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FUMONISINS PRODUCTION POTENTIAL OF *Fusarium verticillioides* ISOLATED FROM SERBIAN MAIZE AND WHEAT KERNELS

ABSTRACT: The production of fumonisins by potentially toxigenic *Fusarium verticillioides* isolates originating from Serbian maize and wheat kernels was tested *in vitro*. A total of six *F. verticillioides* isolates were incubated on yeast extract sucrose medium (YESA) for 4 weeks at 25 °C in the dark. Their toxin production potential was tested by applying a modified HPLC method for determination of fumonisins in cereals, since the TLC method gave no results. Analyses were performed on a HPLC-FLD system after sample extraction from YESA and extract clean-up on a SPE column.

Although the isolates were tested for fumonisin B_1, B_2 and B_3, only fumonisin B_1 was detected. The results showed that all tested isolates had toxigenic potential for fumonisin B_1 production. The average fumonisin B_1 production of the isolates ranged from 7 to 289 µg/kg, thus indicating a highly variable toxigenic potential among the isolates. Isolate 1282 expressed the highest toxigenic potential for fumonisin B_1 production (289 µg/kg), while isolate 2533/A showed a questionable potential for fumonisin production (7 µg/kg).

KEYWORDS: fumonisin, *Fusarium verticillioides*, cereals, toxigenic potential

INTRODUCTION

Cereals are commonly invaded by *Fusarium* spp. and often contaminated by their secondary metabolites that have a major impact on human and animal

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health, welfare and productivity. Among *Fusarium* toxins, zearalenone, fumonisins (FUMs) and trichothecenes are the most toxicologically important and occur most frequently (Cetin and Bullerman, 2005). The most important fumonisins (FUMs) are FB_1, fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3), which are predominantly produced by *F. verticillioides*, *F. proliferatum*, *F. anthophilum*, and *F. nygamai* (Yazar and Omurtag, 2008). FUMs are usually present in maize and maize products, but they also can be found in wheat and non-corn based foodstuffs (Cirillo *et al*. 2003; Kushiro *et al*. 2009).

FUMs are structurally similar to sphingosine, a component of sphingolipids. Sphingolipids are typically found as myelin components. It is believed that FUMs toxicity is a result of sphingolipid biosynthesis blockage. It has been known that FUMs cause leucoencephalomalacia in horses, pulmonary edema in swine, and hepatotoxicity in rats (Gelderblom *et al*. 2001). There is a report about an outbreak of FUMs toxicosis in Serbia (Jovanović *et al*. 2015). In feed samples from the premises, fumonisin B_1 and B_2 were found in the following concentrations: 6.0 mg/kg FB_1 and 2.4 mg/kg FB_2 in the milled maize samples, and 6.05 mg/kg FB1 and 1.68 mg/kg FB_2 in maize grain, respectively. IARC classified FB_1 as a group 2B carcinogen that is possibly carcinogenic to humans (Bray *et al*. 2002).

The occurrence of *F. verticillioides* and other toxigenic *Fusarium* spp. as well as the production of their secondary metabolites (FUMs and other fusariotoxins) are determined by environmental factors in the field, and also by transportation and storage. In wheat harvested in southern Brazil, Mendes *et al*. (2015) found that 54% of the samples were contaminated with FB_1 at levels ranging from 958 to 4,906 μg/kg. Natural FUMs presence in common wheat grains in Argentina was even higher (Cendoya *et al*. 2014). Out of the total number of samples collected during the 2011 harvest, 93% showed FUM contamination with levels ranging from 0.15 to 1,304.39 μg/kg. In 2015, there were five notifications (three in 2014) related to the presence of FUMs in maize and maize products (one of which combined with a high level of aflatoxins) (RASFF, 2016).

Studies in Serbia have shown different results of FB_1 occurrence in wheat among different production years. Stanković *et al*. (2012) reported high incidence (82.1% and 92.0%) and levels (up to 5,400 μg/kg) of FB_1 in wheat kernels from the 2005 and 2007 harvests, while Jakšić *et al*. (2012) indicated significant presence of FB_1 (50.7%), but at lower levels (27–614 μg/kg), in the samples from the 2010 harvest. *F. verticillioides* and *F. proliferatum* isolates originated from wheat kernels had a high FB_1 production potential (Stanković *et al*. 2012).

