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# Mycotoxins produced by *Fusarium* species associated with maize ear rot in Iran

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## Abstract

Mycotoxins contamination is one of the most important problems worldwide in maize that can cause serious threat for human and animal health. The aim of this study was to determine the ability of *Fusarium* species associated with maize ear rot to produce diverse mycotoxins. The results showed, One out of three isolates of *F. subglutinans* produced detectable level of beauvericin (BEA); the only isolate of *F. temperatum* produced 302 µg/g of BEA. Two out of five isolates of *F. redolens* produced enniatin B1and four isolates of this species produced high levels of BEA. As well, 21 isolates of *Fusarium incarnatum-equiseti* species complex (FIESC) and one isolates of *F. brachygibbosum* were evaluated for production of trichothecens (T-2 toxin, HT-2 toxin, Diacetoxyscirpenol (DAS), nivalenol (NIV) and deoxynivalenol (DON)), zearalenone (ZEN), enniatins (ENN A, A1, B and B1) and BEA by HPLC. Production of trichothecens, ZEN and BEA with one, three and four isolates of FIESC, respectively were observed and one isolate of *F. brachygibbosum* only produced detectable level of DAS and ENN B1. Analyzing of moniliformin production of *F. proliferatum* (26 isolates), FIESC (21 isolates) and *F.thapsinum* (10 isolates) showed none of them produce this toxin. These results revealed the ability of *Fusarium* spp. from maize to produce a varied range of mycotoxins which are harmful to human and animal's health. Therefore, the occurrence of such broad number of different species on Iranian maize could be reason of great concern because of the toxigenic risk associated to these species.

**Key words**: *Fusarium*, maize ear rot, HPLC, toxigenic risk

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## Introduction

Mycotoxins have significant influence in food and feed safety. Challenges in mycotoxin and toxigenic fungi research are still enormous, due to the frequency. Maize ear rot is one of the serious diseases caused by *Fusarium* spp. that significantly reduces the quantity and quality of maize (Gallo *et al*., 2015; Stoev, 2015). Each *Fusarium* species has its own mycotoxin profile. As a consequence, mycotoxin contamination in maize kernels from fields and silos is often high (Aguın *et al*., 2014; Alizadeh *et al*., 2012). Fumonisins (FBs), zearalenone (ZEN) and trichothecenes (Type A: T-2 and HT-2 toxins and Diacetoxyscirpenol (DAS); type B: deoxynivalenol (DON) and nivalenol (NIV)) are the most important classes of mycotoxins that are produced by *Fusarium* species. Besides, *Fusarium* genera produce emerging mycotoxins such as fusaproliferin (FUS), beauvericin (BEA), enniatins (ENNs) and moniliformin (MON), which have been recently discovered and less studied (Escriva *et al*., 2015; Moretti *et al*., 2007). Fumonisins have been related to several animal and human diseases such as esophageal cancer (EC), as reported in several countries worldwide (Desjardins *et al*., 2006). A recent study on the fungal mycoflora associated with maize kernels in some maize-producing areas of Iran revealed that *F. verticellioides* and *F. proliferatum* are prevalent species in Iranian maize (Fallahi *et al*., 2019;Ghiasian *et al*., 2000; Zamani-Zadeh *et al*., 1995), since they are both the main producing species of the carcinogenic fumonisins in maize (Fallahi *et al*., 2019). Moreover, there are other reports in the literatures on the *Fusarium* incidence, FBs production and levels of fumonisin in Iranian maize and maize –based products (Amirahmadi *et al*., 2017; Ghiasian *et al*., 2006;Yazdanpanah *et al*., 2000). Trichothecenes is other important group of *Fusarium* toxins related to several livestock diseases. There is poor information about these toxins and related fungi on maize in Iran. On the other hand, ZEN is an estrogenic mycotoxin produced by several species of *Fusarium*. A high risk of ZEN-contaminated maize for animal and human health was also reported in Iran (Rashedi *et al*. 2012; Nuryono *et al*., 2005; Oveisi *et al*., 2005; Hadiani *et al*., 2003). Since maize is an important cereal crop in Iran and worldwide, its quality and safety is of major concern. Hence, evidence about mycotoxin profiles of *Fusarium* species as an important pathogen of this crop can help us in the development of strategies to reduction of mycotoxin production and prediction the

presence of them in maize. Therefore, this work is aimed to assess the mycotoxin production of *Fusarium* isolates from Iranian maize.

