non-trichothecene producing isolates (Table 4). There were negative results with all genotype-specific primers in Table 4.

Distribution of genotypes

Among different wheat samples of various cultivars in the investigated regions, Galikesh farms showed high distribution of NIV producer isolates. This trichothecene genotype was detected in all sampling regions. Among the isolates, 3-ADON was detected from different fields of Gorgan, Azadshar, Ali Abad, Bandar gaz and Agh Ghala, while 15-ADON was detected from different fields of Gorgan and Agh Ghala in the northern region of Iran. Also we observed isolates, which based on PCR assay results should be able to produce NIV, DON, 3-ADON and/or 15 A-DON simultaneously (Table 4).

Table 4 Isolates, origin, species-specific PCR, presence of trichothecene-specific markers detected by TRI-based multiplex PCR assays and trichothecene genotype

Isolate code	Cultivar	Sample site	Species-specific PCR	Trichothecene genotype	PCR assay results											
					Tri5	*Tri3*			*Tri5-Tri6*		*Tri7*	*Tri12*			*Tri13*	
					Tri P	15-ADON	3-ADON	NIV	DON	NIV	DON	NIV	15-ADON	3-ADON	DON	NIV
FH1	Koohdasht	Gorgan	*F. graminearum*	NIV	+	+	+	+	−	−	−	+	−	+	−	+
FH2	Unknown	Gorgan	*F. graminearum*	NIV	+	−	−	+	−	−	−	+	−	−	−	−
FH3	Unknown	Gorgan	*F. graminearum*	NIV	+	−	−	+	−	−	−	+	+	−	−	−
FH4	Tajan	Gorgan	*F. meridionale*	3-ADON	+	−	+	−	−	−	−	−	−	−	−	−
FH5	Tajan	Gorgan	*F. graminearum*	15-ADON	+	+	−	−	−	−	−	−	−	−	+	−
FH6	Unknown	Gorgan	*F. meridionale*	NIV	+	−	−	−	−	−	−	+	−	+	−	−
FH7	N8720	Gorgan	*F. subglutinans*	NIV	+	−	+	−	−	−	−	+	−	−	−	+
FH8	Tajan	Azadshar	*F. proliferatum*	NIV	+	−	+	+	−	−	−	−	+	−	−	−
FH9	Tajan	Azadshar	*F. culmorum*	NIV	+	−	−	+	−	−	−	−	+	−	−	−
FH10	Unknown	Azadshar	*F. graminearum*	3-ADON	+	−	−	−	−	−	−	−	−	+	+	−
FH11	Tajan	Minoodasht	*F. graminearum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH12	Tajan	Minoodasht	*F. graminearum*	NIV	+	−	−	+	−	−	−	−	−	−	−	+
FH13	Unknown	Minoodasht	*F. graminearum*	NIV	+	−	−	−	−	+	−	+	−	−	−	+
FH14	Gonbad	Minoodasht	*F. proliferatum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH15	Tajan	Minoodasht	*F. proliferatum*	NIV	+	−	−	−	−	+	−	−	−	−	−	+
FH16	Koohdasht	Ali Abad	*F. graminearum*	3-ADON	+	−	+	−	−	−	+	−	−	−	−	−
FH17	Tajan	Ali Abad	*F. graminearum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH18	Tajan	Ali Abad	*F. graminearum*	NIV	+	−	+	+	−	−	−	−	−	−	−	−
FH19	N8720	Galikesh	*F. proliferatum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH20	N8720	Galikesh	*F. proliferatum*	NIV	+	−	+	+	−	−	−	−	−	−	−	−
FH21	N8720	Galikesh	*F. proliferatum*	NIV	+	−	−	+	−	−	−	−	−	+	−	−
FH22	Unknown	Galikesh	*F. graminearum*	NIV	+	−	−	−	−	+	−	+	−	−	−	−
FH23	Unknown	Galikesh	*F. culmorum*	NIV	+	−	−	+	−	−	−	−	−	+	−	+
FH24	Tajan	Bandar gaz	*F. graminearum*	3-ADON	+	−	−	−	−	−	−	−	−	+	+	−
FH25	Unknown	Bandar gaz	*F. graminearum*	NIV	+	−	−	+	−	−	−	+	−	−	−	+
FH26	Gonbad	Daland	*F. graminearum*	NIV	+	−	−	+	−	−	−	−	−	−	−	+
FH27	Koohdasht	Daland	*F. graminearum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH28	Koohdasht	Daland	*F. asiaticum*	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 4 (continued)

