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

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ABSTRACT

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 B₁ (FB₁), B₂ (FB₂) and B₃ (FB₃) are found in maize kernel, and the level of FB₁ is generally higher than in FB₂ and/or FB₃ (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.

| 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               | M11     | Maize - Isfahan             |
| MG5     | Maize – Gorgan              | MK7     | Maize - Karaj               | MK17    | Maize - Karaj               | M12     | Maize - Isfahan             |
| MG6     | Maize – Gorgan              | MK8     | Maize - Karaj               | MK18    | Maize - Karaj               | M13     | 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, Ilkirch, 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 (10⁵) 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 color 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 mon- 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 peritheciun 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 (Nₑ) was evaluated according to the number of different mating type strains [Nₑ(mt)], which was expressed as the actual count percentage, and the number of hermaphrodite and male strains [Nₑ(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:

\[
[Nₑ(mt)] = \frac{(4NₘN₃)}{(Nₘ + N₃)}
\]

Where, Nₘ = the total number of isolates (strains) with one mating type (MAT-1), and N₃ = the total number of
isolates (strains) with another mating type (MAT-2) within the same population. The effective population number (size) \(N_e\), based on the relative frequency of female-sterile and hermaphrodite strains, was determined using the following equation:

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

Where, \(N\) = the total number of female-sterile strains, and \(Nh\) = the total number of hermaphrodite strains.

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

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

Where, \(N\) = the total number of female-sterile strains, and \(Nh\) = the total number of hermaphrodite strains.

**Molecular identification**

**DNA extraction:** *Fusarium* isolates were sieved and subcultured 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.

**Table 2. Sequences of primers used in the experiments.**

| Primer name   | Primer sequence (5' → 3')                               | Species-specificity |
|---------------|----------------------------------------------------------|--------------------|
| PRO1/PRO2     | CTTTCCGCGCCAAATTTCTTC/                                   | *F. proliferatum*  |
|               | TGTCAGAATCCAGGTTG/                                       |                    |
|               | CTTCTCGATTTCTTC/                                         |                    |
|               | AATTGGCCATTTGGAATATATATAGTAATATAGTA                      |                    |
| VERT-1/VERT-2 | GTGACGATACCGCAGAAGC/                                     | *F. verticillioides*|
|               | CACCCGACGCAATCATCAG/                                     |                    |

**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 GCC GCG AAA CCG 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:

\[
\text{arcsin}\sqrt{\frac{x}{100}}
\]

*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 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-pionnnotal 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 macroconidia 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               | colour              | 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 shaped | 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 | Cotty              |
| 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 | + | + | - | + | - | A-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 |
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 macroconidia 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 2D). 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 $N_{(mt)}$ up to 100% of the actual count. One isolate out of the 13 fertile isolates of population D was a hermaphrodite resulting in $N_{(mt)}$ 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 $N_{(mt)}$ 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 $N_{(mt)}$ 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* allele, and the other should contain the *MAT* 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 crossfertile 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, $Ne$ | $N_{e(v)}$ |
|-------------------|---------------------------------------|----------------------|-----------------------------------|------------|
| A                 | 10:5                                  | 88.89                | 0                                 | 100        |
| D                 | 2:2                                   | 100                  | 51.2                              | 64         |

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

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 \textit{F. proliferatum} using PRO1/ PRO2 primers was 585 bp (Figure 3). To confirm these results, 15 isolates of \textit{F. verticillioides} and five isolates of \textit{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 \textit{Fusarium} isolates. The molecular identification results were consistent with the morphological identification results. Additionally, the DNA sequencing analysis confirmed the 24 isolates of \textit{F. verticillioides} and 13 isolates of \textit{F. proliferatum}. Similar results using VERT1/2, VER1/2 and PRO1/2 primers were found in various investigations of \textit{Fusarium} spp. (Reyneri, 2006).

**Figure 3.** Species-specific PCR assays (1.25% of agarose gel electrophoresis). (A) \textit{F. verticillioides} (800 base pairs) with VERT1/2 primers; (B) \textit{F. verticillioides} (578 base pairs) with VER1/2 primers; (C) \textit{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 \textit{F. verticillioides} and \textit{F. proliferatum} isolates to produce fumonisin. The expected 400-bp single fragment was detected with the DNA from all 24 isolates of \textit{F. verticillioides} (Figure 4). Nonetheless, no DNA single fragment was found in the \textit{F. proliferatum} that was used as a negative control. The results suggested that all of the \textit{F. verticillioides} isolates, based on their positive amplification with specific VERTF1/VERTF2 primers, were fumonisin-producers. All 24 \textit{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 \textit{F. verticillioides} isolates. The PCR amplified 400 bp DNA single fragments, which confirmed the fumonisin producer potential of the \textit{F. verticillioides} isolates.