## Materials and methods

### Sampling and fungal isolation

Maize samples of two crop seasons, 2015 and 2016, were collected in September–October from fields and maize grain silos of ten provinces of Iran: Khuzestan, Fars, Golestan, Ardabil, Alborz, Qazvin, Zanjan, Kermanshah, Lorestan and Isfahan. The sampling method in every field was based on hierarchical method (McDonald *et al*., 1999). Kernels were surface sterilized for 1 min in 3% sodium hypochlorite solution, rinsed twice in sterile distilled water, dried on filter paper and placed on petri dishes containing Nash & Snyder medium (1 L of distilled water, 15 g of peptone, 1 g of K2HPO4, 0.5 g of MgSO4.7 H2O, 15 g of agar, 1 g of PCNB (Terraclor 75% WP) (pentacloronitrobenzene)). Petri dishes containing kernels were incubated at 25 °C in the dark for 5–7 days. All the developed cultures from the kernels were transferred to potato dextrose agar (PDA) using a single-spore technique (Leslie and Summerell, 2006) and incubated at 25 °C for 7 days. Morphological and molecular identification was done based on methods previously used by Fallahi *et al*., (2019).

## Mycotoxins analysis

### *In vitro* mycotoxin production and toxicity

Selected 57 *Fusarium* isolates, representative of the *Fusarium* species belonging to *F.proliferatum* (26 isolates), *F. subglutinans* (three isolates), *F. temperatum* (one isolates), *Fusarium incarnatum-equiseti* species complex (FIESC) (21 isolates), *F. brachygibbosum* (one isolates), *F.redolens* (5 isolates) were examined for mycotoxin production.

For mycotoxin production assays, we used 30 g rice in PYREX Glass Erlenmeyer Flasks, added with 13.5 ml of distilled water, standing overnight, and then autoclaved at 121 °C for 30 min. The flasks containing autoclaved rice were inoculated with piece of fungal cultures grown on PDA and incubated at 25 °C for 21 days in order to allow fungal development and mycotoxin production. High-performance liquid chromatography (HPLC) was used to detect mycotoxins trichothecenes, ZEN , BEA and ENNs. Standards of these toxins were obtained from Sigma Aldrich (Milan, Italy) and stored at 4° C in darkness.

**Determination Zearalenone (ZEN), beauvericin (BEA), enniatins (ENNA, ENNA1, ENNB and ENNB1) and Moniliformin (MON) production**

ZEN production was analyzed for FIESC (21 isolates) and *F. brachygibbosum* (one strain). In the same way, FIESC (21 isolates), *F. brachygibbosum* (one strain), *F. redolens* (five isolates), *F.subglutinans* (three isolates), and *F.temperatum* (one isolate) were assayed for BEA and ENNs production. As well, production of MON for *F. proliferatum* (26 isolates), *F.incarnatum-equiseti* species complex (21 isolates) and *F.thapsinum* (10 isolates) was analyzed. One gram of inoculated rice culture was used for toxin extraction with 5 mL of methanol/water (70: 30, v/v). Samples were placed for 60 min in an orbital shaker, and then was filtered using Whatman no. 4 filters (Maidstone, UK). The sample (100 µL) was diluted with 900 µL ultrapure water (Millipore, Bedford, MA) and filtered using RC through 0.20 µm regenerated cellulose filter (Phenomenex, Torrance, CA, USA). A volume of 100 µL of extract was injected into HPLC apparatus (Agilent 1260 Series, Agilent Technology, Santa Clara, CA, USA) that equipped with a binary solvent manager. The analytical column was a Gemini (150 x 4.6 mm, 5 µm, Phenomenex) preceded by a Security Guard™ cartridge Gemini (4 x 3 mm, Phenomenex). Retention time of the ZEN was about 7.8 min, 11.4 min for BEA, 9 min for ENN B, 10.3 min for ENN $B_1$, 12 min for ENN $A_1$ and 13 min for ENN A. MON respectively was evaluated according to the analysis method of BEA and ENN with a slight change. The retention time of MON was about 2.7 min.