Isolate code	Cultivar	Sample site	Species-specific PCR	Trichothecene genotype	PCR assay results											
					Tri5	*Tri3*			*Tri5-Tri6*		*Tri7*	*Tri12*			*Tri13*	
					Tri P	15-ADON	3-ADON	NIV	DON	NIV	DON	NIV	15-ADON	3-ADON	DON	NIV
FH29	Tajan	Agh Ghala	*F. graminearum*	NIV	+	−	+	−	−	−	−	+	−	−	−	−
FH30	Tajan	Agh Ghala	*F. graminearum*	NIV	+	+	−	+	−	−	−	+	−	−	−	−
FH31	Unknown	Agh Ghala	*F. meridionale*	NIV	+	−	−	−	−	−	−	+	−	+	−	−
FH32	Unknown	Agh Ghala	*F. graminearum*	3-ADON	+	−	+	−	−	−	+	−	−	−	+	−
FH33	Koohdasht	Agh Ghala	*F. graminearum*	NIV	+	−	−	+	−	−	−	−	−	−	−	+
FH34	Koohdasht	Agh Ghala	*F. graminearum*	15-ADON	+	+	−	−	+	−	−	−	−	−	−	−
FH35	Koohdasht	Ismail Abad	*F. subglutinans*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH36	Unknown	Ismail Abad	*F. graminearum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH37	Unknown	Ismail Abad	*F. culmorum*	−	−	−	−	−	−	−	−	−	−	−	−	−
FH38	Tajan	Kordkuy	*F. graminearum*	NIV	+	−	−	−	−	−	−	−	−	−	−	+
FH39	Tajan	Kordkuy	*F. culmorum*	NIV	+	−	−	−	−	−	−	+	−	−	−	+
FH40	Unknown	Fazel Abad	*F. subglutinans*	NIV	+	+	−	+	−	−	−	−	−	−	−	−
FH41	Unknown	Fazel Abad	*F. graminearum*	NIV	+	−	−	−	−	−	−	+	−	−	−	−

Tri P: trichothecene producers, +: Presence, −: Absence, 3ADON: 3-acetyldeoxynivalenol + deoxynivalenol, 15ADON, 15-acetyldeoxynivalenol + deoxynivalenol, NIV: nivalenol + acetylated derivatives (Miller *et al*. 1991; Ward *et al*. 2002)

Mycotoxin analysis

Data of trichothecene production by *Fusarium* isolates on rice medium are shown in Table 5. For FGSC, 53.6 %, 28.6 %, 42.9 % and 21.4 % of the analysed samples contained NIV, DON, 3-ADON and 15-ADON, in levels ranging from 437 to 1205 μg kg^{-1}; 269 to 1248 μg kg^{-1}; 178 to 1183 μg kg^{-1} and 240 to 1155 μg kg^{-1}, respectively. Most of the *F. culmorum*, *F. proliferatum* and *F. subglutinans* isolates were NIV producers (75 %, 66.7 % and 66.7 %), at levels between 700 and 1075 μg kg^{-1}, 260 and 1199 μg kg^{-1} and 313 to 1202 μg kg^{-1}, respectively (Table 5). Isolates FH1 of *F. graminearum* and FH8 of *F. proliferatum* produced NIV, 3-ADON and 15-ADON simultaneously (Table 5).

Analysis CWDEs activity

Analysis of CWDEs showed that all *Fusarium* isolates were capable of producing CWDEs. According to the results obtained, for most of the isolates tested the maximum levels of cellulase, xylanase, pectinase and lipase activity were observed at 72, 96, 144 and 192 h post-culturing (hpc) on liquid medium, respectively. Then, the activity decreased gradually with time, until it remained at constant levels at the end of culture time. At the time point in which most of the isolates showed maximum activity for each enzyme, the level of CWDEs activity among isolates varied from 232 to 938 μg ml^{-1} for cellulase, 589 to 1215 μg ml^{-1} for xylanase, 3340 to 4695 μg ml^{-1} for pectinase, and 13 to 28 μg ml^{-1} for lipase (Table 5).

The FH1 and FH8 isolates showed the maximum cellulase activity *in vitro* among all isolates. The lowest cellulase activity was observed for FH17 and FH36 isolates, respectively. The lowest xylanase activity belonged to FH19 and FH11 isolates, and also maximum xylanase activity with 1215 ± 7.1 and 1187 ± 11.3 μg ml^{-1}, respectively, was observed for FH1 and FH8 isolates. With regard to pectinase, FH8, FH29, FH7 and FH1 isolates showed the maximum cellulase activity. The lowest pectinase activity belonged to FH11, FH19, FH37 and FH28 isolates, respectively. Maximum lipases activity was observed for FH1 and FH8 isolates, respectively and the isolates FH37 and FH11 had the lowest lipase activity (Table 5).

Virulence and aggressiveness assay

Comparison of the data obtained from inoculation of *Fusarium* isolates on wheat seedlings, wheat spikes and leaf segments revealed that different isolates tested had various virulence capabilities (Table 6). Based on the results obtained, significant differences in disease index on seedlings, wheat spikes and also leaf lesion length were observed among isolates tested.

