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Detection of the fumonisin–producing *Fusarium fujikuroi* species complex (FFSC), associated with wild grasses in Iran

Kh. Chehri ✉

Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran

Abstract: In order to determine the fumonisin producing isolates of the *Fusarium fujikuroi* species complex (FFSC), associated with infected spikes, and ear rot of grass plants, we examined 78 samples collected from Kermanshah province, Iran. Based on morphological characters, twenty two isolates assigned to the FFSC and were identified as *F. proliferatum* (ten isolates), *F. verticillioides* (seven isolates), and *F. subglutinans* (five isolates). This was additionally approved using the specific primers of *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*, i.e. VER1/VER2, PRO1/PRO2, and SUB1/SUB2, respectively. PCR–based detection of the fumonisin–producing isolates of FFSC was also achieved using the primers FUM1 F/FUM1 R. Out of twenty two isolates, eleven isolates (50%) determined as fumonisin chemotype.

Key words: *Fusarium* spp., grasses, molecular detection, mycotoxins, Iran

INTRODUCTION

Poaceae is a large family of monocotyledonous flowering plants known as grasses. The Poaceae are among the most economically important plant families owing to their usages in food production, industry, and lawns (Inch & Gilbert 2003). Since the 1950s, grasses have been considered as the main source of food for domesticated animals in Iran. Northern and western parts of Iran are the main growth areas of rangeland plants in Iran (Parsa 1950).

Mycotoxigenic fungi which are natural contami–nants of cereals worldwide (Goswami & Kistler 2004, 2005) have become also threatening for grass plants through several genera including *Fusarium* spp., *Ustilago* spp., and *Aspergillus* spp. (Inch & Gilbert 2003; Postic et al. 2012).

Fusarium ear rots are the most commonly and widely studied diseases of the Poaceae (Akinsanmi et al. 2003; Boutigny et al. 2011; Postic et al. 2012). At least 18 species of *Fusarium* Link have been assigned to *Fusarium* ear rots (Bottalico an&d Perrone, 2002). Among these, members of the *Fusarium fujikuroi* species complex (formerly the *Gibberella fujikuroi* species complex) are the most important and prevalent species, producing different mycotoxins including moniliformin (MON), fumonisins (FUMB), and beauvericin (BEA) (Logrieco et al. 1995, 2002).

The *Fusarium* mycotoxin contamination of grasses is a potential health hazard for animals grazing these plants (Desjardins & Proctor 2011; Postic et al. 2012). The mycotoxin–production ability of *Fusarium* spp. is diverse and particular strains may produce different mycotoxins (Goswami & Kistler 2005). Fumonisins are one of the most important carcinogenic mycotoxins that are mainly produced by *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg and *F. verticillioides* (Sacc.) Nirenberg (Logrieco et al. 1995, 2002). More than 25 structurally related fumonisin analogues have been identified, with fumonisin B1 (FB1) as the most prevalent one (Logrieco et al. 1995; Desjardins 2006). So, rapid and accurate identification of *Fusarium* spp, as well as detection of their mycotoxigenic properties is vital to reduce their harmful effects (Eskola et al. 2001).

The aims of this study were to (i) identify the members of the FFSC associated with grass plants in Iran using morphological and molecular approaches; (ii) determine the genetic potential of FFSC isolates to produce fumonisin.

MATERIALS AND METHODS

Sample collection, isolation, and identification of *Fusarium* isolates

A survey was carried out in five agro–ecological zones of Kermanshah province of Iran, including Sarpol–e Zahab, Mahidasht, Eslamabad–e Gharb, Bisotun, and Kermanshah districts. In each agro–ecological zone, 12 farms were randomly selected and the infected grassy plants containing root, spike, and crown rot symptoms were collected. To identify the members of Poaceae, plants were transferred to the herbarium of Razi University. To isolate *Fusarium* isolates, the infected roots, crown, and head were cut into small pieces, surface sterilized with 96% ethanol for 30 sec followed with a fine rinse and subsequently

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✉ Corresponding Author E-mail: khchehri@gmail.com

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plated on water agar (WA) (1.5%). The resulting single–spore *Fusarium* colonies were transferred to fresh potato dextrose agar (PDA) plates and maintained at 4°C for further studies. To study the growth rates and pigment production of *Fusarium* spp. all the isolates were transferred onto PDA plates and incubated at ambient temperature. For microscopic observations, all the isolates of *Fusarium* were transferred to carnation leaf agar (CLA) (Fisher et al. 1982) media. *Fusarium* species were identified based on morphological characters according to the *Fusarium* laboratory manual (Leslie & Summerell 2006).

