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Influence of low-temperature stress on the production of zearalenone by three *Fusarium* species in *vitro*

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ABSTRACT

Purpose: Comparison and determination of ZEN production capacity of *F. oxysporum, F. graminearum,* and *F. solani*. The influence of low temperature stress and incubation time on the production of ZEN was also investigated. **Research Method**: Production of ZEN was evaluated by incubating media cultures at a constant temperature of 25°C for a period of 14 days before being incubated at 8°C for 14 days (Low-temperature stress/ LTS). The second set of the samples was incubated at constant 25°C for 4 weeks' time (constant-temperature/ CT). HPLC and a fluorescence detector were employed to measure the concentration of ZEN. The species-specific pair of primers was used to perform a PCR assay on the fungal DNA. **Findings:** The higher levels of ZEN were seen at LTS compared with CT in three *Fusarium* species. The highest ZEN was produced in *F. oxysporum* media cultures. The significant production of ZEN was seen when *F. oxysporum* incubated at LTS compared with CT ($P \leq 0.05$). The highest and the lowest levels of ZEN were seen in *F. oxysporum* and *F. solani* and the difference of ZEN production by them were significant in CT and LTS, respectively ($P \leq 0.05$). ZEN production for the *F. graminearum* was in the intermediate level. Presence of PKS4 gene was confirmed by PCR in these *Fusarium* species. **Limitations**: Higher cost of ZEN detection by HPLC was a limitation. **Originality/Value:** Production of ZEN in LTS and CT by the *Fusarium* evaluated species was confirmed. However, LTS stimulate ZEN production in *Fusarium* media cultures, especially for *F. oxysporum*.

INTRODUCTION

Mycotoxins are worldwide problems that affect animals and humans through contaminating feeds and foods. The mycotoxins produced by *Fusarium* sp. are zearalenone (ZEN), trichothecenes [including deoxynivalenol (DON) and T-2 toxin (T-2)] and fumonisin B1 (FB1) (Antonissen et al., 2015) among which ZEN is a typical field contaminant of crops. ZEN is a non-steroidal estrogenic mycotoxin occurring in feeds and foods such as corn, wheat, barley, sorghum, and oats and contaminated 36% of feed and food ingredients based on a global survey in the period of 2004–2011 (Streit et al., 2013). An important property of ZEN is its thermo-stability during storage, milling, processing and cooking (Döll & Dänicke, 2011). Muthomi et al. (2008) reported the co-occurrence of ZEN, DON and T-2 in about 35% of the wheat samples. The *Fusarium* species inducing head blight which is associated with mycotoxicosis to produce chronic adverse health effects to human and livestock fed on the contaminated wheat products. The quality of animal derived foods as well as the health, production and performance of animals may be compromised by *Fusarium* toxins (Döll & Dänicke, 2011). The complete growth of fungi and mycotoxin production are closely associated with several environmental factors. Various species of *Fusarium* are capable of producing different toxins in different incubation times and temperatures (Kokkonen et al., 2010). The present study was aimed to determine ZEN production capacity of three species of *Fusarium* including *F. oxysporum*, *F. graminearum* and *F. solani*. Moreover, the in vitro effects of low-temperature stress and incubation time on ZEN production were also observed.

MATERIALS AND METHODS

Fungal source

The *F. oxysporum* PTCC 5115 and *F. solani* PTCC 5284 were obtained from the stock collection maintained at the Iranian Research Organization for Science and Technology (IROST) and *F. graminearum* was obtained from Department of Agriculture, Golestan University, Gorgan, Iran. The stock cultures were kept on Sabouraud Dextrose Broth (SDB; Merck®, Germany) at 25 °C at Department of Animal Health Management, School of Veterinary Medicine, Shiraz, Iran.

Preparation of culture media

SDB powder (Merck Company, Germany) was dissolved in distilled water (30 grams in 1000 ml). The solution was autoclaved at 121 ºC for 15 minutes and then was allocated in universal tubes (4 ml each). Each fungus was cultivated in 6 tubes, which were incubated at 25ºC for 2 weeks. Then, three cultures of each fungus were transferred to refrigerator (8 ºC) for 2 weeks and the rest were kept at 25 ºC.