**Determination of Trichothecenes group A (T-2, HT-2 toxins and Diacetoxyscirpenol (DAS)) and group B (nivalenol (NIV) and deoxynivalenol (DON))**

FIESC (21 isolates) and *F. brachygibbosum* (one isolates) were evaluated for production of trichothecenes group A (T-2, HT-2 toxins and DAS) and group B (NIV and DON). Then 1 g of rice culture that inoculated with fungi was used for extraction of toxin with 5 mL of acetonitrile/water (84:16, v/v) with 1 % of acetic acid by orbital shaking for 2 h. After filtration through Whatman no. 4 filters (Maidstone, UK), 100 µL of sample was diluted with 900 µL ultrapure water. The residue was filtered using RC through 0.20 µm regenerated cellulose filter. Finally, 10 µL of extract was injected into to HPLC apparatus (Agilent 1260 Series) for trichothecenes group A and 50 µL for group B. The analytical column was a ZORBAX Eclipse Plus C18 (50 mm × 2.1 mm i.d., 1.8

μm) for group A and Synergi Hydro-RP 80A (150 x 3 mm, 4 μm, Phenomenex) for group B. The retention time of the HT-2 was about 1.97 min, 4.9 min for T-2, 14.6 min for DAS, 8.2 min for DON and 4.1 min for NIV.

## Results

### Mycotoxin production

The mycotoxin production of isolates is reported in Tables 1 and Table 2. In Table 1 the values of the ENNs and BEA production by *F. subglutinans*, *F. temperatum* and *F. redolens* isolates are reported. As well, In table 2 the values of the Trichothecenes, ZEN, ENNs and BEA production by FIESC and *F. brachygibbosum* isolates are described.

One of three isolates of *F. subglutinans* could produce detectable level of BEA. The only isolate of *F. temperatum* produced 302.1 μg/g of BEA. Two out of five isolates of *F. redolens* produced detectable level of ENN B1. As well, BEA production was detected by four isolates of *F. redolens* varied from 688 μg/g to 7936 μg/g (table 1).

The results showed that only one isolate of FIESC can produce each of T-2, HT-2 toxin and DAS. Three and four isolates of FIESC produced ZEN and BEA respectively. One isolate of *F. brachygibbosum* produced detectable level of DAS and ENN B1. None of these 22 isolates produced NIV, DON, MON, ENN A, ENN A1 and ENN B1 (table 2).

In other part, the moniliformin production of *F. proliferatum* (26 isolates) and *F.thapsinum* (10 isolates) was evaluated and none of them produces this toxin.

**Table 1: Enniatins (ENNs) and beauvericin (BEA) production of *Fusarium subglutinans*, *F. temperatum* and *F. redolens* isolates, obtained from maize kernels in Iran.**

| Species | ITEM code | Location | ENN A (μg/g) | ENN A1 (μg/g) | ENN B (μg/g) | ENN B1 (μg/g) | BEA (μg/g) |
|---|---|---|---|---|---|---|---|
| *F. subglutinans* | ITEM18269 | Storage silo | n.d. | n.d. | n.d. | n.d. | 16.0 |
| | ITEM18270 | Storage silo | n.d. | n.d. | n.d. | n.d. | n.d. |
| | ITEM18271 | Fars | n.d. | n.d. | n.d. | n.d. | n.d. |
| *F. temperatum* | ITEM18272 | Golestan | n.d. | n.d. | n.d. | n.d. | 302.1 |
| *F. redolens* | ITEM18205 | Khuzestan | n.d.[a] | n.d. | n.d. | n.d. | n.d. |
| | ITEM18207 | Khuzestan | n.d. | n.d. | n.d. | 124.2 | 5274.7 |
| | ITEM18208 | Khuzestan | n.d. | n.d. | n.d. | 123.8 | 3756 |
| | ITEM18265 | Esfahan | n.d. | n.d. | n.d. | n.d. | 688.4 |
| | ITEM18266 | Esfahan | n.d. | n.d. | n.d. | n.d. | 7935.7 |

[a]n.d. = not detected

**Table 2: Secondary metabolite production by strains of *Fusarium incarnatum-equiseti* species complex (FIESC) *and F. brachygibbosum* isolated from maize in Iran**.

| ITEM code | Locatio | Species | NIV (µg/g) | DON (µg/g) | T-2 (µg/g) | HT-2 (µg/g) | DAS (µg/g) | ZEN (µg/g) | ENN A (µg/g) | ENN A1 (µg/g) | ENN B (µg/g) | ENN B1 (µg/g) | BEA (µg/g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18181 | Golestan | FIESC[a] | n.d[b]. | n.d[b]. | n.d. | n.d. | n.d. | 175.1 | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18186 | Qazvin | FIESC | n.d. | n.d. | 16.3 | 9.5 | 42.6 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18190 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18195 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18190 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 21.8 |
| 18200 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18206 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18209 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18210 | Fars | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18215 | Fars | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 26.4 |
| 18218 | Ardabil | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18221 | Ardabil | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1028.2 |
| 18223 | Ardabil | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | 5123.9 | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18230 | Golestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 109.6 | 2012.3 |
| 18236 | Golestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18239 | Golestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18261 | Alborz | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18262 | Zanjan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18268 | Lorestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18274 | Khuzestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 44.3 |
| 18275 | Golestan | FIESC | n.d. | n.d. | n.d. | n.d. | n.d. | 6493 | n.d. | n.d. | n.d. | n.d. | n.d. |
| 18211 | Fars | *F. brachygibbosum* | n.d. | n.d. | n.d. | n.d. | 20.3 | n.d. | n.d. | n.d. | 186.6 | n.d. | n.d. |