Pathogenicity tests on seedlings showed that the lowest disease index was observed for the FH11 isolate. The FH1 isolate caused the highest level of disease progress on seedlings with average DI of 63 ± 1.2 (Table 6, Fig. 3a). The results of pathogenicity test on wheat spikes showed that the longest lesions were produced by FH1 isolate with an average DI of 69.3 ± 0.3 (Table 6, Fig. 3b). The shortest lesions were produced by FH11 and FH19 isolates among all *Fusarium* isolates. FHB index of other isolates ranged from 66.3 ± 1.3 to 12.7 ± 0.3. Leaf assay revealed that the highest lesion length was produced by the FH1 isolate. The lowest lesion length was produced by FH19 and FH28 isolates, respectively. Other isolates tested fell between these with various levels of virulence on wheat leaf segments (Table 6, Fig. 3c).

The results of aggressiveness test on seedlings, wheat spikes, and detached-leaves showed earlier development of disease symptoms by FH1 isolate compared to other isolates tested (Table 6).

Correlation between activity of CWDEs and virulence

We compared the activity of CWDEs produced by some of the *Fusarium* isolates, which caused maximum or minimum level of virulence on wheat leaves, seedlings, or spike (Fig. 4) for finding possible association between CWDEs and virulence. The isolates FH1 and FH8, which showed the highest levels of virulence in three different bioassays on leaf, seedling and spike, had considerably higher levels of enzyme activity at various time points investigated. Whereas, the isolates FH11 of *F. graminearum* and FH19 of *F. proliferatum*, which had the lowest virulence capability (on wheat leaf, spike and seedling), revealed the lowest level of CWDEs activity at most of the time points tested (Fig. 4).

Table 5 Mycotoxin production by *Fusarium* isolates in rice cultures analyzed by GC-MS and maximum of enzyme activity

