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Research Article

Detection of Fumonisin Chemotype Produced by *Gibberella fujikuroi* Species Complex Isolated From Barely in Western Iran Using Specific PCR Assays

Khosrow Chehri* and Rasoul Godini

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

***Corresponding author**

Khosrow Chehri, Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran, Post Code: 6714115111, Email: khchehri@gmail.com

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Abstract

To identify fumonisin chemotype of *Gibberella fujikuroi* species complex (GFSC) isolated from barely, 75 barely samples were collected from different markets in Western Iran. Based on morphological characters, 40 *Fusarium* isolates were obtained from the grains and grouped into four species *F. proliferatum* (14) and *F. subglutinans* (3) as the two known GFSC members, *F. graminearum* senso lato (13), and *F. equiseti* (10). The identification of *F. proliferatum* and *F. subglutinans* isolates were confirmed molecularly using species-specific of PRO1/PRO2 and SUB1/SUB2 primers, respectively, which selectively amplified the partial calmodulin gene of rDNA. PCR-based detection of a mycotoxin-synthesis-pathway gene was also used to determine the potential of the analyzed strains to produce fumonisin using FUM1 F/FUM1 R primers. Of 17 tested isolates, 8 isolates (47%) are fumonisin chemotype. To the best of our knowledge, this is the first report on molecular identification and mycotoxigenic capacity characterization of GFSC isolated from barely in Western Iran.

INTRODUCTION

Fusarium contamination is a major problem in agriculture and the food industry which have involved human life for a long time. *Fusarium* species invade vast numbers of the economically important crops such as wheat, rice, maize and barley, and consequently result in significant economic losses [1,2]. *Fusarium* pathogens infect not only plants, but also their products and as a consequence may impress the downstream industries such as the food industry. In addition to destructive effects, some of *Fusarium* species secret mycotoxins on their substrates which is considered as an important factor for the evaluation of healthy of agricultural products and foods [2]. Mycotoxins are secondary metabolites of fungi with harmful effects on consumers, either human or other organisms. *Fusarium* species synthesize mycotoxins such as trichothecenes, fumonisins, moniliformin, beauvericin, and enniatins. Plus *Fusarium*, species belong to other genera such as *Aspergillus*, *Penicillium*, *Alternaria* and *Claviceps* are potentially able to produce important mycotoxins [3].

Gibberella fujikuroi species complex (GFSC) encompasses many important pathogenic fungi that their counterpart anamorphs are placed in *Fusarium* genus. There are at least 36 described species in GFSC including important fusaria such as *F. circinatum*, *F. fujikuroi*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* [4]. Members of GFSC cause diseases such as pitch canker on Pines, Bakana on rice seedlings, Mango malformation disease, root rot of Soybean, and ear and stalk rot of maize [5-7]. Secondary metabolites such as gibberellin, a plant hormone, are biosynthesized by some species of GFSC particularly *F. fujikuroi* [8]. Moreover, some of them are potential producers of mycotoxins such as fumonisins, moniliformin, and fusarin C [9-11].

Fumonisins, as an important group of mycotoxins, are produced by species from GFSC and have extremely important impression on the agriculture and food industries. Fumonisns are accumulated in plants such as cereals and their consumption results in hazardous affects such as cancer and neural tube defects on animals and human [12-14]. Fumionisins consist of at least 16 defined compounds including: B1, B2, B3, B4, A1, A2, A3, AK1,

C1, C3, C4, P1, P2, P3, PH1a and PH1b [15]. Mmolecular studies revealed that there is a gene cluster (*FUM*) with 15 genes, which is responsible for fumonisins production in GFSC. Expression pattern of these genes are related to fumonisin biosynthesis [16].

Barley is among three cereal crops (wheat, maize and barley) that the total yields of them are more than two-third of cereal production throughout the world [17]. In Iran, barley (*Hordeum vulgare* L.) is a strategic plant that the under culture area of this crop in Kermanshah and Hamedan provinces is around 290 thousands hectare. Aims of the present study are firstly, to isolate and identify, both morphologically and molecularly, *Fusarium* species belong to GFSC isolated from the barley grains collected from Western Iran, and secondly, to detect fumonisin producer isolates by considering species-specific genes.