Maize infections by *Fusarium* species and its contamination with FUMs in Serbia are not so rare. Stanković *et al*. (2011) found a very high occurrence of FB1 (70.7%) in 203 maize samples. However, Krnjaja *et al*. (2011) reported about not so high occurrence of *Fusarium* spp. in two hybrids of maize grains for silage (3.89% and 42.00%). These authors also found that *F. verticillioides* was the dominant mould from *Fusarium* genera. The toxigenic potential of some maize isolates has also been confirmed. Namely, Tančić *et al*. (2012) investigated 16 maize isolates of *F. verticillioides* for the ability to produce FB_1. It was reported that all isolates produced FB_1 in concentrations ranging from 88.60 to 1,300.60 mg/kg.

Hence, the aim of this research was to investigate *in vitro* production of FUMs by *F. verticillioides* isolates that originated from Serbian maize and wheat kernels, by using screening (TLC) and confirmatory (HPLC) analytical methods.

MATERIALS AND METHODS

Isolation of Fusarium verticillioides

Potentially toxigenic *F. verticillioides* cultures were isolated from Serbian maize and wheat kernels collected in the Province of Vojvodina (northern part of Serbia) (Table 1). Coarsely ground kernel samples were placed on DG 18 (dichloran 18% glycerol agar) and incubated at 25 °C for 7 days in the dark. *Fusarium* spp. isolates obtained from growing colonies on DG 18 were transferred individually on PDA (potato dextrose agar) for further purification and identification. Fragments of the colonies developed on PDA were transferred to SNA (synthetic nutrition agar) (Nirenberg and O' Donnell, 1998). Determinations of the fusaria were done according to Nelson *et al*. (1983). The stock cultures of *F. verticillioides* were maintained on PDA at 4–6 °C as a part of the Maize Research Institute "Zemun Polje" collection.

Table 1. Origin of the cereal samples

Ord. No.	Cereal	Locality	Sample designation
1.	Wheat	Despotovac	825
2.	Corn	Zemun Polje	914
3.	Corn	Bačka Topola	1282
4.	Corn	Zemun Polje	2533

Production and TLC analysis of fumonisins

F. verticillioides isolates were cultivated on yeast extract-sucrose agar (YESA – 2% yeast extract, 15% sucrose, and 2% agar, pH 6.5) (Samson and van Reenen-Hoekstra, 1988). The capacity for toxin production of the tested isolates was determined after 28 days of cultivation at 25 °C in the dark according to a rapid screening method (Bočarov-Stančić *et al*. 2009). The agar plugs (diameter 5 mm) were cut out from the colony center with a sterile metal borer, removed from the agar plate and placed with sterile tweezers in a sterilized Petri dish with the mycelial side up. The circular plugs were wetted with 10–20 µl of chloroform/methanol (2:1 v/v) and after few minutes the rapidly extracted mycelial side was gently applied against the TLC plate (Alugram SIL G/UV 254, Macherey-Nagel). After drying the application spot, another agar plug of the same colony was applied nearby, together with 30 µl of the working FB1 standard (100 µg/ml). The thin-layer chromatography was performed in tanks with a saturated toluene-ethyl acetate-formic acid developing solvent

(50+40+10, v/v). After developing plates and air drying in a dark fume extractor, the plates were sprayed with 20% $AlCL_3$, dried in the oven (120 °C) and examined under long wave UV light (366 nm). Three replicates were performed for all analyses.

Extraction of fumonisins from YESA

Reagents

HPLC gradient grade methanol (MeOH) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Reagent solution o-phthaldialdehyde (OPA) for derivatization was prepared as follows: 40 mg of OPA (Sigma-Aldrich, Darmstadt, German) was dissolved in 1 ml of MeOH, diluted with 5 ml of 0.1 mol/l $Na_2B_4O_7$, and finally 50 µL of 2-mercaptoethanol (Serva Electrophoresis, Heidelberg, Germany) was added.