Isolate code	Trichothecene production *in vitro* (μg kg^{-1})				Maximum of enzyme activity (μg ml^{-1})			
	NIV	3-ADON	15-ADON	DON	Cellulase 72 hpc	Xylanase 96 hpc	Pectinase 144 hpc	Lipases 192 hpc
FH1	910	498	253	ND	938 ± 2.8 a	1215 ± 7.1 a	4620 ± 6.3 a	29 ± 0.9 a
FH2	1105	ND	ND	ND	488 ± 0.4 g	833 ± 8.0 kl	4255 ± 3.8 f	23 ± 0.6 d
FH3	1007	243	ND	ND	618 ± 21.7 f	950 ± 3.6 efg	4295 ± 5.2 ef	24 ± 0.2 d
FH4	ND	501	ND	1248	725 ± 0.8 c	823 ± 4.6 l	3558 ± 16.7 h	20 ± 0.5 fg
FH5	ND	ND	1001	1000	672 ± 19.6 d	876 ± 5.6 ij	4253 ± 4.6 f	24 ± 0.9 d
FH6	499	108	ND	ND	633 ± 4.0 ef	890 ± 10.8 hi	4324 ± 6.8 ef	16 ± 0.2 h
FH7	1202	308	ND	ND	667.8 ± 5.9 d	1041 ± 3.8 c	4620 ± 6.3 a	27 ± 0.5 b
FH8	438	997	240	ND	930 ± 7.8 a	1187 ± 11.3 a	4695 ± 33.3 a	28 ± 0.5 a
FH9	751	ND	325	ND	770 ± 25.2 bc	1001 ± 5.4 cd	4597 ± 39.1 b	26.8 ± 0.2 b
FH10	ND	1098	ND	269	630 ± 2.3 ef	820 ± 12.6 l	3553 ± 21.3 h	20 ± 0.2 fg
FH11	–	–	–	–	324 ± 8.8 j	620 ± 10.8 pq	3375 ± 21.3 i	13 ± 0.5 i
FH12	754	ND	ND	ND	670 ± 0.4 d	871 ± 13.5 ijk	4259 ± 9.3 f	23 ± 2.5 d
FH13	620	ND	ND	ND	649 ± 0.6 de	843 ± 15.3 jkl	3711 ± 6.5 1 g	23 ± 0.6 d
FH14	–	–	–	–	452 ± 1.3 h	725 ± 20.0 mno	3519 ± 16.0 h	19 ± 0.1 g
FH15	1199	ND	ND	ND	628 ± 6.2 ef	1008 ± 14.3 cd	4452 ± 35.4 bc	24 ± 0.0 d
FH16	ND	1030	ND	1023	599 ± 6.1 f	827 ± 13.5 l	3584 ± 10.4 h	22 ± 0.4 de
FH17	–	–	–	–	232 ± 5.0 k	650 ± 4.6 p	3517 ± 14.2 h	19 ± 0.1 g
FH18	701	315	ND	ND	723 ± 1.0 c	911 ± 1.2 ghi	3726 ± 8.82 g	20 ± 0.0 fg
FH19	–	–	–	–	378 ± 2.1 i	589 ± 16.4 q	3367 ± 4.4 i	16 ± 0.3 h
FH20	1098	974	ND	ND	732 ± 20.8 c	1094 ± 1.3 b	4426 ± 18.2 c	26 ± 0.0 b
FH21	260	1089	ND	ND	824 ± 7.4 b	1001 ± 7.8 d	4413 ± 41.5 cd	24 ± 0.0 d
FH22	1001	ND	ND	ND	726 ± 3.6 c	974 ± 2.0 de	4323 ± 47.5 ef	24 ± 0.1 d
FH23	806	355	ND	ND	649 ± 25.3 de	898 ± 9.2 hi	4354 ± 4.4 ef	24.4 ± 1.6 d
FH24	ND	279	ND	1077	673 ± 10.2 d	825 ± 5.4 l	3577 ± 45.3 h	22 ± 0.3 de
FH25	739	ND	ND	ND	738 ± 2.0 c	896 ± 5.6 hi	4345 ± 24.7 de	24 ± 1.2 d
FH26	437	ND	ND	ND	686 ± 14.6 d	929 ± 27.8 fgh	4267 ± 22.5 ef	24 ± 0.9 cd
FH27	–	–	–	–	497 ± 1.2 g	752 ± 11.9 mn	3577 ± 44.4 h	20 ± 0.1 fg
FH28	–	–	–	–	475 ± 0.4 gh	704 ± 17.8 o	3340 ± 3.8 i	16 ± 0.1 h
FH29	1230	1183	ND	ND	726 ± 3.6 c	1012 ± 0.2 cd	4620 ± 6.3 a	26 ± 0.4 bc
FH30	480	ND	1155	ND	678 ± 36.6 d	961 ± 10.5 ef	3585 ± 42.5 h	20 ± 0.2 fg
FH31	1211	980	ND	ND	712 ± 5.2 c	1080 ± 8.2 b	4510 ± 21.8 b	26 ± 0.3 b
FH32	ND	178	ND	895	611 ± 17.2 f	759 ± 13.4 m	3544 ± 33.3 h	20 ± 0.0 fg
FH33	494	ND	ND	ND	725 ± 0.8 c	828 ± 0.8 l	3508 ± 7.12 h	19 ± 0.5 g
FH34	ND	ND	293	1000	632 ± 13.8 ef	730 ± 20.5 mno	3511 ± 9.61 h	19 ± 0.2 fg
FH35	-	–	–	–	296 ± 18.5 j	706 ± 17.3 o	3510 ± 10.0 h	19 ± 0.3 g
FH36	–	–	–	–	254 ± 12.3 k	714 ± 20.5 no	3563 ± 23.9 h	20 ± 0.1 fg
FH37	–	–	–	–	249 ± 10.9 k	646 ± 7.7 p	3350 ± 45.1 i	12.9 ± 0.4 i
FH38	1088	ND	ND	ND	629 ± 20.9 ef	874 ± 2.1 ig	3568 ± 29.2 h	21 ± 0.5 ef
FH39	1075	ND	ND	ND	725 ± 0.8 c	884 ± 12.1 i	3576 ± 28.4 h	20 ± 0.0 fg
FH40	313	ND	1088	ND	725 ± 0.8 c	940 ± 5.7 fg	4287 ± 5.8 ef	24 ± 0.5 d
FH41	1205	1180	ND	ND	614 ± 5.0 f	958 ± 36.1 ef	4402 ± 54.9 cd	26 ± 0.0 bc

ND: not detected, −: without genotype of trichothecene biosynthesis. Average ± standard error, Different letters indicate significant differences according to Duncan analysis using SPSS software ($P = 0.05$), each experiment was repeated two times with similar results

Table 6 Virulence and aggressiveness of *Fusarium* isolates on seedling, wheat spike and leaf segments inoculated

