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MORPHO-MOLECULAR IDENTIFICATION, PATHOGENICITY VARIATION, MATING BIOLOGY, AND FUMONISIN PRODUCTION OF *FUSARIUM* SPECIES IN *ZEA MAYS* L.

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A B S T R A C T

Fusarium verticillioides and *Fusarium proliferatum* cause a wide range of maize diseases. These fungi produce dangerous mycotoxins, such as fumonisin B1, which are important threats to humans and animals. Given this predicament, the present study aimed to identify the fungi both molecular-morphologically and also investigate the pathogenicity variation and mating type of 41 *Fusarium* strains in maize (*Zea mays* L.) samples with sifting their fumonisin contents. Furthermore, species-specific primers for the molecular identification of distinct strains amplified 2 fragments of 578 and 800 bp in *Fusarium verticillioides*, while a single 585 bp band was amplified in *Fusarium proliferatum*. Accordingly, 24 isolates out of 41 were identified as *F. verticillioides*, and 13 isolates were identified as *F. proliferatum*. The fumonisin-producing and non-producing *Fusarium* strains were identified using the VERTF-1/VERTF-2 primers. A total of 24 isolates of *F. verticillioides* were positively scored based on the amplification of a single 400 bp fragment. The highest and lowest fumonisin content, as measured using an enzyme-linked immunosorbent assay (ELISA), belonged to strains MS1 and MG3, respectively, and ranged from 960-12673 and 4.07-23 ppm, respectively. Additionally, the mating type test showed that the sexual form of the studied *Fusarium* species could possibly belong to the A and D mating populations. *In vivo* and *in vitro* pathogenicity tests revealed a high susceptibility.

Keywords: *Fusarium verticillioides, Fusarium proliferatum,* fumonisin, ELISA.

INTRODUCTION

Plants as sessile organisms are faced with a variety of threats during their life cycle, such as biotic and abiotic stressors (Abiri *et al.*, 2015). The adverse effects of these stressors leads to a negative impact on plant growth and development, which ultimately reduce 75% of plant production (Peng *et al.*, 2014). Biotic stressors, including bacteria, insects, fungi, weeds, and viruses have adverse effects on the plants yield as well. Approximately 8,000 fungal species can infect crops during their life cycle (Ciarmiello *et al.*, 2011; Peng *et al.*, 2014). *Fusarium* is a large genus of filamentous fungi that is present as a saprophyte in soil and as a pathogen in crops (Chelkowski, 2014). Typically, the fungus initially infects plant root systems. Consequently, the vascular system is obstructed. This, in turn, prevents or reduces the water flow from the roots to the upper regions and causes plant wilting (Summerell and Leslie, 2011). *Fusarium verticillioides* (*Fusarium moniliforme*) and *Fusarium proliferatum* are the most commonly reported fungal species that infect plants, such as maize, and cause stalk, root and ear rot, mostly in subtropical and

tropical areas (Kouadio *et al.*, 2007; Minervini *et al.*, 2005). These *Fusarium* species induce the production of carcinogenic mycotoxins, such as fumonisins, which are low-molecular-weight and highly toxic secondary metabolites (Karami-Osboo *et al.*, 2010; Wan *et al.*, 2013). Fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) are found in maize kernel, and the level of FB_1 is generally higher than in FB_2 and/or FB_3 (Alizadeh *et al.*, 2012; De Curtis *et al.*, 2011). Fumonisin is associated with a variety of human and animal health diseases, including acute toxicity, cancer, birth defects, immunity suppression, inhibition of normal growth, and the reduction of the nutritional quality of the infected grains (Babič *et al.*, 2015; Ingle *et al.*, 2008).

Drier weather and higher temperature during the flowering stage, a key step of ear infection by *F. verticillioides*, and rainfall near harvest increase fumonisin and ear rot levels (Kuhnem *et al.*, 2015). Interestingly, fumonisin production does not significantly correlate with the ability of *F. verticillioides* to cause maize ear rot (Desjardins *et al.*, 2002; Torelli *et al.*, 2012).

Secondary metabolites that are responsible for *Fusarium*-related mycotoxicoses in animals and humans were identified and chemically characterized in the early 1960s (Summerell and Leslie, 2011). To date, immunochemical methods, capillary zone electrophoresis (CZE), gas chromatography, enzyme linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC) have been used to quantify the amount of fumonisin and also to assess the resistance to *Fusarium*-induced diseases (Karbancioglu-Guler and Heperkan, 2009). To evaluate mycotoxin, immunological approaches are commercially available that use specific mono- and polyclonal antibodies produced against various toxins, and these antibodies are essential for ELISA tests and immunoaffinity column-based analyses (Sforza *et al.*, 2006). Morphological identification of fungi pathogenicity in the plant is the initial and most complicated step in the identification process. The current investigation represents an original approach to identify the fungal isolates collected from different regions of Iran using morphological and molecular methods. Furthermore, pathogenicity, mating-type, and mycotoxicity measurements were performed.

MATERIALS AND METHODS

Plant materials: The maize (*Zea mays* L.) samples were collected from the widespread *Fusarium* regions of Iran and are presented in Table 1.

Table 1. The maize samples which collected from different regions in Iran.

Isolates	Host and Geographical origin	Isolates	Host and Geographical origin	Isolates	Host and Geographical origin	Isolates	Host and Geographical origin
MS1	Maize – Sary	MQ1	Maize - Qazvin	MK10	Maize - Karaj	MK20	Maize - Karaj
MS2	Maize – Sary	MK1	Maize - Karaj	MK11	Maize - Karaj	MK21	Maize - Karaj
MS3	Maize – Sary	MK2	Maize - Karaj	MK12	Maize - Karaj	MK22	Maize - Karaj
MG1	Maize – Gorgan	MK3	Maize - Karaj	MK13	Maize - Karaj	MK23	Maize - Karaj
MG2	Maize – Gorgan	MK4	Maize - Karaj	MK14	Maize - Karaj	MK24	Maize - Karaj
MG3	Maize – Gorgan	MK5	Maize - Karaj	MK15	Maize - Karaj	MK25	Maize - Karaj
MG4	Maize – Gorgan	MK6	Maize - Karaj	MK16	Maize - Karaj	MI1	Maize - Isfahan
MG5	Maize – Gorgan	MK7	Maize - Karaj	MK17	Maize - Karaj	MI2	Maize - Isfahan
MG6	Maize – Gorgan	MK8	Maize - Karaj	MK18	Maize - Karaj	MI3	Maize - Isfahan
MG7	Maize – Gorgan	MK9	Maize - Karaj	MK19	Maize - Karaj	MA1	Maize - Ardabil
						MA2	Maize - Ardabil

Infected sample collection: Each field was classified into five plots (100 m in diameter), and then five seeds of corn with initial *Fusarium* symptoms (Figure 1) were separately and randomly sampled from each plot. Based on *Fusarium* morphological identification, the collected infected seeds were harvested and surface sterilized in 1% sodium hypochlorite solution for 3 minutes using the protocol in (Harrigan, 1998; Leslie and Klein (1996); Summerell *et al.*, 2010). The sterilized seeds were rinsed three times with distilled water and dried with filter paper (Whatman filter paper No. 1) in a laminar flow cabinet.

Initial identification and purification of fungal

isolates: To identify *Fusarium* strains, the sterilized maize seeds were directly transferred onto Petri dishes containing potato dextrose agar (PDA) (Difco, Fisher Bioblock Scientific, Illkirch, France) (Nelson et al., 1983). The samples were also tested on peptone PCNB agar (PPA) and Nash Snyder Media (SNM) (Summerell *et al.*, 2003) to double confirm the PDA culture results. The Petri dishes were incubated in the dark at 25 °C for 5-7 days and were then transferred to a germinator adjusted at 25-30 °C. The induced fungi were sub-cultured on the same media. To purify the isolates, the spore suspensions were sub-cultured on water agar (WA) media. To prepare the spore suspension, 5 mL of distilled water was added to each petri dish, then the suspension was measured using a hemocytometer to determine the amount that was sufficient (10^6) for the purification step.