MATERIAL AND METHODS

Isolation and Identification of *Fusarium* spp.

Seeds of barley were collected from different markets of Kermanshah and Hamedan provinces and transferred to the lab in plastic bags. Following hand-selection, seeds were placed onto plates of water-agar (WA) media and incubated at 25 °C for 4 days. Then, single spores of *Fusarium* were placed onto Potato Dextrose Agar (PDA) and Carnation Leaf Agar (CLA) media. Afterward, the morphological characteristics, both macroscopic and microscopic, of resulted colonies were studied and compared to species description of the *Fusarium* laboratory manual book to identify species [18].

DNA extraction

Briefly, following culturing *Fusarium* isolates in Potato Dextrose Broth (PDB, Sigma) shaking at 150 rpm at 25 ± 2 °C for 5 days, mycelia were harvested by filtration through Whatman paper 1 and freeze-dried for 20 h. DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's protocol to extract DNA.

Molecular identification using species-specific PCR

To confirm morphological identification, *Fusarium* isolates were considered molecularly using species-specific primers of PRO1/PRO2 and SUB1/SUB2 [19]. Primers are presented in (Table 1). Amplification reactions were done in a total volume of 25 µl, by mixing 1 µl of template DNA with 17.8 µl ddH_2O, 1 µl of deoxynucleotide triphosphate (dNTP) (Promega); 0.5 µl of $MgCl_2$ (Promega); 1 µl of each primer; 0.2 µl of *Taq* DNA polymerase (Promega) and 2.5 µl of PCR 5X reaction buffer (Promega, Madison, Wl, USA). PCR amplification was performed in the Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) with the following programs: an initial denaturation step at 94 °C for 5 min, 35 cycles of 94 °C (1 min) / 56 °C (1 min) /72 °C (3 min), and a final extension step at 72 °C for 10 min. To visualize the PCR products 1×TBE electrophoresis in ethidium-bromide-stained and 1% agarose gel were used.

Molecular analyses for Fusarium strains producing fumonisins

To investigate potential ability of fumonisin production in the strains FUM1 F/FUM1 R primers were applied (Table 1) [20]. PCR amplification was carried out in the Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) according to temperature profiles described by Bluhm and colleagues [21]. To visualize PCR products 1×TBE electrophoresis in ethidium-bromide-stained and 1% agarose gel were used.

RESULTS

Seventy-five barely samples were collected from different markets in Western Iran (Table 2). Based on morphological characterizations, 40 *Fusarium* isolates were recovered from the infected barely grains. Macroscopic and microscopic characteristics showed that all of the isolates belonged to species *F. proliferatum* (14) and *F. subglutinans* (3), *F. graminearum* senso lato (13), and *F. equiseti* (10) (Table 2). *Fusarium*

Table 1: Primers used for detection of the GFSC potential to produce fumonisins.

Primer name	Sequence 5′-3′	Product size (bp)	Target sequence	Source
SUB1	CTGTCGCTAACCTCTTTATCCA	631	Calmodulin gene	Mulé et al, (2004).
SUB2	CAGTATGGACGTTGGTATTATATCTAA			
PRO1	CTTTCCGCCAAGTTTCTTC	585	Calmodulin gene	Mulé et al, (2004).
PRO2	TGTCAGTAACTCGACGTTGTTG			
FUM1 F	CCATCAC AGTGGGACACAGT	183	FUM1 gene	Bluhm et al, (2004).
FUM1 R	CGTATC GTCAGCATGATGTAGC			

Table 2: Place of sample collection, number of potentially toxigenic (FUM1) isolates isolated from barely grains in western Iran.