Isolate code	Virulence and aggressiveness analysis					
	leaf		seedling		spike	
	Virulence (LL, mm)	Aggressiveness (hpi)	Virulence (DI)	Aggressiveness (hpi)	Virulence (FHB index)	Aggressiveness (hpi)
FH1	39 ± 1.1 a	12	63 ± 1.2 a	72	69.3 ± 0.3 a	48
FH2	29 ± 1.6 fg	48	32 ± 1.0 hijk	168	29.7 ± 0.3 gh	120
FH3	30.5 ± 1.9 efg	48	33 ± 0.7 ghij	168	30 ± 2.3 gh	120
FH4	18 ± 0.0 i	72	20 ± 0.6 no	216	24.3 ± 2.7 ij	144
FH5	23.75 ± 0.6 h	60	27 ± 0.3 m	216	41 ± 1.2 cd	120
FH6	29 ± 1 fg	48	35 ± 0.3 efg	144	42.3 ± 2.6 cd	72
FH7	33.25 ± 1.2 cde	36	37 ± 0.3 cd	96	42 ± 2.5 cd	72
FH8	37 ± 0.8 ab	24	40 ± 0.3 b	72	52.7 ± 2.8 b	60
FH9	31 ± 0.9 ef	48	35 ± 0.3 efg	144	42 ± 0 cd	84
FH10	19 ± 1.3 i	72	18 ± 0.6 p	228	18.3 ± 4.3 kl	156
FH11	7.75 ± 0.94 lm	96	4 ± 0.6 s	264	7 ± 2.3 n	180
FH12	29.25 ± 1.6 fg	48	31 ± 0.3 jk	180	21.3 ± 1.3 jk	168
FH13	29.5 ± 0.5 fg	48	31 ± 0.3 jk	180	27.7 ± 0.3 hi	120
FH14	10.25 ± 0.7 kl	96	10 ± 0.6 q	240	16. ± 0.6 lm	168
FH15	30 ± 0.8 efg	60	35 ± 0.3 ef	144	42 ± 0 cd	72
FH16	20 ± 0.7 i	72	22 ± 0.9 n	216	27 ± 0 hi	120
FH17	11 ± 0.6 kl	96	8 ± 0 qr	240	16.3 ± 2.4 lm	168
FH18	29 ± 1 fg	48	34 ± 0.6 efgh	144	31 ± 1 fgh	120
FH19	5.75 ± 0.6 m	108	9 ± 1.7 qr	240	7.3 ± 0.3 n	180
FH20	36.5 ± 0.9 abc	24	39 ± 0.7 bc	84	68.7 ± 0.9 a	60
FH21	32 ± 1.2 def	36	36 ± 0.3 de	108	43.3 ± 0.3 c	72
FH22	29.5 ± 0.6 fg	48	34 ± 0.3 efghi	168	30.7 ± 0.9 fgh	120
FH23	29 ± 1.1 fg	48	34 ± 0.6 ghij	180	45.3 ± 0.3 c	72
FH24	19.25 ± 0.5 i	72	18 ± 0.7 op	216	14.3 ± 0.9 lm	168
FH25	28.25 ± 1.0 g	60	33 ± 0.3 ghij	180	38.3 ± 0.9 de	72
FH26	29.75 ± 0.5 fg	48	32 ± 0.7 ijk	180	45.7 ± 0.3 c	72
FH27	15 ± 0.4 j	96	10 ± 0.6 q	228	13 ± 0.6 m	168
FH28	6.75 ± 1.7 m	108	7 ± 0.6 r	240	14.3 ± 0.7 lm	168
FH29	34.5 ± 1.5 bcd	24	38 ± 0.3 bc	96	66.3 ± 1.3 a	60
FH30	29.25 ± 1.2 fg	48	35 ± 0.6 ef	120	43.3 ± 0.7 c	72
FH31	35.5 ± 1.0 bc	24	39 ± 0.6 bc	96	45 ± 1.1 c	72
FH32	19.75 ± 1.2 i	72	21 ± 0.7 n	216	16 ± 0 lm	168
FH33	29 ± 1.2 fg	48	31 ± 1.0 jk	180	36 ± 3 e	120
FH34	25 ± 1.1 h	72	28 ± 0.6 l	216	29 ± 0.6 hi	144
FH35	12.5 ± 1.0 j	96	9 ± 0.7 q	240	12.7 ± 0.3 m	168
FH36	10 ± 0.4 kl	96	9 ± 1.1 qr	240	29.3 ± 1.3 h	144
FH37	5.75 ± 0.6 m	108	9.3 ± 0.6 qr	240	12.7 ± 0.3 m	168
FH38	30 ± 1.9 efg	48	30 ± 0.6 k	180	34.3 ± 0.3 efg	120
FH39	28.5 ± 0.5 fg	48	33 ± 0.3 ghij	168	26.3 ± 0.3 hi	144
FH40	29.5 ± 1.3 fg	48	33 ± 0.7 ghij	168	26.3 ± 0.3 hi	144
FH41	29.75 ± 0.6 fg	48	33 ± 0.4 ghi	168	35.3 ± 1.2 ef	120

hpi: hours post inoculation, LL: Lesion length, DI: Disease index, FHB index: Fusarium head blight index. Average ± standard error, Different letters indicate significant differences according to Duncan analysis using SPSS software ($P = 0.05$), each experiment was repeated two times with similar results

Fig. 3 Disease symptoms on wheat seedlings (**a**), spikes (**b**) and leaf (**c**) by *Fusarium* isolates. Control, *F. graminearum* (FH1) and *F. proliferatum* (FH8). Arrows marked symptoms disease and necrotic lesions