Measuring of ZEN

The cultures of *F. oxysporum*, *F. graminearum* and *F. solani* prepared as above were centrifuged (750g, 10 minutes) and the quantities of ZEN were determined in the supernatant of the cultures with high performance liquid chromatography (HPLC) according to the instruction number 9239 of Iranian National Standards Organization (apparatus: Agilent®, USA). The HPLC system consisted of a pump and a fluorescence detector. ZEN was separated in HPLC column (C_{18} octadecylsilane; 5µm ×10cm ×4.6mm) with a mobile phase of water: methanol: acetonitrile (34:56:10, v/v/v). The fluorescent detection was used at both 275 nm and 450 nm wavelengths to respectively show the excitation and emission. ZEN

retention times with 1 ml/min flow rate were 7–8 minutes. The total run time was 10 minutes. The total recovery of ZEN was 85%.

DNA Extraction

The fungal genomic DNA was extracted and was purified for PCR experiment using a DNA extraction kit (Bioneer®, South Korea). Two hundred milligrams of each sample was processed as recommended by the manufacturers. The quantity of DNA was recorded using ANG 100 spectrophotometer (NanoDrop Technologies®, USA).

Polymerase chain reaction (PCR) assay

The primers (PKS4-PS.1, forward 5'-GTGGGCTTCGCTAGACCGTGAGTT-3' and PKS4-PS.2, reverse 5'-ATGCCCTGATGAAGAGTTTGAT-3') (Genfanavaran, Tehran, Iran) were used to amplify the 16S rRNA gene fragments. 12.5 µL master mix (Amplicon, Fermentas, Iran), 13M of each primer, 2µL of DNA and 8.5 µL of PCR grade water were mixed for PCR reaction (final volume of 25 µL). The PCR condition was as follow: denaturation at 94 °C for 5 min and then 35 cycles of 94 °C for 30 sec., 60 °C for 1 min. and 72 °C for 2 min., followed by a final extension at 72 °C for 9 min. (Lysøe et al., 2006) [by Thermal Cycler-LifePro (Bioer Technology, China)]. The PCR products (400 bp) were separated by electrophoresis in a 1.2% [wt/vol] agarose gel containing Safe Mode DNA stain (SinaClon, Tehran, Iran) to visualized DNA using a UV transilluminator.

Statistical analysis

SPSS (version 21.0) was employed to analyze the data. The differences between the amounts of ZEN (ppb) produced by *Fusarium* species and between two groups of each fungus after incubation were performed using Kruskal–Wallis one-way analysis of variance and Mann-Whitney U Test, respectively. $P \leq 0.05$ was considered as significant. Data were presented as mean±standard deviation of the mean (SD).

RESULTS

The highest amounts of ZEN were detected in *F. oxysporum* cultures. All examined *Fusarium* species produced higher amounts of ZEN under LTS condition compared with CT (Table 1). Significant rises were seen in ZEN production by *F. oxysporum* after transferring of cultures to LTS condition ($P \leq 0.05$; Fig. 1). Incubation of *F. graminearum*, and *F. solani* in LTS condition resulted in the production of numerically higher amounts of ZEN compared with CT condition (Fig. 1). The mean amounts of ZEN produced by *F. oxysporum*, *F. graminearum* and *F. solani* in all replicates are shown in Fig. 2. The highest and the lowest amounts of ZEN were seen in *F. oxysporum* and *F. solani* cultures, respectively; with significant differences between CT and LTS conditions for all examined species ($P \leq 0.05$). The presence of PKS4 gene was confirmed by PCR method in all examined fungi (Fig. 3).

DISCUSSION

An old study has stated that *F. oxysporum* and *F. solani* do not produce ZEN, but *F. graminearum* and *F. culmorum* are capable of producing this toxin (Caldwell et al., 1970). However, the production of ZEN by *F. solani* and *F. oxysporum* was confirmed by Richardson et al. (1985) and Jimenez, et al. (1996), respectively. According to the results of the present study, *F. graminearum*, *F. oxysporum* and *F. solani* all can produce ZEN. In the

study of Beev et al. (2013) while, *F. graminearum* was the main producer of ZEN, 35% of isolates of *F. oxysporum* were able to produce it.