Figure 1. The infected samples of Maize in the field.

After 16 hours of incubation, the germinated single spores were transferred to PDA using a sterilized needle (Santiago et al., 2015). Each colony of fungi was identified based on the morphological characteristics, such as growth rate, pigmentation, shape, and colour of mycelium, to determine the type of the strains. Each petri dish was measured on the 3rd, 7th, 10th and 14th day to determine the growth rate (23).

Morphological identification: The purified isolates of *Fusarium* were sub-cultured on Spezieller Nährstoffarmer agar (SNA) for 14 days at 20-22 °C in the dark, on carnation leaf agar (CLA) for 5-10 days at 20 °C with alternating 12 hours of fluorescent light, on PDA for 7-14 days at 25 °C in the dark and on carrot agar (CA) for 1-2 mounts at 22-23 °C under near UV light of 320-400 nanometers (Summerell *et al.*, 2003).

Various characteristics were evaluated for each media separately. The shape, presence or absence of micro- and macro-conidia colonies and false head, observation of short and long chains, and the type of phialide, including mono- or poly-phialide, were determined on SNA media. The observation of orange sporodochia on CLA media suggests the presence of *Fusarium* infection, while no chlamydospore and colony chains should be observed. Furthermore, PDA media was used to determine the shape and colour of the colonies.

Mating type idiomorphs identification: To determine the frequency of the mating type idiomorph, two mating type testers, including MAT-1 and MAT-2, were used in both mating populations of A and D. The tester isolates were then cross-fertilized with the purified isolates. The procedures of the sexual crosses were carried out on carrot agar (CA) with the aforementioned standard testers as maternal parents and the purified isolates as paternal parents. The cross-fertilized isolates were maintained in an incubator (ES2000 UV) under permanent cool-white light near-UV with a frequency of 320-400 nanometers, 22-23 °C and sufficient humidity for 1-2 months. A cross was considered fertile when a cirrhis of ascospores oozing from a mature perithecium were observed 2–6 weeks after fertilization. The perithecia production was evaluated as the sexual reproduction in CA media in 2 replicates.

Reciprocal crosses were performed between the isolates as female parents and testers as male parents. The process was confirmed using fertility determination of the tested isolate or male parent. In such cases, an isolate formed perithecia or a fertile progeny interspecies cross was performed, and the same isolate was applied for both male and female parents (Leslie and Klein, 1996; Venturini *et al.*, 2011). The effective population size concept was used to investigate the population genetics of the *Fusarium* species. The effective population size (N_e) was evaluated according to the number of different mating type strains [$N_{e(mt)}$], which was expressed as the actual count percentage, and the number of hermaphrodite and male strains [$N_{e(f)}$], which also expressed as the actual count percentage (Harrigan, 1998).

The effective population size according to the mating type's frequency was evaluated using the following equation:

$$[Ne(mt)] = \frac{(4NmNf)}{Nm + Nf}$$

Where, N_m = the total number of isolates (strains) with one mating type (*MAT-1*), and N_f = the total number of

isolates (strains) with another mating type (*MAT*-2) within the same population.

The effective population number (size) [$N_{e(f)}$], based on the relative frequency of female-sterile and hermaphrodite strains, was determined using the following equation:

$$[Ne(f)] = \frac{(4N_2Nh)}{(N+Nh)^2}$$

Where, N = the total number of female-sterile strains, and N_h = the total number of hermaphrodite strains.

The variance of the effective population size was determined using the following equation:

$$[Ne(v)] = \frac{N_2}{(N+Nh)}$$

Where, N = the total number of female-sterile strains, and N_h = the total number of hermaphrodite strains.

Molecular identification

DNA extraction: *Fusarium* isolates were sieved and sub-cultured on a modified PDB culture (Fisher Bioblock Scientific, Illkirch, France), and 100 mg of mycelia of each isolate were taken for DNA extraction after 7 days. The harvested mycelia were ground in liquid nitrogen, and DNA was isolated using the CTAB protocol (Mulè *et al.*, 2004) and then stored in 1× Tris-EDTA at 4 °C.

PCR condition: The primer pairs VER1/2 (5′-CTTCCTGCGATGTTTCTCC-3/5′-AATTGGCCATTGGTATTATATATCTA-3′), VERT1/2 (5′-GTCAGAATCCATGCCAGAACG-3)/(5′-CACCCGCAGCAATCCATCAG-3′) and PRO1/2 (5′-CTTTCCGCCAAGTTTCTTC-3′/5′-TGTCAGTAACTCGACGTTGTTG-3′) were used as the species-specific primers to identify *F. verticillioides* and *F. proliferatum* (Table 2). The reaction mixtures were prepared in a total volume of 25 µL with a final concentration of 2.5 µL of PCR buffer, 17 µL of deionized (DI) distilled water, 0.5 µL of each dNTP and 0.75 µL of $MgCl_2$. For each reaction, 0.25 µL of Ampli Taq polymerase, 1 µL of each primer and approximately 2 µL of fungal template DNA were used (Qiagen, Germany). Reactions were performed in a GeneAmp PCR System 9700 thermal cycler using the following PCR conditions: denaturation at 95 ºC for 5 min, 35 cycles of denaturation at 94 °C for 50 s, annealing at 56 °C for 50 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min, followed by cooling at 4 °C until the recovery of the samples. The amplified products were visualized on 1.2% agarose gels stained with midori (Danielsen *et al.*, 1998).

Table 2. Sequences of primers used in the experiments.

Primer name	Primer sequence (5′ → 3′)	Species-specificity
PRO1/ PRO2	CTTTCCGCCAAGTTTCTTC/ TGTCAGTAACTCGACGTTGTTG	*F. proliferatum*a
VER1/ VER2	CTTCCTGCGATGTTTCTCC/ AATTGGCCATTGGTATTATATATCTA	*F. verticillioides*a
VERT-1/ VERT-2	GTCAGAATCCATGCCAGAACG/ CACCCGCAGCAATCCATCAG	*F. verticillioides*a

Identification of fumonisin producing strains: Among all isolates, 24 *F. verticillioides* isolates were evaluated for their potential to produce fumonisin using their genomic DNA. The VERTF-1 (5′-GCG GGA ATT CAA AAG TGG CC -3′) /VERTF-2 (5′-GAG GGC GCG AAA CGG ATC GG -3′) set of primers were used for a PCR-specific assay to amplify a multi-copy IGS sequence (Reid *et al.*, 1999). The PCR conditions, except for primer extension condition (72 °C for 45 sec) and the annealing temperature (60 °C for 30 s), were similar to those described for the VERT-1/VERT-2 primers.

Evaluation of the pathogenicity in a greenhouse: Inoculation was performed using the toothpick method by inserting the infected toothpicks (*F. verticillioides* and *F. proliferatum*) into maize stems when the seedlings were two-months-old. The pathogenicity of each isolate was determined based on the length of the necrosis observed in the stems at 10 days after the inoculation (Vigier *et al.,* 1997). In the greenhouse, the maximum temperature varied between 28 and 30 °C, and the minimum temperature varied between 17 and 20 °C. To verify the presence of *F. verticillioides*, two pieces of stalk from below and above the insertion point were incubated on a PDA plate for four to five days and analyzed for the existence of *F. verticillioides*. The percentage of humidity was 88% in the greenhouse.