**Figure 4.** Species-specific PCR assays (1.25% of agarose gel electrophoresis). \textit{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≤0.01) for all of the independent factors (Table 5). Although information on the most effective factors for \textit{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.
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   | 39.5 ns    | 45.4 ns      | 0.052 ns                | 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.
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 fumonisins 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 | low | 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 | 5 |
| 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 | 5 | 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 1 cm = very low, between 1-2 cm = 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 fumonisins for each isolates.](image)

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).

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

Figure 9. Various amount of fumonisins for each isolates. Different letters indicate significant difference among isolates by using Duncan’s multiple range tests at p≤0.05.
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 analysis identifies six groups of isolates, where MS1, MA1, MK18 and MK21 isolates comprised the first cluster. The *Fusarium* species of this group were 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 fumonisins of 21 to 37 isolates was tested using ELISA.

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 subsamples 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 composed higher amount of the overall fumonisin content in compare with FB2 and FB3. 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.

REFERENCES

Abiri, R., A. Valdiani, M. Maziah, N. A. Shaharuddin, M. Sahebi, Z. N. B. Yusof, N. Atabaki and D. Talei. 2015. A critical review of the concept of transgenic plants: Insights into pharmaceutical biotechnology and molecular farming. Current Issues on Molecular Research, 18: 21-42.

Alizadeh, A. M., G. Roshandel, S. Roudbarmohammadi, M. Roudbary, H. Sohanaki, S. A. Ghiasian, A. Taherkhani, S. Semnani and M. Aghasi. 2012. Fumonisin B1 contamination of cereals and risk of esophageal cancer in a high risk area in northeastern Iran. Asian Pacific Journal of Cancer Prevention, 13: 2625-2628.

Babič, M. N., P. Zalar, B. Ženko, H.-J. Schroers, S. Džeroski and N. Gunde-Cimerman. 2015. Candida and Fusarium species known as opportunistic human pathogens from customer-accessible parts of residential washing machines. Fungal Biology, 119: 95-113.

Bentley, A. R., J. F. Leslie, E. C. Y. Liew, L. W. Burgess and B. A. Summerell. 2008. Genetic structure of Fusarium pseudograminearum populations from the Australian grain belt. Phytopathology, 98: 250-255.

Burger, H. M., G. S. Shephard, W. Louw, J. P. Rheeder and W. C. A. Gelderblom. 2013. The mycotoxin distribution in maize milling fractions under experimental conditions. International Journal of Food Microbiology, 165: 57-64.

Bush, B. J., M. L. Carson, M. A. Cubeta, W. M. Hagler and G. A. Payne. 2004. Infection and Fumonisin Production by Fusarium verticillioides in developing maize kernels. Phytopathology, 94: 88-93.

Chelkowski, J. 2014. Fusarium: Mycotoxins, Taxonomy, Pathogenicity. Elsevier.

Ciarmiello, L. F., P. Woodrow, A. Fuggi, G. Pontecorvo and P. Carillo. 2011. Plant Genes for Abiotic Stress, Abiotic Stress in Plants-Mechanisms and Adaptations. InTech Publishers.

Cumagun, C. J. R. 2008. Female fertility and mating type distribution in a Philippine population of Fusarium verticillioides. Journal of Applied Genetics, 49: 123-126.

Da Silva, V. N., F. M. C. Fernandes, A. Cortez, D. H. B. Ribeiro, A. P. de Almeida, R. H. Hassegawa and B. Corrêa. 2006. Characterization and genetic variability of Fusarium verticillioides strains isolated from corn and sorghum in Brazil based on fumonisins production, microsatellites, mating type locus, and mating crosses. Canadian Journal of Microbiology, 52: 798-804.

Danielsen, Meyer and J. Funk. 1998. Genetic characteristics of Fusarium verticillioides isolates from maize in Costa Rica. Plant Pathology, 47: 615-622.

De Curtis, F., V. De Cicco, M. Haidukowski, M. Pascale, S. Somma and A. Moretti. 2011. Effects of agrochemical treatments on the occurrence of Fusarium ear rot and fumonisin contamination of maize in Southern Italy. Field Crops Research, 123: 161-169.

Desjardins, A. E., G. P. Munkvold, R. D. Plattner and R. H. Proctor. 2002. FUM1-A gene required for fumonisin biosynthesis but not for maize ear rot and ear infection by Gibberella moniliformis in field tests. Molecular Plant-Microbe Interactions, 15: 1157-1164.

Gargees, M. T. and A. M. Shareef. 2009. Reducing liver aflatoxin M1 residues in chicks with mycofix plus 3.0 during aflatoxicosis. Iraqi Journal of Veterinary Sciences, 23.