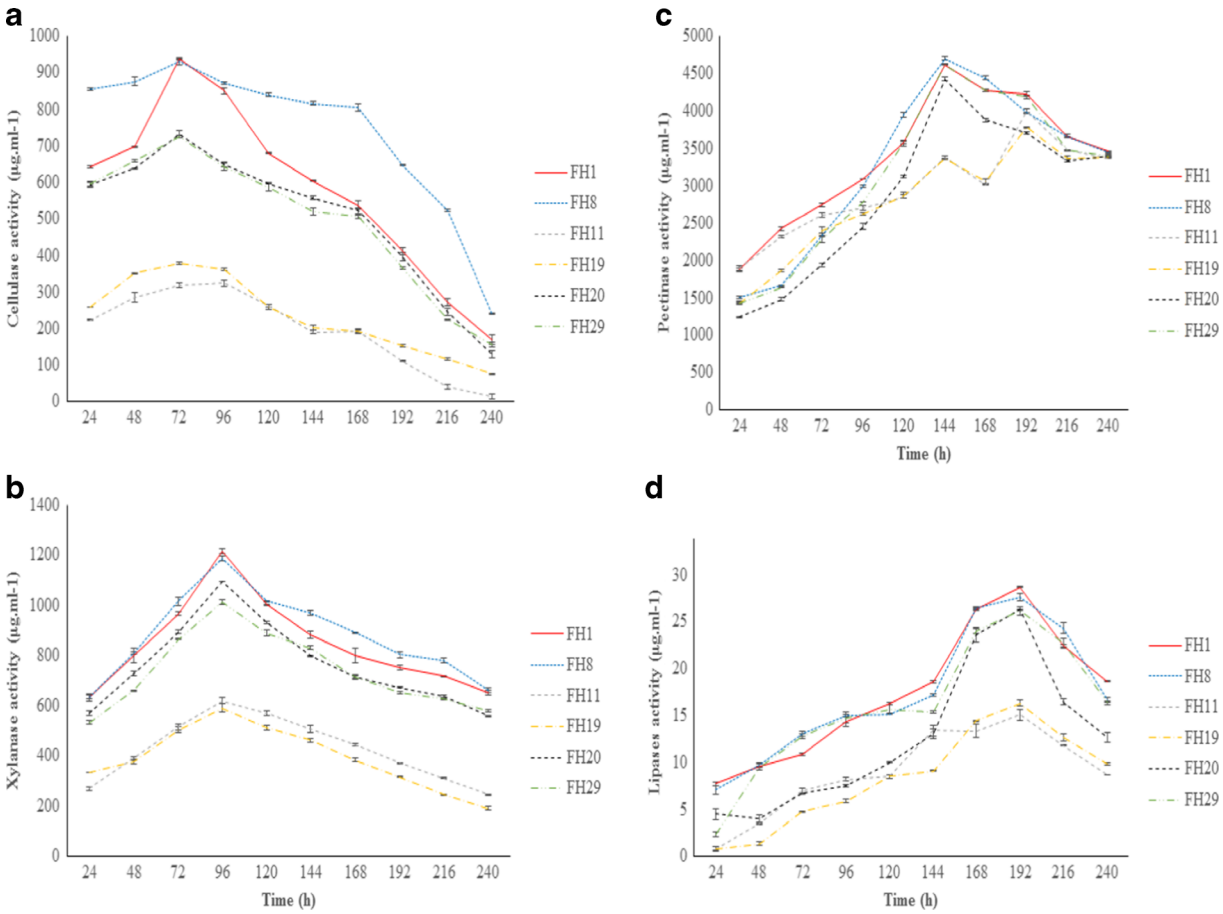

Fig. 4 Analysis activities of CWDEs produced by *Fusarium* isolates over an incubation maximum period of 240 h. cellulase activity (**a**), xylanase activity (**b**), pectinase activity (**c**) and lipase activity (**d**). Values are means of 3 replicates. FH1; FH8; FH11; FH19; FH20; FH29

Discussion

In this study, to identify and determine the frequencies of *Fusarium* spp. causing wheat head blight in northern region of Iran, a total of 41 *Fusarium* spp. isolates were obtained from infected wheat heads showing disease symptoms such as ear blight and bleached grains and the isolates were characterized using morphological and molecular methods. Morphological identification was confirmed using a set of species-specific primers. This is the first detailed report of trichothecene genotypes in populations of *Fusarium* spp. collected from the northern region of Iran and quantification of trichothecenes using GC-MS. Also, we evaluated the activities of CWDEs, which are involved in plant-pathogen interactions during FHB infection and investigated the correlation between virulence factors, aggressiveness and virulence capability of *Fusarium* isolates on seedlings, spikes and leaves of wheat plants.