***In vivo* evaluation of pathogenicity:** To evaluate the pathogenicity in the field, the *Fusarium*-infected

toothpick (*F. verticillioides* and *F. proliferatum*) was inserted into the initial inter-node of the 2-month-old maize stems. The isolate pathogenicity was investigated based on the necrosis length at 10 days after the inoculation (Vigier *et al.*, 1997). The corn ears were infected with spore suspension of *F. verticillioides* and *F. proliferatum* isolates using the silk channel and ear inoculation (Nail Punch) method. The disease severity (DS) was measured for both methods using the visual disease severity rating with scales 1-7 (Reid and Zhu, 2005). The severity of ear rot symptoms was calculated using a 7-class rating scale, where 1 = 0%, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–50%, 6 = 51–75%, and 7 = >75% of the kernels exhibiting visible symptoms of infection such as rot and mycelial growth (Okamoto *et al.*, 2013). The minimum temperatures during the field pathogenicity were 30 °C, whereas the maximum temperature was 34 °C. The recorded humidity was 24-26% in the field. For statistical analysis, the data of diseases severity (DS) was converted to the following:

$$\frac{arcsin\sqrt{\times}}{100}$$

In vitro evaluation of pathogenicity: During the flowering stage, the maize stems were cut using a sterilized scalper and moved to the laboratory. The inoculated toothpicks from the PDB media containing *F. verticillioides* and *F. proliferatum* were penetrated into the detached stems. The necrosis length was monitored at 10 days after the inoculation. For all of the above experiments, 2 control treatments were used (untreated and sterile toothpick-stained with distilled water). To evaluate the diseases severity range, the Reid and Zhu (2005) protocol was used. The temperature in the *in vitro* conditions ranged between 25-30 °C. The quantification of total fumonisin using an ELISA test: To assess the total fumonisin production, autoclaved maize grains were inoculated with spore suspensions of 20 isolates and incubated for 4 weeks at 25 °C. After incubation, 70% methanol was added, and the extract was filtered and diluted with distilled water. The final products were subjected to ELISA using an AgraQuant Fumonisin Kit (Romer Labs, Austria).

RESULTS AND DISCUSSION

Morphological identification of fungal isolates: Based on an initial morphological analysis of the 41 isolates originating from maize, 24 isolates were identified as *F. verticillioides*. A total of 13 isolates were reliably identified as *F. proliferatum,* and 4 isolates could not be identified.

Regardless of the genus of the fungus isolates, the initial pigmentation of the *F. verticillioides* and *F. proliferatum* isolates on the PDA, PPA and SNM media included a diverse spectrum of colours, such as whitish, creamy, light and dark purple and violet grey (Table 3). The growth pattern of the isolates was diverse. In some cases, a radial growth pattern was observed, while others were expanded as vertical plates that were arranged radially. This radial pattern of the fungus may be the result of the day/night cycle (Somma *et al.*, 2010). An analysis of the radial growth rate as an initial differentiation index was performed for various types of *Fusarium* in the experiment. The radial growth length of some isolates at 25 and 30 °C was between 21-30 and 22-35 mm, respectively. These radial growths lengths were consistent with the morphological characteristics of *F. verticillioides*. On the other hand, the radial growths length of some isolates at 25 and 30 °C were between 25-35 and 25-32 mm, respectively.

The latter result of the radial growths length range confirmed that the aforementioned isolates belong to *F. proliferatum* (Leslie and Summerell, 2008). The differences in the colour may be influenced by the plant genotype and the soil and environmental conditions. Geographical parameters, including climate, is important for the occurrence of *Fusarium* infection and the pattern of infestation. The colour and shape of the isolates of *Fusarium* species in wheat, barley, maize and sorghum are often correlated with different climatic conditions (rainfall and temperature) in diverse geographic locations (Kim *et al.*, 2003).

The initial physiological characterization of the *Fusarium* isolates was conducted on CLA media. The *F. verticillioides* isolates produced two asexual distinct conidial forms of conidia in sporodochia. The sporodochia were orange, yellow or tan and formed a pseudo-pionnotal mass or were discrete entities. The *F. verticillioides* isolates induced more microconidia (5.9–11 µm × 1.8–2.7 µm) and fewer macroconidia (29.2–56.3 µm × 2.8–3.4 µm) on CLA media.

The microconidia were formed from monophialides on the CLA and SNA media, which might appear as V-shaped pairs to present a “rabbit ear” appearance and were presented in chains that could be quite long. The macroconidia were slender and long, slightly straight or falcate, and thin walled. Twenty-one isolates from 24 isolates of *F. verticillioides* produced more macroconidia, and 3 isolates produced more microconidia.

Table 3. Morphological characteristics of the isolates.

I.C	*Fusarium* specie	colo**u**r	Mycelia	Microconidia	Radial growth shape
MS1	*F. verticillioides*	white-Light purple	rabbit ear	Abundant- Single celled-Oval	cottony
MS2	*F. verticillioides*	white to purple	rabbit ear	Abundant- Single celled-Oval	cottony
MS3	*F. verticillioides*	grayish orange	rabbit ear	Abundant- Single celled-Oval	Velvet
MG1	*F. proliferatum*	Light yellow	monophialides	form in chains - club shape	fluffy
MG2	*F. verticillioides*	orange to violet grey	rabbit ear	Abundant- Single celled-Oval	radial
MG3	*F. verticillioides*	dark violet or dark magenta	rabbit ear	Abundant- Single celled-Oval	radial
MG4	*F. proliferatum*	Light pink	polyphialides	club shaped	Concentricity
MG5	*F. proliferatum*	Deep purple brown	Proliferate polyphialides	form in false heads- club shaped	Radial
MG6	*F. proliferatum*	Light pink	monophialides	form in chains - club shaped	Smooth-downy
MG7	*F. proliferatum*	Light pink, concentric rings	monophialides	form in chains -club shaped	Smooth-downy
MK1	*F. verticillioides*	dark violet	rabbit ear	Abundant- Single celled-Oval	Radial
MK2	*F. verticillioides*	dark violet	rabbit ear	Abundant- Single celled-Oval	Radial
MK3	*F. proliferatum*	Light purple-brown	monophialides	form in false heads- club shaped	Concentric ring
MK4	*F. proliferatum*	Light yellowish	Monophialides	form in false heads- club shaped	Woolly
MK5	*F. proliferatum*	Deep purple, concentric rings	Proliferate polyphialides	club shaped- form in chains	Concentricity
MK6	*F. verticillioides*	orange to violet grey	rabbit ear	Abundant- Single celled-Oval	Concentricity
MK7	*F. proliferatum*	Velvet- grayish orange	monophialides	form in chains - club shaped	Radial
MK8	*F. verticillioides*	dark magenta	rabbit ear	Abundant- Single celled-Oval	Radial
MK9	*F. verticillioides*	white to purple	rabbit ear	Abundant- Single celled-Oval	Concentric ring
MK10	*F. proliferatum*	purple-violet with age	Proliferate polyphialides	form in chains - club shaped	Cottony
MK11	*F. proliferatum*	White to light yellow	monophialides	club shaped- form in chains	Concentricity
MK12	*F. verticillioides*	orange to violet grey	rabbit ear	Abundant- Single celled-Oval	Downy
MK13	*F. proliferatum*	Light purple-brown	Proliferate polyphialides	form in false heads- club shaped	Fluffy
MK14	*F. verticillioides*	grayish orange	rabbit ear	Abundant- Single celled-Oval	Downy
MK15	*F. proliferatum*	White to light yellow	monophialides	form in chains - club shaped	smooth
MK16	*F. verticillioides*	violet pigments with age	rabbit ear	Abundant- Single celled-Oval	Downy
MK17	*F. verticillioides*	white-Light purple	rabbit ear	Abundant- Single celled-Oval	Downy
MK18	*F. verticillioides*	dark violet or dark magenta	rabbit ear	Abundant- Single celled-Oval	Concentricity
MK19	*F. verticillioides*	orange to violet grey	rabbit ear	Abundant- Single celled-Oval	Concentricity
MK20	*F. verticillioides*	grayish orange	rabbit ear	Abundant- Single celled-Oval	Velvet
MK21	*F. verticillioides*	white to purple	rabbit ear	Abundant- Single celled-Oval	Downy
MK22	*F. verticillioides*	grayish orange	rabbit ear	Abundant- Single celled-Oval	Velvet

MK23	*F. verticillioides*	violet pigments with age	rabbit ear	Abundant- Single celled-Oval	Downy
MK24	*F. verticillioides*	violet grey	rabbit ear	Abundant- Single celled-Oval	Radial
MK25	*F. verticillioides*	violet grey	rabbit ear	Abundant- Single celled-Oval	Radial
MA1	*F. verticillioides*	dark violet or dark magenta	rabbit ear	Abundant- Single celled-Oval	Radial
MA2	*F. verticillioides*	Light purple	rabbit ear	Abundant- Single celled-Oval	Downy

I.C: Isolate's Code, MS: Maize Sari, MG: Maize Gorgan, MK: Maize Karaj, MA: Maize Ardabil.