[a]*Fusarium incarnatum-equiseti* species complex

[b]n.d. = not detected

**Discussion**

Due to the importance of quality and quantity of maize as a significant source in human and animal nutrition, we evaluated the *Fusarium* mycotoxin production in the maize-producing areas in Iran. Most of the studies have emphasized on "traditional" mycotoxins, such as aflatoxins, ochratoxin A and trichothecenes, ZEN and fumonisins. However, *Fusarium* spp. is also capable of producing other toxic secondary metabolites the so-called emerging mycotoxins such as beauvericin, enniatins, and moniliformin. So far, only limited data is available on these metabolites. This is not only due to their late recognition but especially the late understanding of their role as mycotoxins (Jestoi, 2008).

*F. temperatum* was reported from maize in several studies; but formerly, there was no report of occurrence of it on Iranian maize. This species has potential to produce diverse mycotoxins among which beauvericin and enniatins (Wang *et al*., 2014; Varela *et al*., 2013; Summerell and Leslie, 2011). Previous studies (Moretti *et al*., 1995) reported the BEA production by *F. subglutinans* isolates from different geographical areas, but this contrast with further report (Moretti *et al*., 2007) was due to the identification of *F. temperatum* as a different species than *F. subglutinans* meanwhile. Afterwards Moretti *et al*. (2008) and Scauflaire *et al*. (2012) showed that *F. temperatum* and *F. subglutinans* can be distinguished based on the BEA production. In our study the only isolate of *F.temperatum* produced high level of BEA while only one of three isolates of *F.subglutinas* produced low level of this toxin. Certainly being able to analyze more isolates of *F.subglutinans* and *F.temperatum* from Iran, it could be possible to give detailed information about their mycotoxin profile. A species very closely related to *Fusarium fujikuroi* species complex (FFSC) is *Fusarium redolens*, formerly identified as *F. oxysporum*. All *F. redolens* analyzed isolates produced ENNs and high level of BEA. ENN and BEA have demonstrated not only toxicity on human cell lines, but also on other models. BEA is structurally similar to the ENN and BEA, but BEA differs in the nature of the N-methylamino acid. Owing to this difference between BEA and the ENN, their bioactivities are obviously different. BEA has antimicrobial and anti-tumor activities. Also, antibacterial activity and antifungal capacity of ENN were demonstrated (Wang *et al*., 2012; Blesa *et al*., 2012). Based on the *EF-1α* gene sequences, 21 isolates were identified as

belonging to FIESC, a complex of 30 phylogenetic species (O'Donnell *et al*. 2009, 2012) and one isolate as *F. brachygibbosum*, a closely related species. Previously only few studies reported FIESC isolates from maize in, India and Malaysia (Aiyaz *et al*., 2016; Zainudin *et al*., 2011). FIESC reported as specially trichothecenes and a number of other mycotoxins, such as butenolide, beauvericin, equisetin, fusarochromanone and zearalenone producer (Desjardins, 2006). We analyzed the ability of 21 strains of FIESC to produce different mycotoxin and only few isolates of this species could produce each of T-2, HT-2 toxin, DAS, ZEN and BEA with no production for NIV,DON, ENN and MON.

Previous literatures referred to production of MON by *F.proliferatum* and *F.thapsinum* (Leslie *et al*., 2005; Marasas *et al*., 1986) but none of these two species produced MON in this research. The results obtained in the present study showed that, the mycotoxin production of the isolates from different regions was varied and there was no significant relationship between mycotoxins production and geographic origins of isolates.

Although distribution of mycotoxins in different regions depended on several factors such as environmental conditions, endogenous and exogenous factors, the analysis of mycotoxin production in vitro can be useful in forecast the mycotoxins contamination in the field (Ferrigo *et al*., 2016). So, determination the mycotoxin profiles of the *Fusarium* species, as the most common toxigenic species on maize, provide useful elements to evaluate the potential risk of mycotoxin contamination in Iranian maize. These results are beneficial for more efficient management of these pathogens in fields and silos.

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