Geiser, D. M., M. del Mar Jiménez-Gasco, S. Kang, I. Makalowska, N. Veeraraghavan, T. J. Ward, N. Zhang, G. A. Kulda and K. O’Donnell. 2004. Fusarium-ID v. 1.0: A DNA sequence database for identifying Fusarium. Molecular diversity and PCR-detection of toxigenic Fusarium species and ochratoxigenic fungi. Springer Netherlands, p. 473-479.

Harrigan, W. F. 1998. Laboratory Methods in Food Microbiology. Gulf Professional Publishing.
Hsuan, H., L. Zakaria and B. Salleh. 2010. Characterization of *Fusarium* isolates from rice, sugarcane and maize using RFLP-IGS. Journal of Plant Protection Research, 50.

Ingle, A., A. Gade, S. Pierrat, C. Sonnichsen and M. Rai. 2008. Mycosynthesis of silver nanoparticles using the fungus *Fusarium acuminatum* and its activity against some human pathogenic bacteria. Current Nanoscience, 4: 141-144.

Karami-Osboo, R., M. Mirabolfathi and F. Aliakbari. 2010. Natural deoxynivalenol contamination of corn produced in Golestan and Moqan areas in Iran. Journal of Agricultural Science and Technology, 12: 233-239.

Karbancioglu-Guler, F. and D. Heperkan. 2009. Comparison of enzyme linked immunoassay and high performance liquid chromatography for determination of fumonisins in dried figs. Zbornik Matice srpske za prirodne nauke: 37-43.

Kim, J.-A., G. K. Agrawal, R. Rakwal, K.-S. Han, K.-N. Kim, C.-H. Yun, S. Heu, S.-Y. Park, Y.-H. Lee and N.-S. Jwa. 2003. Molecular cloning and mRNA expression analysis of a novel rice (*Oryza sativa*) MAPK kinase, OsEDR1, an ortholog of Arabidopsis AtEDR1, reveal its role in defense/stress signalling pathways and development. Biochemical and Biophysical Research Communications, 300: 868-876.

King, S. B. 1981. Time of infection of maize Kernels by *Fusarium moniliforme* and *Cephalosporium acremonium*. Phytopathology, 71: 796.

Kouadio, J. H., S. D. Dano, S. Moukha, T. A. Mobio and E. E. Creppy. 2007. Effects of combinations of *Fusarium* mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon, 49: 306-317.

Kovacevic, T., J. Levic, S. Stankovic and J. Vukojevic. 2013. Mating populations of *Gibberella fujikuroi* (Sawada) S. Its species complex isolating from maize, sorghum and wheat in Serbia. Genetika, 45: 749-760.

Kuhnem, P. R., P. Spolti, E. M. Del Ponte, J. A. Cummings and G. C. Bergstrom. 2015. Trichotheccene genotype composition of *Fusarium graminearum* not differentiated among isolates from maize stubble, maize ears, wheat spikes, and the atmosphere in New York. Phytopathology, 105: 695-699.

Leslie, J. F. and K. K. Klein. 1996. Female fertility and mating type effects on effective population size and evolution in filamentous fungi. Genetics, 144: 557-567.

Leslie, J. F. and B. A. Summerell. 2008. The *Fusarium* Laboratory Manual. John Wiley and Sons.

Lim, C. J., J. E. Hwang, H. Chen, J. K. Hong, K. A. Yang, M. S. Choi, K. O. Lee, W. S. Chung, S. Y. Lee and C. O. Lim. 2007. Over-expression of the Arabidopsis DRE/CRT-binding transcription factor DREB2C enhances thermotolerance. Biochemical and Biophysical Research Communications, 362: 431-436.

Lim, S.-H., S.-H. Yun and Y.-W. Lee. 2001. Mating behavior, mycotoxin production, and vegetative compatibility of *Gibberella fujikuroi* species complex from sorghum in Korea. The Plant Pathology Journal, 17: 276-280.

Melake-Berhan, A., L. G. Butler, G. Ejeta and A. Menkir. 1996. Grain mold resistance and polyphenol accumulation in sorghum. Journal of Agricultural and Food Chemistry, 44: 2428-2434.

Minervini, F., A. Giannoccaro, A. Cavallini and A. Visconti. 2005. Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters. Toxicology Letters, 159: 272-283.

Mulè, G., A. Susca, G. Stea and A. Moretti. 2004. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides, F. proliferatum* and *F. subglutinans*. Molecular Diversity and PCR-detection of Toxigenic *Fusarium* Species and Ochratoxigenic Fungi. Springer Netherlands, p. 495-502.

Nelson, P. E., T. A. Toussoun and W. F. O. Marasas. 1983. *Fusarium* species. An illustrated manual for identification.

Okamoto, S., K. Furuya, S. Nozaki, K. Aoki and H. Niki. 2013. Synchronous activation of cell division by light or temperature Stimuli in the dimorphic yeast *Schizosaccharomyces japonicus*. Eurakotic Cell, 12: 1235-1243.