Based on morphological observations, a total of 41 isolates belonging to four *Fusarium* species were isolated from wheat grain samples. Twenty eight isolates were identified as FGSC, six isolates as *F. proliferatum*, three isolates as *F. subglutinans* and one isolate as *F. culmorum*. The FHB disease was observed in different regions of Golestan province. Gorgan and Agh Ghala showed the highest percentage of infected samples. It is possible that planting the sensitive cultivar Tajan and crop rotation with maize and sorghum are the causes of FHB prevalence in this region (Table 4). The main species associated with FHB disease are *F. graminearum*, *F. culmorum* and *F. proliferatum*, among which *F. graminearum* and *F. proliferatum* are known to produce toxins (Karami-Osboo et al. 2010). Davari et al. (2013) reported that 96 % of the isolates recovered from FHB affected wheat in Ardabil province of Iran belong to *F. graminearum*, which is in accordance with our data.

The results of morphological identification were in accordance with the reports of Sarver et al. (2011) and Starkey et al. (2007). *F. graminearum* formed narrow conidia with 4.0–4.5 μm width, similar to *F. asiaticum*. But, the width of *F. meridionale* isolates was less than 4.5 μm. *F. graminearum* and *F. asiaticum* did not form beaked conidia in contrast to *F. meridionale*. Conidia of *F. graminearum* and *F. asiaticum*, however, were gradually curved and, in addition, those were most frequently widest above the mid-region. In contrast, conidia of *F. meridionale* were gradually curved and most frequently widest at the mid-region (Sarver et al. 2011; Starkey et al. 2007).

Species-specific PCR analysis showed that among 41 isolates used in this study, 24 isolates were identified as *F. graminearum*, six were *F. proliferatum*, four were *F. culmorum*, three were *F. meridionale*, three were *F. subglutinans* and one isolate of *F. asiaticum* was identified. The products of DNA amplification corresponded to sizes reported for species-specific PCR products and confirmed the morphological identification (Alkadri et al. 2013; Castañares et al. 2014; Williams et al. 2002). Based on our molecular analysis, morphological identification of all 4 isolates belonging to *F. culmorum* was confirmed using the OPT18-F470/OPT18-R470 primers and gave a PCR product of about 472 bp. But according to Schilling et al. (1996), this primer pair does not give a positive signal with all *F. culmorum* isolates.

Results of the present study indicated that *F. graminearum s.s.* was the most frequently isolated species (58.6 %), confirming other reports on this species as one of the most often isolated *Fusarium* species from the FGSC not only in Iran (Haratian et al. 2008), but also in many other countries including the United States (Schmale et al. 2011; Alvarez et al. 2011; Prodi et al. 2011).

The *Tri5* gene encodes trichothecene synthase as the initial product in the trichothecene biochemical pathway (Neissen and Vogel 1997), which could be used for developing a PCR-based assay to detect trichothecene-producing *Fusarium* species (Doohan et al. 1999). The results of trichothecene genotype detection revealed 83.4, 66.7, 75, 66.7 and 100 % amplification of the *tri5* gene for the isolates belonging to *F. graminearum*, *F. proliferatum*, *F. culmorum*, *F. subglutinans* and *F. meridionale*, respectively. The isolate of *F. asiaticum* did not produce trichothecene.

Production of NIV required *Tri13* and *Tri7* genes that produce the acetylation and oxygenation of the oxygen at C-4 to produce nivalenol and 4-acetyl nivalenol, respectively (Lee et al. 2009). Our results showed that NIV was produced by 60.9 % of the isolates. Whereas, 41.5 % of the isolates produced 3-ADON, 17.1 % 15-ADON, and 17.1 % DON. In most isolates, NIV was found simultaneously with 3-ADON and 15-ADON. Except for 16 isolates, NIV was always produced in larger amounts than the other trichothecenes. Our data are in accordance with observations of Gale et al. (2011), which

reported that the NIV chemotype was prevalent on wheat in Southern Louisiana.

Trichothecene genotype detection of the FGSC population showed that the NIV genotype was the most frequent (57.2 %) followed by 3-ADON (17.9 %) and 15-ADON (7.2 %) genotypes. With regard to *F. proliferatum* and *F. subglutinans* the analysis showed only presence of the NIV (66.7 %) and absence of 3-ADON and 15-ADON chemotypes. Two isolates of *F. culmorum* belonged to the NIV genotype. So, investigation of trichothecene chemotypes revealed that the NIV chemotype was the most prevalent in *Fusarium* isolates obtained from wheat farms of the northern region of Iran, followed by 3-ADON and 15-ADON chemotypes. Similar results were obtained by Abedi-Tizaki and Sabbagh (2013). Also, Haratian et al. (2008) reported that the NIV chemotype was dominant in Mazandaran province in the northern part of Iran. Results similar to our findings were obtained in southern Louisiana (Gale et al. 2011), England and Wales (Jennings et al. 2004), Netherlands (Waalwijk et al. 2003) and South Africa (Sydenham et al. 1989). Observation of a wide variation in trichothecene production *in vitro* among *Fusarium* isolates in this study was supported by results reported by other investigators using GC-MS analysis (Alvarez et al. 2009; Covarelli et al. 2014).