I.C	Macroconidia	F.H	Mn.Ph	P.Ph	S	Ch	M.P&MAT
MS1	Present-sickle shaped to straight, 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MS2	Present- Relatively long and slender , 5 septate, curved apical cell	+	+	-	+	-	A-*MAT2*
MS3	Present- slender, slightly falcate 5 septate, curved apical cell	+	+	-	+	-	A-*MAT2*
MG1	Present-Slender, thin-walled, relatively straight, 4 septate	+	-	+	+	-	D-*MAT1*
MG2	Present- slender, slightly straight 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MG3	Present- slender long, slightly falcate, thin walled. 3 septate	+	+	-	+	-	A-*MAT2*
MG4	Absent	+	-	+	+	-	D-*MAT2*
MG5	Absent	+	-	+	+	-	D-*MAT1*
MG6	Present- slender, almost straight, and to 5-septate	+	-	+	+	-	D-*MAT1*
MG7	Absent	+	-	+	+	-	D-*MAT2*
MK1	Present- slender long, slightly falcate, thin walled. 5 septate	+	+	-	+	-	A-*MAT1*
MK2	Present- Relatively long and slender , 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MK3	Absent	+	-	+	+	-	D- -
MK4	Absent	+	-	+	+	-	D-*MAT1*
MK5	Present- - Slender, thin-walled, straight, 5 septate	+	-	+	+	-	D-*MAT2*
MK6	Present- slender long, slightly falcate, thin walled. 4 septate	+	+	-	+	-	A-*MAT1*
MK7	Present- - Slender, thin-walled, straight, 3 septate	+	-	+	+	-	D-*MAT2*
MK8	Present- slender long, slightly falcate, thin walled. 4 septate	+	+	-	+	-	A-*MAT2*
MK9	Present- slender, slightly falcate 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MK10	Present- - Slender, thin-walled, straight, 4 septate	+	-	+	+	-	D-*MAT1*
MK11	Absent	+	-	+	+	-	D-*MAT2*
MK12	Present- Relatively long and slender , 4 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MK13	Present- - Slender, thin-walled, straight, 4 septate	+	-	+	+	-	D-*MAT2*
MK14	Present- slender long, slightly falcate, thin walled. 3 septate	+	+	-	+	-	A-*MAT2*
MK15	Absent	+	-	+	+	-	D-*MAT1*
MK16	Present- slender, slightly straight 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*

MK17	Present- slender long, slightly falcate, thin walled. 4 septate	+	+	-	+	-	A-*MAT1*
MK18	Present- Relatively long and slender , 5 septate, curved apical cell	+	+	-	+	-	A-*MAT2*
MK19	Present- Relatively long and slender , 4 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MK20	Present- Relatively long and slender , 4 septate, curved apical cell	+	+	-	+	-	A-*MAT2*
MK21	Present- slender long, slightly falcate, thin walled. 5 septate	+	+	-	+	-	A-*MAT2*
MK22	Present- slender, slightly falcate 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MK23	Present- Relatively long and slender , 4 septate, curved apical cell	+	+	-	+	-	A-*MAT2*
MK24	Present- Relatively long and slender , 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MK25	Present- slender long, slightly falcate, thin walled. 5 septate	+	+	-	+	-	A-*MAT1*
MA1	Present- slender, slightly straight 5 septate, curved apical cell	+	+	-	+	-	A-*MAT1*
MA2	Present- slender, slightly falcate 4 septate, curved apical cell	+	+	-	+	-	A-*MAT2*

I.C: Isolate's Code, MS: Maize Sari, MG: Maize Gorgan, MK: Maize Karaj, MA: Maize Ardabil. Mn.p: Monophialidic, P.ph: Polyphialidic, F.h: Fals head, S: Sporodochia, Ch: Chlamidosporia, M.p; Mating population, MAT: Mating type

The sporodochia colours were tan to pale orange for *F. proliferatum*. The *F. proliferatum* isolates tended to produce more microconidia (4.9–12 µm × 2.5–3.4 µm) and less macroconidia (1.8–2.6 µm × 25.8–43.4 µm and 2.5–3.4 µm × 42.7–48.8 µm) on carnation leaf agar (CLA) medium. All 13 isolates of *F. proliferatum* produced microconidia and macroconidia, but the production of microconidia was greater. The septated macroconidia can be produced on short monophialides with special structures called sporodochia (Cumagun, 2008). During the isolate identification procedure, chlamydospores were not produced. Microconidia were less common in chains and false heads from mono and poly-phialides. Similar to the results of *F. verticillioides* isolate identification; chlamydospores were not produced in the *F. proliferatum.* **Mating type idiomorphs identification:** The observation ascospore ooze (Figures 2E, 2F and 2G) and perithecia (Figures 2C, 2D and 2E) indicated that the progeny was fertile. On the other hand, the progeny was not considered to be fertile when the ascospores oozed out of only 1-3 perithecia (Figure 2D). Similarly, the small perithecia covered with mycelium were considered to be non-fertile progenies (Figures 2A, 2C and 2 D). The mating type results confirmed the heterothallic behaviour of *F. verticillioides* and *F. proliferatum*. Accordingly, the field isolates that originated from maize (5 isolates) belonged to population D. The aforementioned five isolates formed mature perithecia with testers originating from Italy (Table 4) (Figure 2B). The MATD-1 to MATD-2 ratio was 6:6 within population D, resulting in the reduction of $N_{e(mt)}$ up to 100% of the actual count. One isolate out of the 13 fertile isolates of population D was a hermaphrodite resulting in $N_{e(f)}$ of 26.21% of the actual count. The reciprocal crossing confirmed that all isolates were female-sterile and male-fertile. The 24 field isolates, including 10 maize isolates, belonged to population A. The mating types of MATA-1 and MATA-2 were segregated at a ratio of 10 among the 24 isolates of population A. The reduction of $N_{e(mt)}$ was up to 97.22% of the actual count in population A. The reciprocal crosses showed that all of the tested field isolates were female-sterile and male-fertile. The determination of hermaphrodites resulted in no change in $N_{e(f)}$ of the actual count (Table 4). The mating type results of population A showed that there was genetic variation and sexual reproduction among these isolates. Interestingly, for the occurrence of sexual reproduction, both isolates must be in the same mating cluster. More specifically, one isolate must carry the *MAT*-1 allele, and the other should contain the *MAT*-2 allele. In other words, one of the isolates should be competent to act as a maternal parent, and the other should play the role of a paternal parent (Kovacevic *et al.*, 2013).

The domination of the mating type depends on the environmental conditions, ecological niche, and hosts within each biological species (Lim *et al.*, 2007). *G. fujikuroi* is heterothallic and facilitates the genetic crossing of the two mating types. Two isolates are cross-fertile if they carry the diverse mating type idiomorphs *MAT*-1 and *MAT*-2. Eight diverse mating populations have been identified in *G. fujikuroi,* of which three mating populations (designated as A, D, and E) are fumonisin producers and pathogens of corn (Da Silva *et al.*, 2006). Reportedly, 79 field isolates of *Fusarium spp*. crossed to standard testers (*MAT*-1 and *MAT*-2) from four mating populations of the *G. fujikuroi* species complex.

Table 4. The size of A and D populations of *Gibberella fujikuroi* species collected from maize.

Biological species	Mating ratio (*MAT*-1 : *MAT*-2)	Mating type, *Ne(mt)*	Effective population number, N_e / Male/hermaphrodite polymorphism, *Ne(f)*	$N_{e(v)}$
A	10:5	88.89	0	100
D	2:2	100	51.2	64

Nfs- female sterile (male fertile) isolates number; *Nh*- hermaphrodite isolates number; *Ne*- effective population number.