Extraction and Clean-up

A 20 g sample of YESA containing 4-week incubated *F. verticillioides* was extracted using 50 ml of chloroform–methanol (2+1, v/v) mixture. After 30 min extraction on a magnetic stirrer (Automatic Science Instrument, China), the extract was filtered through slow running filter paper (Filtros Anoia, Barcelona, Spain) and evaporated to dryness. The residue was dissolved in 25 ml of solvent MeOH–water (75+25, v/v). Clean-up was done with MycoSep 231 Fum columns (RomerLabs, USA), using 5 ml of the dissolved extract (pH adjusted to 8–9 using 12.5% ammonia solution) purified in the column and 3 ml evaporated to dryness and reconstituted in 200 µl of ACN–H_2O (50+50, v/v). The reconstituted extract was derivatized with OPA at a ratio of 1:1 (v/v), and 5 µl was injected into the HPLC system after 1 min.

Quantification of fumonisins by the modified HPLC method

Analytical Standards

Fumonisin mix solution OEKANAL®, analytical standard 50 µg/ml FB1 and FB2 in ACN–water (50+50, v/v), Sigma-Aldrich Article/Product 34143, and FB3 solution OEKANAL®, analytical standard 50 µg/ml in ACN–water (50+50, v/v), Sigma-Aldrich Article/Product 32606, were used to prepare calibrant solutions in the concentrations of 0.25 to 4.0 µg/ml in ACN–water (50+50, v/v) for each toxin.

HPLC–FLD Analysis

The researchers used HPLC Dionex UltiMate 3000 Series system with FLD 3100 (Thermo Scientific, Germany), consisting of an auto sampler WPS-3000, degasser, quaternary pump, and SupelcosilTMLC-18-DB column (250×4.6 mm, particle size 5 µm; Supelco, USA). The system was controlled by Chromeleon® 7 software (Thermo Scientific). The mobile phase was 0.1 mol/l MeOH –

NaH_2PO_4 (80+20, v/v) adjusted to the pH 3.40 with H_3PO_4, filtered through 0.22 μm membrane filter, at a flow rate of 1.0 ml/min. Wavelength of excitation radiation was 335 nm and emission 440 nm.

RESULTS AND DISCUSSION

TLC screening analysis showed that none of the investigated *F. verticillioides* isolates produced FB_1. Therefore, it can be said that this method was not suitable for testing the potential for the biosynthesis of FUMs by *F. verticillioides* isolates under laboratory conditions, although it has given good results in the case of other fusaria and fusariotoxins (deoxynivalenol, zearalenon, diacetoxyscirpenol and T-2 toxin) (Bočarov-Stančić *et al*. 2009).

However, the HPLC method showed that all the isolates possessed a toxigenic potential for FUM production (Table 2). It is important to emphasize that only FB_1 was detected, although FB_2 and FB_3 were also analyzed.

The average FB_1 production in two wheat isolates were 29 and 61 μg/kg, while in four maize isolates the average FB_1 levels ranged from 7 to 289 μg/kg.

Table 2. Fumonisin production (μg/kg) by *Fusarium verticillioides* isolates

Ord. No.	Isolate designation	Fumonisin B1 (μg/kg)
1.	825/A	29
2.	825/B	61
3.	914	99
4.	1282	289
5.	2533/A	7
6.	2533/B	44

Many isolates of *F. verticillioides* could produce FUMs. It was found by Nelson *et al*. (1991) that Australian isolates produced only trace amounts of FB_1. Nevertheless, Miller *et al*. (1993) discovered medium level (147 mg/kg) producing isolates in Southeast Asia. One of the greatest FB_1 yields (17,900 mg/kg) was reported in South Africa. This toxin yield was obtained from whole maize kernels as a culture material, with *F. verticillioides* MRC 826 as inoculum, incubated at 20 °C in the dark for 13 weeks (Alberts *et al*. 1990).

In Europe, the isolate of *F. proliferatum* from maize investigated for FUMs production biosynthesized much larger quantities of this fusariotoxin (31,000 mg/kg) (Castellá *et al*. 1999).

According to Nelson *et al*. (1991), strains producing less than 50 mg/kg of FB1 are low producers, those producing 50–500 mg/kg are intermediate producers and those producing more than 500 mg/kg are high producers. Regarding our results, it can be concluded that all our investigated isolates of *F. verticillioides* are low FB_1 producers.