Molecular methods

Genomic DNA of the FFSC isolates was extracted from 10 mg of freeze–dried mycelia using DNeasy® Plant Mini Kit (Qiagen) according to the manufact–urer's protocol. Polymerase chain reaction (PCR) was performed according to Mulé et al. (2004). VER1/ VER2, PRO1/PRO2, and SUB1/SUB2 (Mulé et al. 2004) were the primers specifically used to identify *F. verticillioides, F. proliferatum*, and *F. subglutinans* isolates (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas, respectively (Table 1). Amplification reactions were performed according to Mulé et al. (2004). The polymerase chain reaction was performed at 95 °C (5 min) for hot start, followed by 35 cycles of 94 °C for 50 sec, 56 °C (50 sec.), 56 °C (50 sec.) and a final extension of 72 °C (1 min). FUM1 F/FUM1 R set of primers (Bluhm et al. 2004, (Table 1) was also used to determine the fumonisin–producing isolates of FFSC, examined in this study. The PCR program for amplification of FUM1 F/FUM1 R was as described by Bluhm et al. (2004). The PCR products were visualized in 1% agarose gel electrophoresis.

Chemical detection of mycotoxins

From selected samples, potential mycotoxigenic *Fusarium* isolates were chemically analysed to determine the concentration of B1 and B2 fumonisins. Detection of mycotoxins was carried out using high performance liquid chromatography (HPLC) assay, according to Chehri et al. (2010).

RESULTS

Seventy–eight samples with mostly spike and crown rot symptoms were collected from different sites of Kermanshah province. Nine species from nine tribes of the Poaceae, including *Avena wiestii*, *Bromus sericeus*, *Dactylis glumerata*, *Aegilops cylindrical*, *Stipa barbata*, *Lolium perenne*, *Melica Jacquemontii*, *Agropyron repens*, and *Ermopoa persica* were identified based on pollen morphology using light microscopy (Table 2).

Based on morphological features, 22 isolates belonging to FFSC were recovered from infected tissues of grassy plants. These isolates were mainly identified as *F. proliferatum* (10), *F. verticillioides* (7) and *F. subglutinans* (5) (Table 2) (Fig. 1).

Table 1. Primers used for detection of the fumonisin–producing *Fusarium fujikuroi* species complex members.

Primers*	Sequence 5′–3′	PCR product (bp)	Target sequence	Source
SUB1 SUB2	CTGTCGCTAACCTCTTTATCCA CAGTATGGACGTTGGTATTATATCTAA	631	Calmodulin gene	Mulé et al. (2004)
VER1 VER2	CTTCCTGCGATGTTTCTCC AATTGGCCATTGGTATTATATATCTA	578	Calmodulin gene	Mulé et al. (2004)
PRO1 PRO2	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG	585	Calmodulin gene	Mulé et al. (2004)
FUM1 F FUM1 R	CCATCAC AGTGGGACACAGT CGTATC GTCAGCATGATGTAGC	183	FUM1 gene	Bluhm et al. (2004)

* SUB: For *Fusarium subglutinans*; VER: For *F. verticilioides*; PRO: For *F. prolofiratum*; FUM: For *Fumonisin*

Table 2. Sampled regions in association with the FFSC isolates recovered from roots, crown, and inflorescences of wild grasses in Kermanshah province, Iran.

Region	Host and identified FFSC species	Fumonisin–producing FFSC species on the host
Sarpol–e Zahab	*Avena wiestii* (a, b, c) *Bromus sericeus* (a, c) *Dactylis glumerata* (c)	a (*A. wiestii*), c (*B. sericeus, D. glumerata*)
Mahidasht	*Aegilops cylindrical* (a, b, c) *Stipa barbata* (c) *Lolium perenne* (c)	b (*A .cylindrical*), c (*S. barbata*)
Kermanshah	*Lolium perenne* (b, c) *Melica Jacquemontii* (a, c)	a (*M. Jacquemontii*), c (*L. perenne*)
Bisotun	*Dactylis glumerata* (b, c) *Agropyron repens* (c) *Ermopoa persica* (c)	a (*A .repens*), c (*D. glumerata*, *E .persica*)
Eslamabad–e Gharb	*Avena wiestii* (a, c) *Aegilops cylindrical* (c)	a (*A .cylindrical*), c (*A. wiestii*)

a = *F. verticillioides*, b = *F. subglutinans*, c = *F. proliferatum*

The macroscopic and microscopic characteristics were applied to identify *Fusarium* species. Ten isolates of *F. proliferatum* were characterized by the production of abundant aerial mycelium. All isolates produced slightly straight macroconidia with 3–5–septate. The conidiogenous cells producer false head and chain microconidia were monophialides and polyphialides.