Peng, X.-C., D.-W. Qiu, H.-M. Zeng, L.-H. Guo, X.-F. Yang and Z. Liu. 2014. Inducible and constitutive expression of an elicitor gene Hrip1 from *Alternaria tenuissima* enhances stress tolerance in Arabidopsis. Transgenic Research, 24: 135-145.

Rahjoo, V., J. Zad, M. Javan-Nikkhah, A. M. Gohari, S. M. Okhovvat, M. R. Bihamta, J. Razzaghi and S. S. Klemsdal. 2008. Morphological and molecular
identification of *Fusarium* isolated from maize ears in Iran. Journal of Plant Pathology: 463-468.

Reid, L. M., R. W. Nicol, T. Ouellet, M. Savard, J. D. Miller, J. C. Young, D. W. Stewart and A. W. Schaaßma. 1999. Interaction of *Fusarium graminearum* and *F. moniliforme*in maize ears: Disease progress, fungal biomass and mycotoxin accumulation. Phytopathology, 89: 1028-1037.

Reid, L. M. and X. Zhu. 2005. Screening Corn for Resistance to Common Diseases in Canada. Agriculture and Agri-Food Canada.

Reyneri, A. 2006. The role of climatic condition on mycotoxin production in cereal. Veterinary Research Communications, 30: 87-92.

Santiago, R., A. Cao and A. Butrón. 2015. Genetic factors involved in fumonisin accumulation in maize kernels and their implications in maize agronomic management and breeding. Toxins, 7: 3267-3296.

Seifert, K. A., T. Aoki, R. P. Baayen, D. Brayford, L. W. Burgess, S. Chulze, W. Gams, D. Geiser, J. de Gruyter, J. F. Leslie, A. Logrieco, W. F. O. Marasas, H. I. Nirenberg, K. O’Donnell, J. Rheeder, G. J. Samuels, B. A. Summerell, U. Thrane and C. Waalwijk. 2003. The Name *Fusarium Moniliforme* should no longer be used. Mycological Research, 107: 643-644.

Sforza, S., C. Dall’Asta and R. Marchelli. 2006. Recent advances in mycotoxin determination in food and feed by hyphenated chromatographic techniques/mass spectrometry. Mass Spectrometry Reviews, 25: 54-76.

Somma, S., C. Alvarez, V. Ricci, L. Ferracane, A. Ritieni, A. Logrieco and A. Moretti. 2010. Trichotheccene and beauvericin mycotoxin production and genetic variability in *Fusarium poaeisolatum* from wheat kernels from northern Italy. Food Additives and Contaminants: Part A, 27: 729-737.

Steenkamp, E. T., B. D. Wingfield, A. E. Desjardins, W. F. O. Marasas and M. J. Wingfield. 2002. Cryptic speciation in *Fusarium subglutinans*. Mycologia, 94: 1032-1043.

Summerell, B. A., L. W. Burgess, S. Bullock, D. Backhouse and N. D. Tri. 1998. Occurrence of perithecia of giberella fujikuroi mating population a (*Fusarium moniliforme*) on maize stubble in northern Vietnam. Mycologia, 90: 890.

Summerell, B. A., M. H. Laurence, E. C. Y. Liew and J. F. Leslie. 2010. Biogeography and phylogeography of *Fusarium*: A review. Fungal Diversity, 44: 3-13.

Summerell, B. A. and J. F. Leslie. 2011. Fifty years of *Fusarium*: How could nine species have ever been enough. Fungal Diversity, 50: 135-144.

Summerell, B. A., B. Salleh and J. F. Leslie. 2003. A utilitarian approach to *Fusarium* identification. Plant Disease, 87: 117-128.

Torelli, E., G. Firrao, G. Bianchi, F. Saccardo and R. Locci. 2012. The influence of local factors on the prediction of fumonisin contamination in maize. Journal of the Science of Food and Agriculture, 92: 1808-1814.

Venturini, G., G. Assante, S. L. Toffolatti and A. Vercesi. 2011. Mating behavior of a northern Italian population of *Fusarium verticillioides* associated with maize. Journal of Applied Genetics, 52: 367-370.

Vigier, B., L. M. Reid, K. A. Seifert, D. W. Stewart and R. I. Hamilton. 1997. Distribution and prediction of *Fusarium* species associated with maize ear rot in Ontario. Canadian Journal of Plant Pathology, 19: 60-65.

Wan, L. Y. M., P. C. Turner and H. El-Nezami. 2013. Individual and combined cytotoxic effects of *Fusarium* toxins (deoxynivalenol, nivalenol, zearalenone and fumonisins B1) on swine jejunal epithelial cells. Food and Chemical Toxicology, 57: 276-283.

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