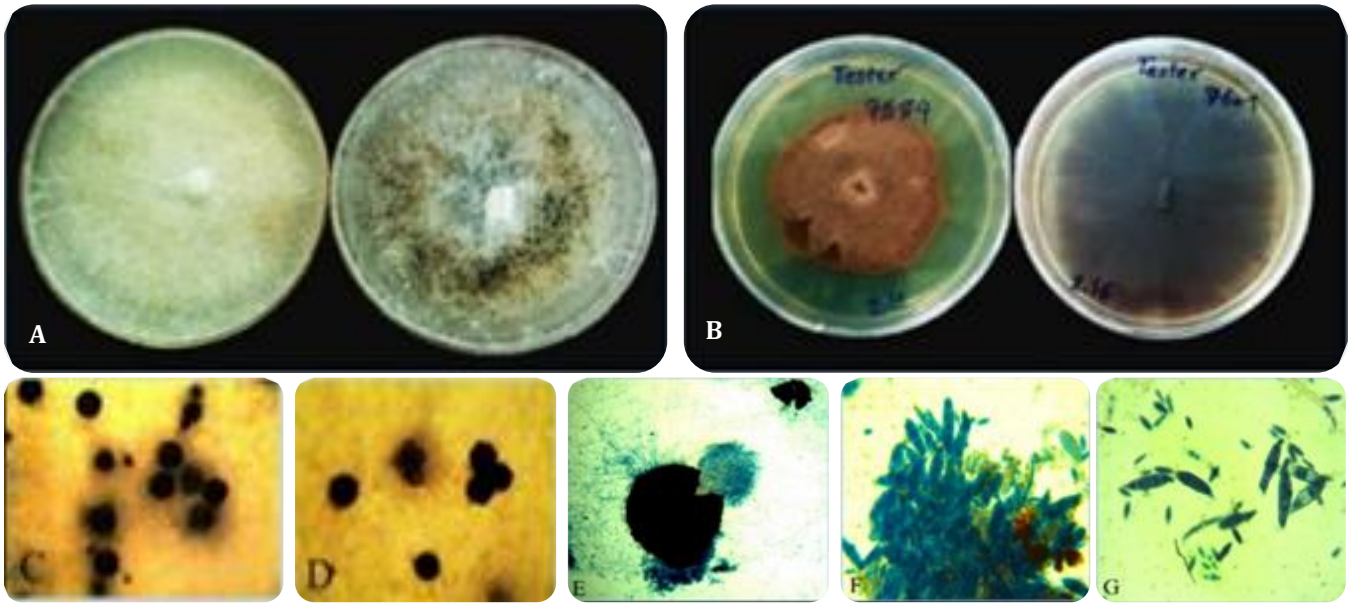

Figure 2. (a) Field isolates and control, (b) testers originating from Italy, (c, d) Perithecia covered with mycelium, (e, f, g) Ascospore-oozing and Asci and ascospores.

Several studies have demonstrated that the sexual reproduction potential of *Fusarium spp*. belongs to the A, D, E and F mating populations (Lim *et al.*, 2001). Consistent with the present results, various population types were identified in the sorghum isolates from Brazil (A) and Korea (populations A, D, and F) (Rahjoo *et al.*, 2008; Summerell *et al.*, 1998). According to Lim *et al.* (2001), a high frequency of hermaphrodites can be detected in the F population of the *MAT*-1 mating type (Rahjoo *et al.*, 2008). In contrast, the hermaphrodite isolate was not observed in 79 field isolates of sorghum, maize and wheat *Fusarium spp*. in Serbia (Lim *et al.*, 2001). Under natural conditions, only the A population forms perithecia (Bush *et al.*, 2004). The *F. verticillioides* isolates of maize collected from Tanzania, the United States and Costa Rica show a higher rate of sexual reproduction and frequencies of hermaphrodites (Harrigan, 1998; Vigier *et al.*, 1997).

Molecular identification

Species molecular identification: The PCR results indicated that 24 isolates belonged to *F. verticillioides*, and 13 isolates belonged to *F. proliferatum*. The single DNA amplicon length was 800 bp and 578 bp for VERT-

1/ VERT-2 and VER1/ VER2 primers, respectively, while the single DNA amplicon from *F. proliferatum* using PRO1/ PRO2 primers was 585 bp (Figure 3). To confirm these results, 15 isolates of *F. verticillioides* and five isolates of *F. proliferatum* were evaluated using PRO1/ PRO2 and VERT-1/ VERT-2 primer sets, respectively. No cross-reactions were observed when nonspecific primers were used for the isolates. Four unknown isolates were examined using VERT1/2, VER1/2 and PRO1/2 primers, and no DNA fragments were observed.

In comparison with other methods, the polymerase chain reaction (PCR) was more sensitive and faster for the identification of *Fusarium* isolates. The molecular identification results were consistent with the morphological identification results. Additionally, the DNA sequencing analysis confirmed the 24 isolates of *F. verticillioides* and 13 isolates of *F. proliferatum*. Similar results using VERT1/2, VER1/2 and PRO1/2 primers were found in various investigations of *Fusarium* spp. (Reyneri, 2006).

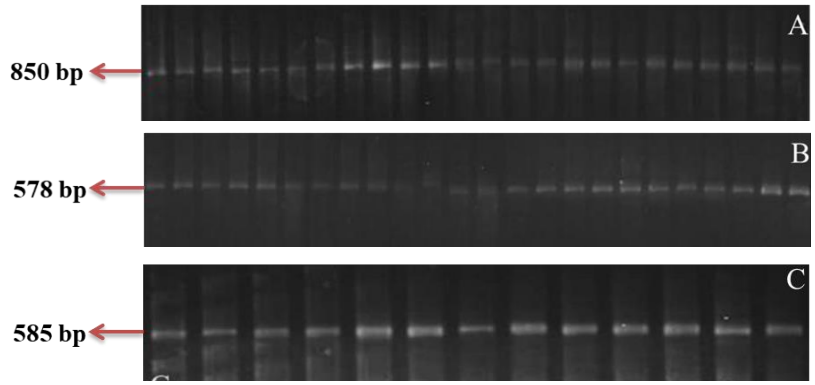

Figure 3. Species-specific PCR assays (1.25% of agarose gel electrophoresis). (A) *F. verticillioides* (800 base pairs) with VERT1/2 primers; (B) *F. verticillioides* (578 base pairs) with VER1/2 primers; (C) *F. proliferatum* (585 base pairs) with PRO1/2 primers.

Fumonisin molecular identification: VERTF-1 and VERTF-2 primers were used to analyze the potential of all *F. verticillioides* and *F. proliferatum* isolates to produce fumonisin. The expected 400-bp single fragment was detected with the DNA from all 24 isolates of *F. verticillioides* (Figure 4). Nonetheless, no DNA single fragment was found in the *F. proliferatum* that was used as a negative control. The results suggested that all of the *F. verticillioides* isolates, based on their positive amplification with specific VERTF1/VERTF2 primers, were fumonisin-producers. All 24 *F. verticillioides* isolates showed amplified products with VER-1/VER-2, VERT-1/VERT-2 and VERTF-1/VERTF-2 sets of primers. Similarly, Seifert et al. (2003) and Gargees and Shareef (2009) used the VERTF-1/2 primer set to analyze *F. verticillioides* isolates. The PCR amplified 400 bp DNA single fragments, which confirmed the fumonisin producer potential of the *F. verticillioides* isolates.

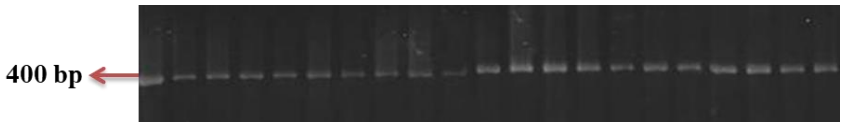

Figure 4. Species-specific PCR assays (1.25% of agarose gel electrophoresis). *F. verticillioides* (400 base pairs) with VERTF1/2 primers.