Five isolates of *F. subglutinans* were characterized by the production of purple to dark purple cottony. All isolates produced slender and falcate macroconidia with 3–septate. The conidiogenous cells producer false head microconidia were monophialides and polyphialides. Also, based on morphological features, seven isolates were belonging to *F. verticillioides*. The colony of all isolates produced white, light purple to dark violet pigmentations. The macroconidia were commonly slender and almost straight with 3–5 septate, mostly 3 septate, with curved apical cell and foot shaped basal cell. The conidiogenous cells producer with false head and chain microconidia were monophialides.

All isolates of *F. verticillioides* and *F. subglutinans* were obtained from infected spikes, whereas five strains of *F. proliferatum* were isolated from infected spikes and the seven from infected root and crown. Members of FFSC were also molecularly distinguished using specific primers. The primers PRO1/2, VER1/2, and SUB1/2 produced DNA fragments of 585, 578, and 631 bp in all isolates of *F. proliferatum*, *F. verticillioides*, and *F. subglutinans*, respectively (Fig. 1–3).

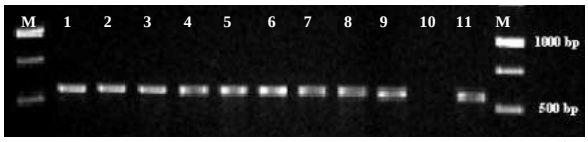

Fig. 1. PCR products (585 bp) obtained with specific primer pairs PRO1/2 from 10 isolates of *F. proliferatum*. Lane M: GeneRuler 1 kb DNA Ladder. 1 = FFSC razi1 weed, 2 = FGSC razi2 weed, 3 = FFSC razi1 weed, 4 = FFSC razi1 weed, 5 = FFSC razi1 weed, 6 = FFSC razi1 weed, 7 = FFSCrazi1weed, 8= FFSC razi1 weed, 9= FFSC razi1 weed, 10 = *F. verticillioides*, 11 = FFSC razi10 weed.

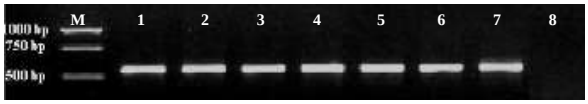

Fig. 2. PCR products (575 bp) obtained with specific primer pairs VER1/2 from seven isolates of *F. verticillioides*. Lane M: GeneRuler 1 kb DNA Ladder. 1 = FFSC razi11 weed, 2 = FFSC razi12 weed, 3 = FFSC razi13 weed, 4 = FFSC razi14 weed, 5 = FFSC razi15 weed, 6 = FFSC razi16 weed, 7 = FFSC razi17 weed, 8 = *F. proliferatum*.

PCR assays were also used to identify the chemotypes of all FFSC isolates. The expected PCR product for FUM1 (183 bp) was amplified in all PCR reactions. Eleven isolates belonging to *F. proliferatum* (seven isolates), *F. verticillioides* (three isolates), and *F. subglutinans* (one isolates), isolated from infected spike samples were identified as the potential fumonisin producers (Fig. 4). The highest amount of fumonisin producing isolates was observed in Sarpol–e Zahab (25%) and Bisotun (25%) regions of Kermanshah province (Table 2). Frequencies of the potential mycotoxigenic isolates are given in Table 2. The HPLC analysis of infected spike samples gave no positive results and fumonisin B1 and B2 were not detected.

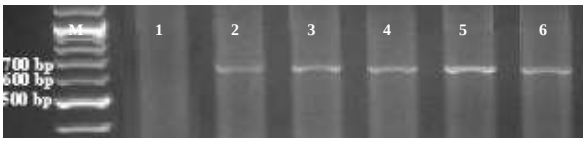

Fig. 3. PCR products (631 bp) obtained with specific primer pairs SUB1/22 from five isolates of *F. subglutinans*. Lane M: GeneRuler DNA Ladder Mix, 100 to 10,000 bp. 1 = *F. proliferatum*, 2 = FFSC razi18 weed, 3 = FFSC razi19 weed, 4 = FFSC razi20 weed, 5 = FFSC razi21 weed, 6 = FFSC razi22 weed.

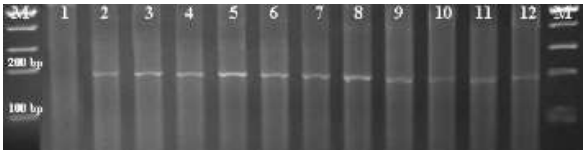

Fig. 4. PCR detection of the fumonisin–producing isolates of FFSC using the specific primer pairs FUM1 F/FUM1R. Lane M: GeneRuler DNA Ladder1 = FFSC razi1 weed, 2 = FGSC razi3 weed, 3 = FFSC razi5 weed, 4 = FFSC razi6 weed, 5 = FFSC razi7 weed, 6 = FFSC razi8 weed, 7 = FFSC razi9 weed, 8 = FFSC razi10 weed, 9 = FFSC razi12 weed, 10 = FFSC razi14 weed, 11 = FFSC razi16 weed, 12 = FFSC razi19 weed.