Wheat and maize in rotation with sorghum are the most important cereal crops in the northern region of Iran. Crop rotation may influence the pathogen population dynamics, especially since it was shown that NIV and DON act as virulence factors on wheat, while only the NIV chemotype is virulent on maize (Maier et al. 2006). Our results showed that in the north of Iran, which is an important region in producing small grain cereal crops such as wheat and rice, FHB-associated *F. graminearum* isolates produced mostly NIV rather than DON derivatives. This finding is in accordance with observations of Davari et al. (2013), who demonstrated association of the NIV chemotype with local rice production. Other studies have also clearly shown this association between rice production and the prevalence of the NIV chemotype (Umpiérrez-Failache et al. 2013; Qiu and Shi 2014; Van der Lee et al. 2015), which confirmed our data. According to these results, it might be concluded that differences in crop rotations and bordering crops may influence the species and chemotypes found in wheat.

In general, NIV chemotypes appeared to be more aggressive than the other chemotypes of *F. graminearum*. This finding is in agreement with the observations of other researchers (Carter et al. 2002; Cumagun et al. 2004). In addition, *Fusarium* isolates producing higher level of NIV and 3-ADON chemotypes were more aggressive than other isolates. Similarly, Von der Ohe et al. (2010) demonstratd that the isolates with 3-ADON chemotype were more aggressive than those with 15-ADON. Also, Puri and Zhong (2010) suggested that the 3-ADON isolates were more aggressive and caused higher FHB severity.

Evaluating the activities of CWDEs, which are involved in the infection process of FHB pathogens on wheat, was performed in this study. Aggressiveness of *Fusarium* spp. involves different mechanisms such as production of extracellular enzymes and mycotoxins (Ortega et al. 2013). Hemibiotrophic (*F. graminearum*) and necrotrophic (*F. subglutinans*, F. *proliferatum* and *F. culmorum*) pathogens often produce different extracellular CWDEs (Stankovic et al. 2007). These enzymes are particularly important for phytopathogenic fungi without specialized penetration structures (Gibson et al. 2011). We focused on detailed investigation of pectinase, cellulase, xylanase and lipase activities in different time points because they are the major CWDEs in *Fusarium* species (Kikot et al. 2009; Ortega et al. 2013). During 10 days of CWDEs investigation, production rate and time of reaching each enzyme to its maximum activity varied among different isolates. Cellulase was the first in reaching its peak, while the maximum activity of xylanase, lipase and pectinase appeared later and at lower magnitude. Similar results were obtained by Ortega et al. (2013), who reported that lipase activity reached to its maximum activity after longer incubation time compared to other enzymes tested. Comparing CWDEs activities and virulence of the isolates revealed that lipase and pectinase had less effect on virulence compared to cellulase and xylanase. Similar results were reported by Phalip et al. (2005), who analyzed exo-proteome of *F. graminearum* grown on plant cell wall.

The results of our study about virulence of *Fusarium* isolates on seedlings, wheat spikes and leaf segments showed that all isolates were pathogenic on wheat (cv. Falat) and differences in virulnce capability were found. According to the results reported by other investigators, strong association has been found between the severity of FHB and mycotoxin concentration (Panthi et al.

2014; Hernandez-Nopsa et al. 2014; Wegulo 2012; Burlakoti et al. 2007) as observed in this study. *F. graminearum* isolates without the ability of producing trichothecenes were unable to cause severe disease on wheat tissues, which was in agreement with the data of Bai et al. (2002). Our results are in accordance with several other studies showing that trichothecenes have a significant role in determining the virulence of *Fusarium* isolates (Hernandez-Nopsa et al. 2014; Purahong et al. 2013; Umpiérrez-Failache et al. 2013).

Since the NIV chemotype is more detrimental for consumers, we have to find a solution to reduce this mycotoxin. The economic and social impact of FHB highlights the necessity of using effective control strategies. Management of FHB to reduce mycotoxin contamination have been developed by utilizing host resistance, use of biological agents, tillage, seed treatment, crop rotation and fungicides application during flowering stage (Brown et al. 2007; Müllenborn et al. 2008; Willyerd et al. 2012; Hollingsworth et al. 2008). Knowledge on *Fusarium* species and chemotypes, CWDEs and virulence levels could be useful in the production of resistant varieties and other management strategies to reduce destructive effects of FHB disease in small grain cereals, especially in wheat growing areas.

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