Evaluation of the pathogenicity

Evaluation of the pathogenicity on maize in a greenhouse: The analysis of variance (ANOVA) and Duncan's multiple range tests of the isolates on maize in the greenhouse showed a significant difference ($p \leq 0.01$) for all of the independent factors (Table 5). Although information on the most effective factors for *F. verticillioides* infection in maize is still limited, climate conditions, plants agronomic characteristics and insect damage may be important.

Table 5. The ANOVA analysis of various region isolates on maize in the greenhouse.

	df	Necrosis length on maize
Isolates	38	296.4**
Replicate	2	18.0 ns
Error	76	2.04
Total	116	-
CV	-	4.22

In the present study, the differences in isolate pathogenicity may be due to the diverse environmental conditions. The highest necrosis lengths were those of isolates MK7, MA2 and MS3. In contrast, the lowest range of pathogenicity was that for the control treatments, which showed no infection. Variable feedback to maize from the different isolates showed that each isolate has a different pathogenicity (Figure 5). In parallel, MG3, MG7, MK2, MK6, MK19, MK21, MK24 and MA1 isolates had higher necrosis lengths in maize (Figure 7). Additionally, the pathogenicity of the MS1 isolate on maize was very high (46.3 mm) (Figure 7). In contrast, the pathogenicity of isolates MG1, MK1 and MK22 were similar. Interestingly, the *Fusarium* isolates of each region had fluctuating reactions in maize (Figure 6). The maize necrosis length was variable for the *Fusarium* isolates. These results support the differences in maize susceptibility in response to *Fusarium* that have been reported (Geiser *et al.*, 2004; King, 1981; Venturini *et al.*, 2011).

Figure 5. Evaluation of pathogenicity on maize in the greenhouse, a) Infected maize stalk in greenhouse, b) necrosis length.

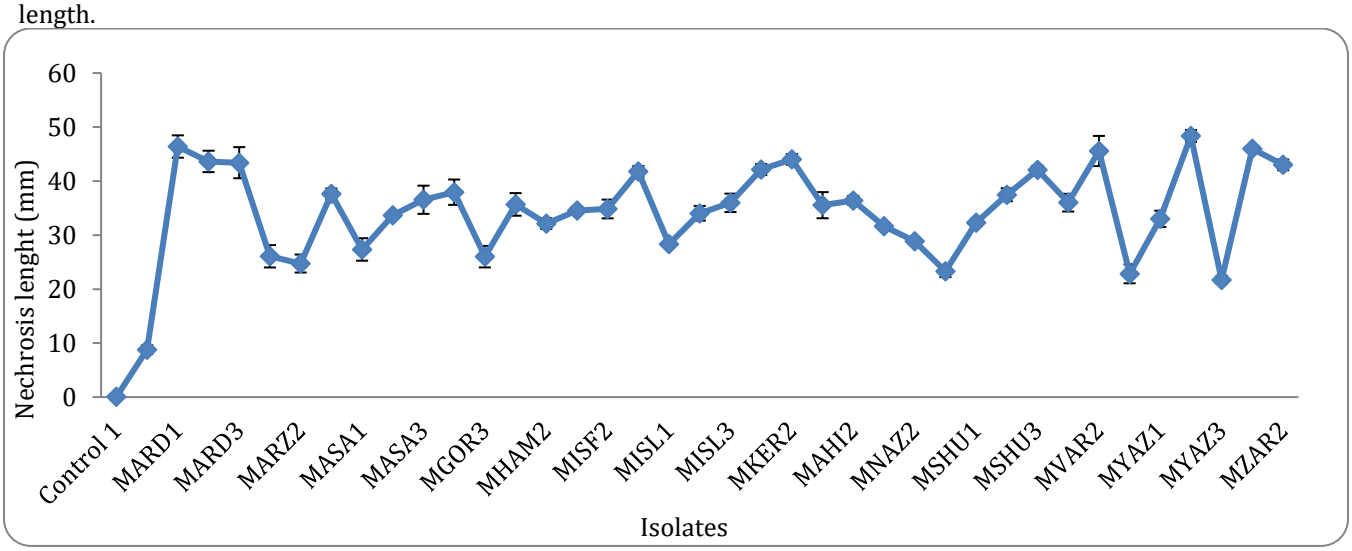

Figure 6. Various responses of maize (MO17) genotype to different *Fusarium* ear rot isolates according to necrosis length (mm). Different letters indicate significant difference among accessions by using Duncan's multiple range tests at p≤0.05.

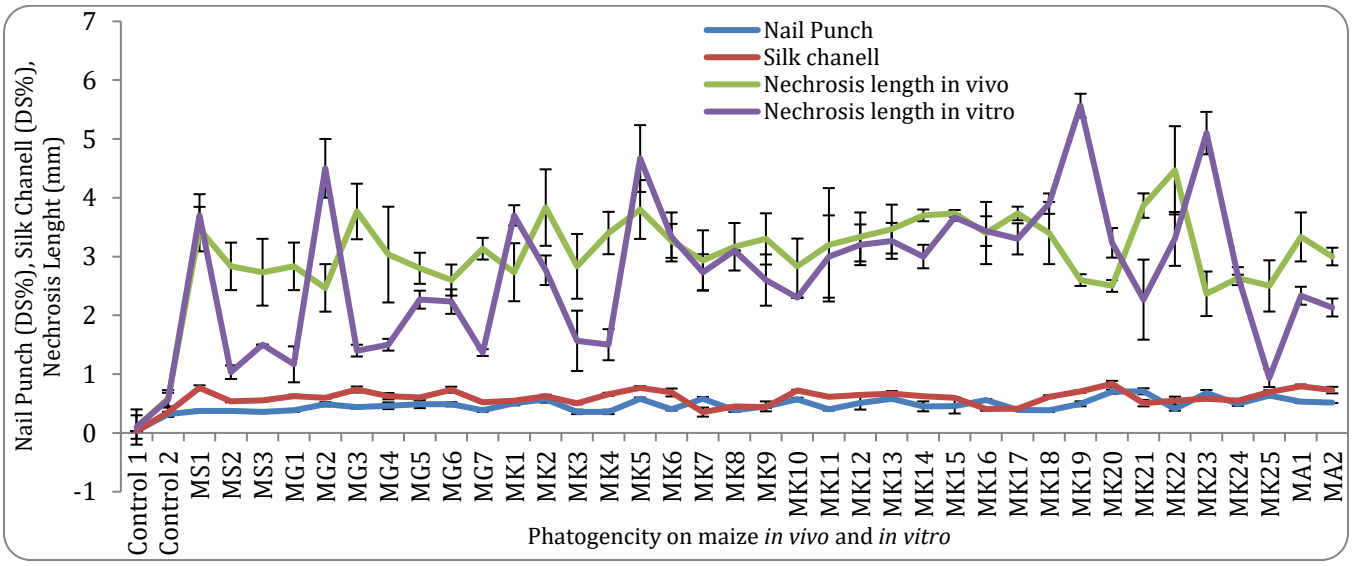

Figure 7. Various responses of maize (MO17) genotypes to different *Fusarium* ear rot isolates according to disease severity (DS%) and necrosis length (mm) *in vitro* and *in vivo* conditions. Different letters indicate significant difference among isolates by using Duncan's multiple range tests at p≤0.

Evaluation of the pathogenicity on maize in vivo and in vitro: The ANOVA and Duncan's multiple range tests results revealed that the pathogenicity of each isolate was significant (p≤0.01) for all of the independent factors of in vitro and in vivo conditions that are shown in Table 6.

The results of the Nail punch (Figure 8C) experiment revealed the different disease severities of the *Fusarium* isolates (Figure 8D). Among the isolates, the highest disease severities were found in the MK20, MK21 and MK25 isolates. In contrast, the lowest disease severities were found for the controls, which were not treated with *Fusarium*. Nevertheless, isolates MS1, MS2, MS3, MG1, MG7, MK8, MK3, MK4 and MK17 showed similarly low disease severities. The MK2, MK5 and MK19 isolates also showed similar disease severities. Other isolates showed a dissimilar or similar disease severity.