Having in mind some previous researches in Serbia, it can be said that Serbian isolates generally showed a significantly less capacity for toxin production than South African or Spanish strains. Namely, maize isolates reported by Tančić *et al*. (2012) produced 88.60–1,300.60 mg/kg of FB_1 on maize grain media, which is a larger magnitude when compared to our results.

CONCLUSION

The fast screening TLC method with cultivation of mycobiota on a YESA medium was not suitable for testing the potential for the biosynthesis of FUMs by *F. verticillioides* isolates under laboratory conditions, probably due to high detection limit.

However, a modified screening method that consisted of the extraction of FUMs from YESA, clean-up of the extract on a SPE column followed by the HPLC-FLD analysis was suitable for detection of FUMs production *in vitro.*

All the tested *F. verticillioides* isolates from maize and wheat kernels biosynthesized FB_1 *in vitro*, although rather low yields of toxin were obtained (7–289 μg/kg).

Future investigations should show whether such low levels of FB_1 are the result of the cultivation procedure or poor extraction efficacy of the modified analytical method.

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REFERENCES

Alberts JF, Gelderblom WCA, Thiel PG, Marasas WFO, Van Schalkwyk DJ, Behrend Y (1990): Effects of temperature and incubation period on the production of fumonisin B_1 by *Fusarium moniliforme*. *Appl. Environ. Microbiol*. 56: 1729–1733.

Bočarov-Stančić AS, Lević TJ, Dimić RG, Stanković ŽS, Salma MN (2009): Investigation of toxigenic potential of fungal species by the use of simple screening method. *Proc. Nat. Sci. Matica Srpska*, 116: 25–32.

Bray F, Sankila R, Ferlay J, Parkin DM (2002): Estimates of cancer incidence and mortality in Europe in 1995. *Eur. J. Cancer* 38: 99–166.

Castellá G, Bragulat MR, Cabañes FJ (1999): Fumonisin production by *Fusarium* species isolated from cereals and feeds in Spain. *J. Food Prot*. 62: 811–813.

Cendoya E, Monge MP, Palacios SA, Chiacchiera SM, Torres AM, Farnochi MC, Ramirez ML (2014): Fumonisin occurrence in naturally contaminated wheat grain harvested in Argentina. *Food Control* 37: 56–61.

Cetin Y, Bullerman LB (2005): Cytotoxicity of *Fusarium* mycotoxins to mammalian cell cultures as determined by the MTT bioassay. *Food Chem. Toxicol*. 43: 755–764.

Cirillo T, Ritieni A, Galvano F, Cocchieri RA (2003): Natural co-occurrence of deoxynivalenol and fumonisins B_1 and B_2 in Italian marketed foodstuffs. *Food. Addit. Contam*. 20: 566–571.

Gelderblom WCA, Abel S, Smuts CM, Marnewick J, Mrasas WFO, Lemmer ER, Ramljak D (2001): Fumonisin-induced hepatocarcinogenesis: Mechanisms related to cancer initiation and promotion. *Environ. Health Perspect*. 109: 291–300.

Jakšić S, Abramović B, Jajić I, Živkov-Baloš M, Mihaljev Ž, Despotović V, Šojić D (2012): Co-occurrence of fumonisins and deoxynivalenol in wheat and maize harvested in Serbia. *Bull. Environ. Contam. Toxicol*. 89: 615–619.

Jovanović M, Trailović D, Kukolj V, Nešić S, Marinković D, Nedeljković-Trailović J, Jakovac Štrajn B, Miličević D (2015): An outbreak of fumonisin toxicosis in horses in Serbia. *World Mycotoxin J*. 8: 387–391.

Krnjaja V, Lević J, Stanković S, Bijelić Z, Mandić, Stojanović LJ (2011): Occurrence of *Fusarium* species in maize grains for silage. *Biotech. Anim. Husbandry* 27: 1235–1240.

Kushiro M, Zheng Y, Nagata R, Nakagawa H, Nagashima H (2009): Limited surveillance of fumonisins in brown rice and wheat harvested in Japan. *J. Food Prot*. 72: 1327–1331.