DISCUSSION

Fusarium head blight (FHB), a destructive disease of the gramineous plants is caused by several *Fusarium* species (Goswami & Kistler 2004). Members of the *F. fujikuroi* species complex, especially *F. proliferatum*, *F. verticillioides*, and *F. subglutinans*, are the most prevalent species involved in Fusarium head blight and root rot of gramineous plants (Goswami & Kistler 2004, 2005; Logrieco et al. 1995, 2002; Goswami & Kistler 2004) ,which was the aim of the present study.

Predominance of *Fusarium* spp. in grassy plants has been reported in Iran. Mojarradi et al. (2006) reported the natural occurrence of *F. anthophilum* on barnyaard grass (*Echinocloa crus–galli*) in Markazi province. Darvishnia et al. (2006) observed *F. sporotrichioides* and *F. tricinctum* from *Aegilops* sp. in Kerman and Mazandaran, respectively which is similar to our results. This is the first comprehensive report of isolation of the FFSC from grassy plants in Iran. The high frequency of the FFSC in this study provides an evidence of the potential importance of *Fusarium* diseases of gramineus weeds which is the most important animal feeds in Iran. Grassy plants may also act as the source of *Fusarium* infection of cultivated

crops (Boutigny et al. 2011; Postic et al. 2012). The widespread nature of FFSC in all grassland sites in western Iran provides further evidence of its ability to interact with various host plants and to produce mycotoxins.

Lenart et al. (2013) applied the positive–negative PCR assay based on the FUM gene to identify the genetic potential of FUM production of the FFSC strains isolated from maize ears in southern Poland. They indicated that three strains of *Fusarium* were the potential FUM producers and FUM gene was present in all strains, showing that they could be responsible for production of significant amounts of fumonisin in wheat ears in Poland (Wolny–Koładka et al. 2015).

In this study, 11 isolates out of 22 (50%) were fumonisin chemotype. These results show that fumonisin is one of the most common chemotypes in western Iran; however, the highest proportion of fumonisin producing isolates was observed in Sarpol–e Zahab (25%) and Bisotun (25%), which are the main growth areas of rangeland plants in western Iran. Haratian et al. (2008) investigated the genetic potential of DON (deoxynivalenol) and NIV (nivalenol) production of *F. graminearum* Schwabe strains isolated from grain cereals in Iran and demonstrated that 11 *Fusarium* strains were the potential DON and NIV producers. They revealed that both DON and NIV chemotypes existed in Iran. Ghiasian *et al.* (2006) reported the natural occurrence of *F. verticillioides* and fumonisins in corn from Iran. The results obtained in this study completed the previous data about genetic potential of fumonisin production of FFSC isolates in Iran and also revealed that grassy plants in addition to grain cereals can raise great concern in Iran.

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ردیابی گونه‌های تولید کننده فومونیزین متعلق به گونه مرکب *Fusarium fujikuroi* species complex مرتبط با غلات وحشی در ایران

خسرو چهری ✉
گروه زیست شناسی، دانشکده علوم، دانشگاه رازی، کرمانشاه

چکیده: به منظور تعیین جدایه‌های گونه مرکب *Fusarium fujikuroi* species complex تولیدکننده فومونیزین مرتبط با بیماری‌های سنبله و طوقه غلات وحشی، ۷۸ نمونه گیاه آلوده از مناطق مختلف کرمانشاه مورد بررسی قرار گرفت. بر اساس مطالعات ریخت شناختی، ۲۲ جدایه متعلق به این گونه مرکب شناسایی شد. این جدایه‌ها متعلق به گونه های *F. proliferatum* (ده جدایه)، *F. verticilioides* (هفت جدایه) و *F. subglutinans* (پنج جدایه) بودند. همچنین گونه‌های *F. verticilioides*، *F. proliferatum* و *F. subglutinans* به روش مولکولی و به ترتیب با استفاده از آغازگرهای اختصاصی نظیر VER1/VER2، PRO1/PRO و SUB1/SUB2 تایید شدند. ردیابی جدایه های *F. fujikuroi* species complex تولید کننده فومونیزین نیز با استفاده از آغازگرهای FUM1 F/FUM1 R انجام شد و نتایج نشان داد که از بین ۲۲ حدایه، ۱۱ جدایه توایدکننده فومونیزین بودند.

کلمات کلیدی: گونه‌های فوزاریوم، غلات وحشی، ردیابی مولکولی، زهرابه‌های قارچی، ایران

مکاتبه کننده: خسرو چهری Email: khchehri@gmail.com
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