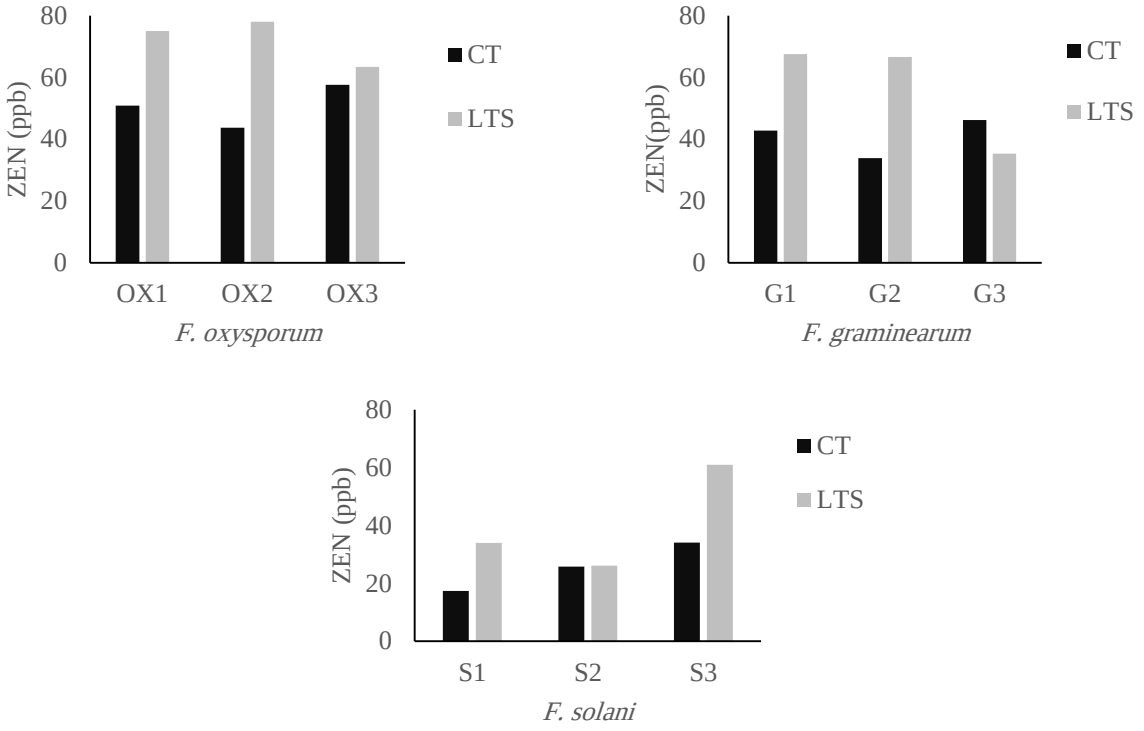

Fig. 1. The amount of ZEN (ppb) produced by *Fusarium oxysporum* (OX), *F. graminearum* (G) and *F. solani* (S) in three replicates; CT (constant temperature, incubation at a constant 25 °C for 4 weeks) LTS (low-temperature stress, incubation at a constant 25 °C for 2 weeks following incubation at 8 °C for 2 weeks).

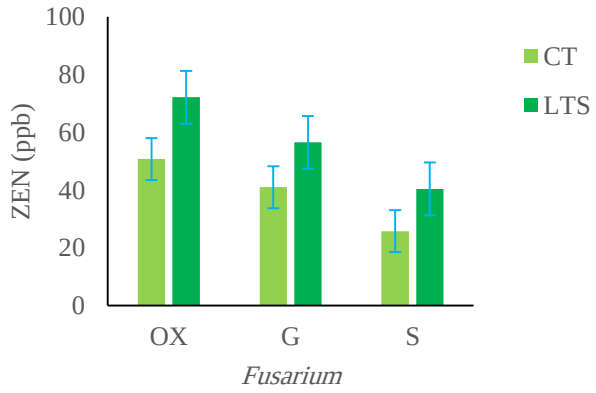

Fig. 2. The mean amount of ZEN (ppb) produced by *Fusarium oxysporum* (OX), *F. graminearum* (G) and *F. solani* (S) in three replicates; Constant temperature (CT): incubation at a constant 25 °C for 4 weeks; Low-temperature stress (LTS): incubation at a constant 25 °C for 2 weeks following incubation at 8 °C for 2 weeks.

Table 1. The amount of ZEN (ppb) produced by *Fusarium* species after incubation under constant temperature condition (CT; 25 °C, 4 weeks) and low-temperature stress (LTS; 25 °C, 2 weeks followed by 8°C for 2 weeks)

Incubation	Replicate	*F. oxysporum*	*F. graminearum*	*F. solani*
CT	1	50.84	42.8	17.39
	2	43.68	33.89	25.8
	3	57.62	46.18	34.1
	Mean±SD	50.71±6.97	40.96±6.35	25.76±8.36
LTS	1	74.99	67.53	34
	2	78.01	66.61	26.12
	3	63.37	35.29	61
	Mean±SD	72.12±7.73	56.48±18.35	40.37±18.29