Mendes RLG, Alves dos Reis T, Correa B, Badiale-Furiong E (2015): Mycobiota and occurrence of fumonisin B_1 in wheat harvested in Southern Brazil. *Cienc. Rural* 45. [Available at: http://dx.doi.org/10.1590/0103-8478cr20140005]

Miller JD, Savard ME, Sibilia A, Rapior S, Hocking AD, Pitt J (1993): Production of fumonisins and fusarins by *Fusarium moniliforme* from Southeast Asia. *Mycologia* 85: 385–391.

Nelson PE, Plattner RD, Shackelford DD, Desjardins AE (1991): Production of fumonisins by *Fusarium moniliforme* strains from various substrates and geographic areas. *Appl. Environ. Microbiol*. 57: 2410– 2412.

Nelson PE, Toussoun TA, Marasas WFO (1983): *Fusarium Species. An Illustrated Manual for Identification*. The Pennsylvania State University Press, University Park and London.

Nirenberg HI, O'Donnell K (1998): New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia* 90: 434–458.

RASFF (2016): The Rapid Alert System for Food and Feed 2015 annual report, In: *RASFF – The Rapid Alert System for Food and Feed online*, May 15, 2017, Available at: [https://ec.europa.eu/food/sites/food/files/safety/docs/rasff_annual_report_2015.pdf]

Samson RA, van Reenen-Hoekstra ES (1988): *Introduction to Foodborne Fungi*. Centralbureau voor Schimmelcultures, Institute of the Royal Netherlands Academy of Art and Sciences, Baarn, Delft.

Stanković S, Lević J, Ivanović D, Krnjaja V, Stanković G, Tančić S (2012): Fumonisin B_1 and its co-occurrence with other fusariotoxins in naturally-contaminated wheat grain. *Food Control* 23: 384–388.

Stanković S, Lević J, Krnjaja V (2011): Fumonisin B_1 in maize, wheat and barley grain in Serbia. *Biotech. Anim. Husbandry* 27: 631–641.

Tančić S, Stanković S, Lević J, Krnjaja V, Vukojević J (2012): Diversity of the *Fusarium verticillioides* and *F. proliferatum* isolates according to their fumonisin B1 production potential and origin. *Genetika* 44: 163–176.

Yazar S, Omurtag G (2008): Fumonisins, trichothecenes and zearalenone in cereals. *Int. J. Mol. Sci*. 9: 2062–2090.

ПОТЕНЦИЈАЛ ЗА ПРОИЗВОДЊУ ФУМОНИЗИНА КОД ИЗОЛАТА *Fusarium verticillioides* СА ЗРНА КУКУРУЗА И ПШЕНИЦЕ ИЗ СРБИЈЕ

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РЕЗИМЕ: Производња фумонизина код потенцијално токсикогених *Fusarium verticillioides* изолата са зрна кукуруза и пшенице пореклом из Србије тестиран је *in vitro*. Укупно шест изолата *F. verticillioides* инкубирано је на агаризованој подлози са екстрактом квасца и сахарозом (YESA) током четири недеље, у мраку на 25 °C. Капацитети одабраних изолата за производњу токсина детектовани су применом модификоване HPLC методе за одређивање фумонизина у зрну житарица, јер брза тријажна TLC метода није дала позитивне резултате. Анализа је изведена на HPLC-FLD систему након екстракције узорка из YESA култура изолата и пречишћавања екстракта на SPE колони. Све анализе су урађене у три понављања. Иако су изолати *F. verticillioides* тестирани на присуство фумонизина $Б_1$, $Б_2$ и $Б_3$, само је фумонизин $Б_1$ био детектован. Резултати су показали да сви тестирани изолати имају потенцијал у синтези фумонизина B_1. Просечна вредност произведеног фумонизина $Б_1$ код изолата *F. verticillioides* кретала се од 7 до 289 μg/kg, што указује на изузетно варијабилан токсигени потенцијал истих изолата. Изолат означен са 1282 показао је највећи потенцијал за биосинтезу фумонизина $Б_1$ (289 μg/kg), док је изолат 2533/A испољио дискутабилан потенцијал за производњу истог фумонизина (7 μg/kg).

КЉУЧНЕ РЕЧИ: фумонизин, *Fusarium verticillioides*, житарице, токсикогени потенцијал