Place of sample collection	*Fusarium* spp. identified	FUM1 producer strain (sources)
Sarpole-Zahab	*F.s (1), F. gr (2), F. eq (3), F. p (4)*	*F.s (1), F. p (2)*
Ravansar	*F.s (1), F. gr (4), F. eq (2), F. p (4)*	*F.s (1), F. p (2)*
Kermanshah	*F.s (1), F. gr (2), F. eq (2), F. p (2)*	*F. p (1)*
Hamedan	*F. gr (3), F. eq (2), F. p (2)*	*F. p (1)*
Eslam Abade-Gharb	*F. gr (2), F. eq (1), F. p (2)*	-

F.gr=F. Graminearum; F.eq.=F.equiseti; F.s=F. Subglutinans; F.p=F. proliferatum

proliferatum and *F. subglutinans*, as the members of GFSC, were also molecularly distinguished. The primers SUB1/2 and PRO1/2 produced DNA fragments 631 and 585bp in all *F. subglutinans*, and *F. proliferatum* isolates, respectively (Figure 1, 2).

PCR-based detection of mycotoxin-synthesis-pathway gene was also carried out to determine the fumonisin biosynthesis potential of the analyzed strains using FUM1 F/FUM1 R primers. PCR assays showed DNA fragments (183 bp) were amplified in 47% of GFSC isolates. Detection of the fumonisin producer isolates showed that 6 and 2 isolates were belonged to *F. proliferatum* and *F. subglutinans*, respectively (Figure 3). The highest proportion of fumonisin producing isolates was observed in Ravansar and Sarpole-Zahab cities. Frequencies of potential mycotoxin producing strains are given in Table 2.

DISCUSSION

The occurrence of mycotoxins produced by *Fusarium* spp. in small cereal grains, particularly in barley, is of great concern worldwide, because their presence in processed feeds and foods seems unavoidable. Consequently, they have been associated with chronic or acute mycotoxicoses in livestock and, to a lesser extent, in humans [22-24]. Barley (*H. vulgare* L.) is one of the highly important crops in Western Iran with considerable impact on the region economy. Previous studies showed that barely grains are sensitive to *Fusarium* pathogens. In this study, our results, in agreement with previous studies, showed that the barley grains are the host of a various fusaria [22,23].

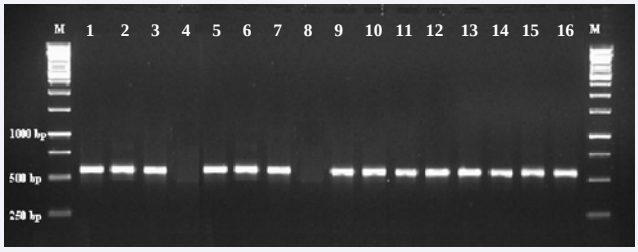

Figure 1 PCR products obtained with specific primer pairs PRO1/2 (band, 585 bp) from 10 isolates of *F. proliferatum*. Lane M: GeneRuler 1 kb DNA Ladder. 1=F.prolirazi1barley, 2= F.prolirazi2barley, 3= F.prolirazi3barley, 4= *F. subglutinans*, 5= F.prolirazi4barley, 6= F.prolirazi5barley, 7= F.prolirazi6barley, 8= *F. verticillioides*, 9= F.prolirazi7barley, 10=, F.prolirazi8barley 11= F.prolirazi9barley, 12= prolirazi10barley, 13= F.prolirazi11barley, 14= F.prolirazi12barley, 15= F.prolirazi13barley, 16= F.prolirazi14barley.

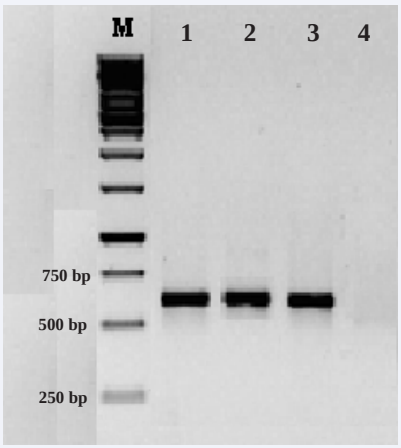

Figure 2 PCR products obtained with specific primer pairs SUB1/2 (band, 631 bp) from 3 isolates of *F. subglutinans*. Lane M: GeneRuler 1 kb DNA Ladder. 1= F.subirazi1barley, 2= F.subirazi2barley, 3= F.subirazi3barley, 4= *F. proliferatum*.