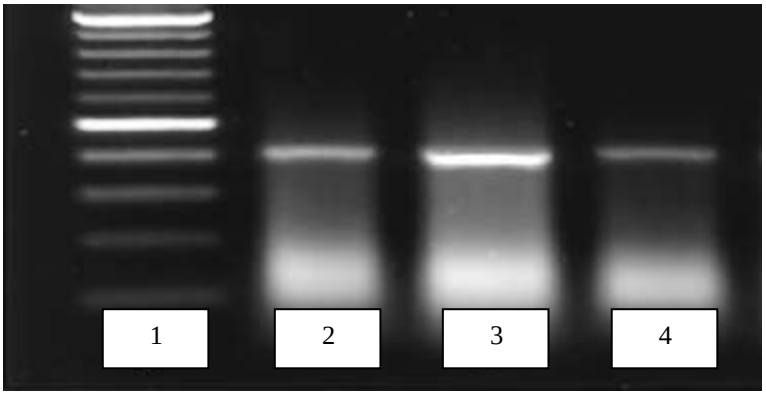

Fig. 3. The bonding shape of PCR; 1) primer bonding, 2) *F. graminearum*, 3) *F. solani*, and 4) *F. oxysporum*.

In the temperate climate zone, *Fusarium* species are important pathogens of maize (Szabó et al., 2016). Production of ZEN on crops under field conditions may be affected by ambient temperature, duration of growth, available substrates and the strain of the fungus. Alternating warm days (20–25 °C) and cool nights (7 °C) during corn maturation and harvesting can stimulate the production of ZEN (Wilson, 2010). The in vitro production of ZEN appears to be influenced by temperature, moisture, pH, oxygen and carbon dioxide concentrations and the size of the culture flask (Greenhalgh et al., 1983). Low temperatures (about 15°C) and alkaline environments can stimulate the ZEN production in vitro (Wu et al., 2017). Caldwell et al. (1970) reported that the ability to produce ZEN by *Fusarium* species was differently related to the isolates of *Fusarium*. Lahouar et al. (2017) found that ZEN production by *F. incarnatum* isolates in sorghum seeds varied from one isolate to the other. *F. culmorum* and *F. graminearum* isolates from wheat, rye and triticale produced ZEN in considerable quantities (Manka et al., 1985). In a study by Molto et al. (1997) 13 out of 27 isolates of *F. graminearum* from Argentinian maize produced ZEN. In vitro production of ZEN was faster on rice medium than on maize or wheat media, but culturing on the rice medium resulted in larger quantities of ZEN (Duverger et al., 2011). Lahouar et al. (2017) did not find any correlation between the growth rate of fungi and production of ZEN. Ryu and Bullerman (1999) studied the effects of LTS on rice cultures of *F. graminearum* and detected the highest amounts of ZEN at 15°C (a 4-week incubation period) after a 2-week incubation at 25 °C. In the study of Martins and Martins (2002), *F. graminearum* cultures produced the highest level of ZEN when they were incubated at 28 °C (16 days) followed by incubation at 12 °C (35 days). Milano and López, (1991), however, reported that incubation of *F. graminearum* and

F. oxysporum at 25°C (4 weeks) enhanced ZEN production, but reducing the temperature to 12–14°C during the succeeding 2 weeks inhibited the toxin production. In the present study, low-temperature stress triggered the production of ZEN. Among the various media cultures, those of *F. oxysporum* produced the highest amounts of ZEN, which could be due to the effect of different conditions, including different *Fusarium* isolates.

In the present study, presence of PKS4 gene, which is necessary for ZEN production (Lysøe et al., 2006) was confirmed by PCR method in all examined *Fusarium* species. Recently, Sim et al. (2018) reported the ability of Multiplex polymerase chain reaction assays (mPCR) for detection of ZEN-production of *Fusarium* species in white and brown rice. It is important that *Fusarium* species have been found to be common among various ruminant feeds. *F. oxysporum* and *F. moniliforme* have been reported as the most prevalent species in dairy cattle feeds with pelleted sugar beet pulp and pelleted malt grains as the most ZEN contaminated feeds (Skrinjar et al., 1995). In addition, ZEN may be present in the straw used as bedding material for animals (Häggblom & Nordkvist, 2015). Additional researches are suggested to study more isolates of *Fusarium* fungi from different regions, crops, granaries and beddings to find the capability of ZEN production by them regarding the main hazards that threaten the food and feed safety.

CONCLUSION

It is concluded that three evaluated *Fusarium* produced ZEN under LTS and CT conditions. However, LTS stimulate ZEN production in *Fusarium* media cultures.

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Conflict of interest

The authors have no conflict of interest to report.

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