In the silk channel experiment (Figure 8A/B), the highest disease severity was found in isolates MS1, MK5, MA1, MK19, MK25, MG3, MG6 and MA2. In contrast, the lowest disease severities were found in isolates MK7, MK8, MK16 and control 1 and 2. However, the disease severities fluctuated in other isolates that were obtained from different regions. The results of the necrosis length analysis of each isolate in the field could divide the isolates into four clusters with the necrosis length for highest (MK4) and for the lowest (control 1 and 2). The necrosis length in the in vitro condition (Figures 8 G and I) was usually lower than the necrosis length in the field condition (Figures 8 E and F). However, the pathogenicity of isolates MG2, MK1, MK5, MK19 and MK23 in vitro was higher than the pathogenicity of those isolates in the field.

Table 6. The ANOVA analysis of various region isolates on maize in the greenhouse.

Source of variation	df	Nail Punch	Silk channel	Necrosis length *in vivo*	Necrosis length *in vitro*
Isolates	38	184.8**	370.4**	1.7**	4.1**
Replicate	2	39.5ns	45.4ns	0.052ns	0.07 ns
Error	76	45.6	37.5	0.28	0.30
Total	116	-	-	-	-
CV	-	25	19.1	17.7	20.8

Ns: non-significant, ** and * the mean difference is significant at 1 and 5% level, respectively.

Figure 8. Evaluation of pathogenicity on maize *in vivo*: (A) Silk channel, (B) Infected maize kernel, (C) Nail punch, (D) Infected maize kernel, (E) Toothpick, (F) necrosis stalk, (G) *in vitro* infected maize stalk and (H) necrosis length.

The pathogenicity of each isolate in different conditions (*in vivo* and *in vitro*) is shown in Table 7. In the current study, we classified all isolates into different groups based on the effect to their pathogenicity (necrosis length) and disease severity percentage according to Reid and Zhu (2005) (Bentley *et al.*, 2008). A necrosis length that is less than 1 cm indicated a very low pathogenicity, while a necrosis length between 1 and 2 cm indicated a low pathogenicity. The necrosis lengths of 2-3 cm, 3-5 cm and more than 5 cm indicated medium, high and very high pathogenicity, respectively. Although control 1 (untreated) and control 2 (sterile toothpick stained with distilled water) did not become infected by a toothpick, less pathogenicity was observed for these groups in all conditions. The necrosis lengths of the control plants resulted from the floating of *Fusarium* spores in the air (Table 7).

Additionally, the necrosis lengths in the *in vitro* condition were lower than most other conditions. These results revealed pathogenicity is decreased after controlling different factors, such as temperature, humidity and irrigation, and external contaminants, such as insects, birds and the wind. In contrast, the pathogenicity of MK23, MK1, MK20 and MK19 were higher *in vitro,* which may be due to unknown factors (Table 7). In the current study, a relationship was found between environmental conditions, necrosis length, and disease severity in the greenhouse, in the field and *in vitro*. In other words, the spread of *F. verticillioides* and *F. proliferatum* spores are mainly dependent on climate factors. Changes in the climate that have occurred over the years may influence "storage fungi" and "field fungi". Although most *Fusarium* species produce inoculum, grow quickly and are pathogenic under humid conditions, the optimal condition for *Fusarium* development is a warm temperature (Burger *et al.*, 2013). Among cereals, maize is the most suited to grow in air temperature during plant development, showing a fast growth rate at up to 30°C with *F. verticillioides* and *F. proliferatum* infection due to ECB (European corn borer) (Burger *et al.*, 2013; Hsuan *et al.*, 2010).

In the pathogenicity experiments, based on the effect in maize under different conditions, all of the isolates were classified into four main groups, where two control treatments comprised two individual groups. Moreover, MK25, MK19, MK23, MK5 and MK10 comprised a group, and the other isolates comprised a fourth group (Figure 8).

The quantification of total fumonisin using an ELISA test: The final results of the fumonisin analysis demonstrated that there were significant differences in the fumonisin accumulation in maize. The results revealed that the fumonisin content in *F. verticillioides* was less variable than that in *F. proliferatum*. Accordingly, isolates, in terms of fumonisin production, could be divided into three groups: high, medium and low groups. Among the 21 fungi isolates analyzed using an ELISA test, MS1, MA1, MK24 and MK4 showed the highest fumonisin content and were classified in the higher level group, as shown in Figure 12. In contrast, the lowest

amounts of fumonisin content were observed in MG2, MG, MK1 and MK6. The results also showed that both *F. verticillioides* and *F. proliferatum* produced various levels of fumonisin. Additionally, *F. verticillioides* showed more variety and a higher amount of fumonisin between the two species. Interestingly, isolates MS1 and MG3 (*F. verticillioides)* with 960 and 4.07 ppm, respectively, had the highest and least amount of total fumonisins among all isolates. Fumonisin levels in *F. proliferatum* isolates ranged from 242 to 687 ppm. The fluctuation trends of total fumonisin revealed that various factors may have an impact on the fumonisin content, such as the environment, isolate strain, genotype and plant type, and the genetic diversity of *Fusarium*. Nonetheless, the factors affecting fumonisin mycotoxin production in plants are not well known (Figure 9).

Table 7. The comparison of necrosis length in different conditions (necrosis length on maize in greenhouse, *in vivo* and *in vitro*).

I.O	Necrosis length on maize (mm)	Necrosis length *in vivo* (mm)	Necrosis length *in vitro* (mm)	Nail Punch (Rate)	Silk channel (Rate)
Control 1	very low	very low	very low	1	1
Control 2	very low	very low	very low	3	4
MS1	high	high	high	4	5
MS2	high	medium	low	4	5
MS3	high	medium	low	4	5
MG1	medium	medium	low	4	5
MG2	medium	medium	low	4	5
MG3	high	high	low	4	5
MG4	medium	high	low	4	5
MG5	high	medium	medium	4	5
MG6	high	medium	medium	4	5
MG7	high	high	low	4	4
MK1	medium	medium	high	4	5
MK2	high	high	low	5	5
MK3	high	medium	low	4	4
MK4	high	high	low	4	5
MK5	high	high	high	5	5
MK6	high	high	high	4	5
MK7	medium	medium	medium	5	4
MK8	high	high	high	4	4
MK9	high	high	medium	4	4
MK10	high	medium	medium	5	5
MK11	high	high	medium	4	5
MK12	high	high	high	4	5
MK13	high	high	high	5	5
MK14	high	high	medium	4	5
MK15	medium	high	high	4	5
MK16	medium	high	high	5	4
MK17	high	high	high	4	4
MK18	high	high	high	4	5
MK19	high	medium	high	4	5

MK20	high	medium	high	5	6
MK21	high	high	medium	5	4
MK22	medium	high	high	4	5
MK23	high	medium	very high	5	5
MK24	high	medium	medium	4	5
MK25	medium	medium	very low	5	5
MA1	high	high	medium	5	6
MA2	high	medium	medium	4	5

Pathogenicity of the necrosis length: less than 1cm= very low, between 1- 2cm= low, between 2- 3 cm= medium, between 3-5 cm = high and more than 5 cm= very high. The disease severity in nail punch and silk channel were measured according to Reid and Zhu (2005) protocol (0% =1, 1-3= 2%, 4-10= 3%, 11-25%= 4, 26-50= 5%, 51-75= 6% and 76-100= 7%).