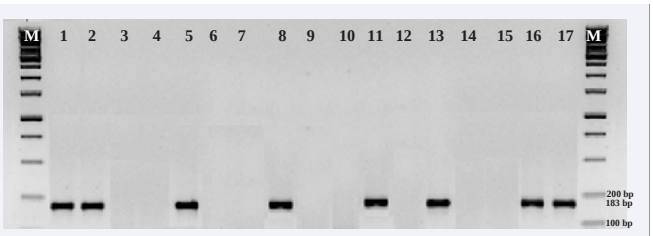

Figure 3 PCR products obtained with specific primer pairs FUM1 F/FUM1 R (band, 183 bp) from 17 isolates of FGSC. Lane M: Gene Ruler DNA Ladder Mix, 100–10,000 bp Ladder. 1=F.prolirazi1barley, 2= F.prolirazi2barley, 3= F.prolirazi3barley, 4= *F. subglutinans*, 5= F.prolirazi4barley, 6= F.prolirazi5barley, 7= F.prolirazi6barley, 8= *F. verticillioides*, 9= F.prolirazi7barley, 10=, F.prolirazi8barley 11= F.prolirazi9barley, 12= prolirazi10barley, 13= F.prolirazi11barley, 14= F.prolirazi12barley, 15= F.subirazi1barley, 16= F.subirazi2barley, 17= F.subirazi3barley.

In the present study, morphological studies showed that all isolates belonged to four potential mycotoxin producer species including *F. graminearum* (sensu lato), *F. equiseti, F. proliferatum* and *F. subglutinans* [24,25]. Mycotoxins, depends on the type, differently impact on consumer health [26], therefore this subject must be considered for mycotoxin control program in Iran.

In the present study, we confirmed the morphological results accuracy using species-specific genes. The results of both morphological and molecular studies showed that *F. proliferatum* is the predominant species associated with the barely grains. *F. proliferatum* have been previously isolated from different crops such as maize, wheat, barley, and rice [27-29]. In addition to the plants, previous studies identified *F. proliferatum* in grain of wheat, maize and barley, as well [30,31]. In Iran, *F. proliferaum* and *F. subglutinans* have been isolated from root, crown, stem and spike of cereals [32-34], but the information of fusaria associated with barley grains is very limited [34]. To the best of our knowledge, we are reporting these species in barley grains collected from Western Iran for first time. In the present study, the number of *F. subglutinans* isolates was only 3 out of 40 that shows limited existence of these species in barley grains, which concurred with the results of previous study by Levic and colleagues [35].

We studied the fumonisins production potency of the isolates belonged to GFSC using molecular PCR-based analysis. Our results showed that approximate 47 percent of GFSC isolates are potential fumonisins producer. Our results concurred with previous study of potential fumonisns producing fungi in barley grains [31]. Also, it has been shown that strains of *F. proliferatum* are major fumonisin B_1 producers [36]. Utilizing PCR-based analysis can help to determine if potentially toxin producer fungi are present in agricultural products, since they can seriously endanger the health of consumers either human or

domestic animals [37]. To our knowledge, in Iran, these findings are reporting in the present article for the first time. In Western Iran, Ravansar and Sarpole-Zahab area are considered as the most important barely plantations and the report of fumonisins chemotypes in these areas definitely will be important to improve the control programs of mycotoxin by ministries of agriculture and health.

In conclusion, our results can make firstly a complementary knowledge of fungi companying barley grains that could be helpful to manage control of possible outbreaks. Secondly, we revealed fumonisins producing isolates belong to GFSC in the barley grains in Western Iran.

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