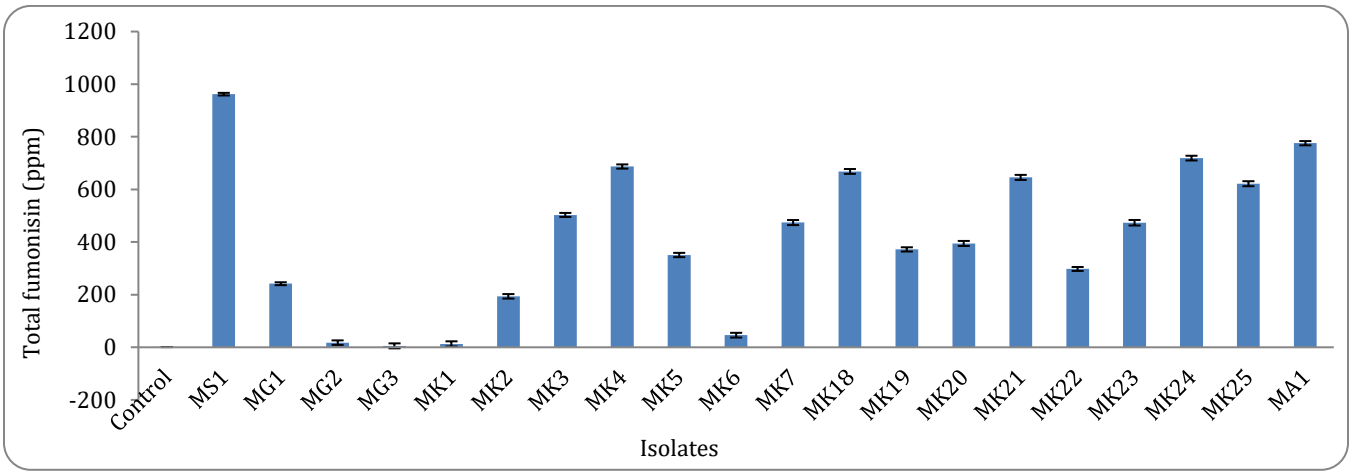

Figure 9. Various amount of fumonisin for each isolates. Different letters indicate significant difference among isolates by using Duncan's multiple range tests at p≤0.05.

The ELISA results of the current study were consistent with the results of kernel infection by *F. verticillioides* (Geiser *et al.*, 2004; Steenkamp *et al.*, 2002). Several investigations have reported that the particle size of the endosperm, endosperm texture, endosperm hardness, kernel density and texture are some of the most sensitive traits to mycotoxin content (Danielsen *et al.*, 1998; Melake-Berhan *et al.*, 1996).

Positive correlations were observed between all of the traits, including maize toothpick in the greenhouse, toothpick in the field, silk channel, detaches and fumonisin content (ELIZA). The highest correlation coefficient was found between the silk channel and other traits such as maize toothpick in the greenhouse (r=0.93) and a toothpick in the field (r=0.87). On the other hand, the lowest correlation coefficients were observed between fumonisin and detach (r=0.09), silk channel and nail punch (r=0.13), and fumonisin and nail punch (r=0.21). The correlation coefficient between maize toothpick in the field and greenhouse were high (Table 8).

Table 8. Correlation coefficients of all the experiments traits.

	Maize toothpick in Greenhouse	Toothpick in Field	Nail Punch	Silk Channel	Detach	Fumonisin
Toothpick in Greenhouse	1					
Toothpick in Field	0.68**	1				
Nail Punch	0.33ns	0.38 ns	1			
Silk Channel	0.93**	0.87**	0.13 ns	1		
Detach	0.50*	0.41 ns	0.45*	0.37 ns	1	
Fumonisin	0.67**	0.34 ns	0.21 ns	0.41 ns	0.09ns	1

In the current study, cluster analysis was applied to determine the similarities of 21 *Fusarium* isolates for all quantitative experiments. Isolates of *F. verticillioides and F. proliferatum* were clustered into separate groups. Some species are very closely related and are regarded as sibling species. The cluster analyses six groups of isolates, where MS1, MA1, MK18 and MK21 isolates comprised the first cluster. The *Fusarium* species of this group was the *F. verticillioides* from different regions. The second cluster was comprised of isolates MK23, MK19, MK20 and MK5. The isolates from this group were from the Karaj region, and the identified *Fusarium* species was *F. verticillioides* that was collected from maize. The MK3, MK4, MK24 and MK25 isolates comprised the third group. The *Fusarium* species of this cluster were *F. verticillioides* and *F. proliferatum* that were collected from maize at the Karaj region. The fourth cluster was comprised of isolates MK22, MG1, MK7, MG2 and MK1 from *F. verticillioides* (Karaj), *F. proliferatum* (Gorgan), *F. proliferatum* (Karaj), *F. verticillioides* (Gorgan) and *F. verticillioides* (Karaj). The isolates in the fifth cluster were MK6 (*F. verticillioides* (Karaj), MK2 *F. verticillioides* (Karaj) and mG3 *F. verticillioides* (Gorgan). The last cluster was comprised of the control treatment group. On the other hand, the clusters could be classified into two major groups with the controls as a group and other isolates as the second group.

The strategies for data collection depended on the fungi species. Although the *Fusarium* isolation technique recovers more than 1 species from a diseased plant material, the method should restrict the recovery of weed and soil *Fusarium*. To identify the *Fusarium* species, morphological, molecular and pathogenicity methods are some of the reliable techniques. In the current study, the first step of identification was to clearly describe the *Fusarium* characteristics using a morphological analysis. Afterwards the morphological results were confirmed using PCR. Subsequently, the pathogenicity of the isolates was evaluated by testing maize in the greenhouse and maize in both *in vitro* and *in vivo* (field) conditions. Finally, the fumonisin of 21 to 37 isolates was tested using ELIZA.

The species of the *Fusarium* genus are variable due to their genetic variation and compositions in the different environmental conditions, where their growth leads to morphological changes. *F. verticillioides* and *F. proliferatum* easily form sporodochia of robust, uniform macroconidia on the carnation leaf-pieces, which is the most common way to identify these species. The PDA media are used initially to evaluate pigmentation and gross colony morphology. Cultures growing on PDA media should usually be regarded as "dead ends" and should not be further subculture. If further subcultures are necessary, mass transfers or single spores from CLA media should be applied instead (Bush *et al.*, 2004; Summerell *et al.*, 2010). In some laboratories, the identification procedures are carried out in accordance with the available equipment. In the current study, all 37 to 41 isolates were tested using PCR primers. The PCR results showed a similar band for each primer, which confirmed the presence of two *Fusarium* based on the sequence length of each band and DNA sequencing. The isolates showed a broad range of pathogenicity in each region, which confirmed that all of the strains have the potential to be pathogenic to maize. The majority of the isolates were identified as *F. verticillioides.* Notwithstanding the fluctuating trends of each isolate under various conditions, in general, maize that was collected from a field in Karaj showed more pathogenicity in comparison with the other isolates. The percentage of non-emerged plants showed that six, three and one isolates expressed low, moderate and high pathogenicity. Various dynamic groups of fungi from different genera lead to *Fusarium* ear rot and grain mold in some plants such as maize and sorghum. To increase the plant tolerance against fungi disease, there are few effective resistance mechanisms under all environmental conditions. Phenolic compound accumulation is also influenced by various environmental features. In the present study, isolates of *F. verticillioides* can be differentiated from isolates of *F. proliferatum* using morphological, molecular and pathogenicity analyses. Some *Fusarium* species were difficult to identify morphologically, and identification based on morphological characteristics is necessary, as it allows for the sorting of *Fusarium* isolates before applying other methods of identification and characterization (Leslie and Summerell, 2008). The extent of the colonization of maize and sorghum by *F. verticillioides* and *F. proliferatum* is influenced by diverse factors, such as water availability and rainfall, or the interaction of several factors. *F. verticillioides* and *F. proliferatum* are the most important fumonisin producers in which the toxins are suspected to be carcinogenic to humans and are implicated in a number of animal diseases(Wan *et al.*, 2013).

In conclusion, the current investigation represents an original approach to identify the fungal isolates collected from different regions of Iran using morphological and

molecular methods. Furthermore, pathogenicity, mating-type, and mycotoxicity measurements were performed. Isolates of maize showed different morphological traits in the same condition *in vivo* and *in vitro*. The pathogenicity assay results and the molecular profiles seem to confirm these observations. For toxin production, FB_1composed higher amount of the overall fumonisin content in compare with FB_2 and FB_3. These could reflect important differences in the ecology and natural history of the *Fusarium* populations from maize and trigger further investigations on the mechanisms of toxin production and pathogenicity within the same situation. Finally, the data obtained by the Morpho-molecular identification, pathogenicity variation, mating biology, and fumonisin production of *Fusarium* species analysis could provide useful status for controlling the development of this species and for its subgroups from maize.

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