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Original scientific paper

DIVERSITY OF THE *FUSARIUM VERTICILLIOIDES* AND *F. PROLIFERATUM* ISOLATES ACCORDING TO THEIR FUMONISIN B_1 PRODUCTION POTENCIAL AND ORIGIN

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Species of the genus *Fusarium* are characterised by the exceptional intraspecies and interspecies variability in respect to morphological, physiological and genetic properties. Intraspecies and interspecies diversity of *Fusarium verticillioides* and *Fusarium proliferatum* isolates in the production of fumonisin B_1 according to their origin from maize and wheat grains was studied. Fumonisin B_1 production potential of

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investigated 42 isolates was assessed by HPTLC and ELISA method. All 22 and 20 investigated *F. verticillioides* and *F. proliferatum* isolates, respectively, had the ability to produce fumonisin B_1 toxin. Fumonisin B_1 production potential of *F. verticillioides* and *F. proliferatum* varied from 88.60 ppm to 1,300.60 ppm and from 2.37 ppm to 1,246.00 ppm, respectively. According to Mann-Whitnev U test, there were no significant differences between the fumonisin B_1 production mean values of isolates of *F. verticillioides* originated from maize and wheat (628.13 ppm and 696.38 ppm, respectively), as well as, fumonisin B_1 production mean values of *F. proliferatum* isolates (212.32 ppm and 158.07 ppm, respectively). Variability coefficient values indicated that fumonisin B_1 production potential variability was more expressed in maize than wheat originated isolates for both species.

Key words: Fusarium verticillioides, *F. proliferatum*, fumonisin B_1 maize, production variability,wheat

INTRODUCTION

Species of the genus *Fusarium* are characterised by the exceptional intraspecies and interspecies variability in respect to morphological, physiological and genetic properties. Due to their great variability, species from the section *Liseola* (*Fusarium verticillioides* Sheldon – syn. *F. moniliforme* Sheld., *F. subglutinans* (Wollen. and Reink.) Nelson, Toussoun and Marasas, *F. proliferatum* (Matsushima Nirenberg) are well known as pathogens of a great number of agricultural host plants such as maize (LOGRIECO *et al*, 2002; FANDOHAN *et al,* 2003), wheat (BIRZELE *et al*, 2002; LOGRIECO *et al,* 2003; FURLONG *et al*, 2005; KRNJAJA *et al*, 2008), barley (BATATINHA *et al*, 2007; STANKOVIĆ *et al*, 2011), oat (BOTTALICO and PERRONE, 2002), hops (STANKOVIĆ *et al*, 2008a), sorghum (DA SILVA *et al,* 2006), asparagus (STĘPIEŃ *et al,* 2011), rice (DESJARDINS *et al*, 2000), bananas (MARASAS *et al*, 1998), mango (BRITZ et al, 2002), pineapple (STĘPIEŃ *et a*l, 2011), etc. Although they are cosmopolites, some species are more frequent in certain climatic regions and certain host plants. They cause root, stem and ear rot with reductions in crop yields estimated between 10% and 30% in Europe (LOGRIECO *et al*, 2002).

Additionally to their economic importance, these species are capable to produce mycotoxins in pre-harvest infected plants or in stored grains. Fumonisins are a group of 15 structurally related mycotoxins, and three of them (FB_1, FB_2 and FB_3) can cause a serious health problem in animals such as equine leukoencephalomalacia (ELEM), pulmonary edema in swine, and liver or kidney cancer in rats or mice. Fumonisins are also associated with human esophageal cancer (DESJARDINS, 2006).

The FB_1 concentration depends on the origin of isolates, including a host plant and a geographic region. Under laboratory conditions, isolates of *F. verticillioides* and *F. proliferatum*, derived from maize, synthesise more FB_1 (average 1259 ppm) than the isolates originating from grains of wheat (average 769 ppm) or barley (average 320 ppm) (VISCONTI *et al*, 1994). NELSON *et al*. (1991) tested strains of *F. verticillioides* from various substrates and geographic areas for FB_1 production and found that the most strains from maize-based feed (16/20) associated with leukoencephalomalacia were high producers, whereas among the strains isolated from millet and sorghum grain, were few producers (4/15). The strains from maize from Nepal had one high producer (1/10), and the most strains from maize of good quality intended for feed of poultry (8/9) and several strains from human tissue (9/13) were mainly high producers. Isolates from sorghum grain and trees, maize, sugar cane and from the Australian soil did not produce fumonisins (0/10).

Fungi produce fumonisin B_1 in the highest concentrations when grain moisture ranges from 27.0 to 32.0% (BARS et al, 1994). High concentrations of fumonisin B_1 (10.242 ppm) and B_2 (3.068 ppm) were obtained when *F. verticillioides* had been cultivated on the maize substrate, rice medium (0.206 ppm FB_1, 0.100 ppm FB_2, respectively), and on the peanut and soybean substrate ($\leq$ 0.05 ppm) (HOLCOMB *et al,* 1993).

The most frequent species in Serbia identified on maize kernels were *F. graminearum* Schwabe, *F. verticillioides* and *F. subglutinans*, while on wheat kernels were *F. graminearum*, *F. poae* (Peck) Wollen. and *F. proliferatum* (LEVIĆ *et al*, 2001; BOČAROV-STANČIĆ *et al*, 2000). Although the frequency of FB_1 producers were determined on maize and wheat kernels in Serbia, a few studies had been carried out with the aim to observe their diversity in the FB_1 production (ĐILAS *et al*, 2003; STANKOVIĆ *et al,* 2008c; STANKOVIĆ *et al*, 2008b).

The aim of our research was to determine genetic FB_1 production potential under the laboratory conditions of 42 *F. proliferatum* and *F. verticillioides* isolates in relation to their origin. This paper reports the intra- and interspecies variability in FB_1 production potential of *F. proliferatum* and *F. verticillioides* isolates originated from maize and wheat kernels collected in 10 different locations in Serbia during the period 2005-2007.

MATERIALS AND METHODS

Plant host samples

With the aim to obtain *F. verticillioides* and *F. proliferatum* isolates for this study, samples of maize and wheat kernels were collected during the 2005, 2006 and 2007 from Northern (Bačka Topola, Kikinda, Kovin, Novi Sad, Sombor, Sremska Mitrovica) and Central/Southern Serbia (Kraljevo, Loznica, Niš, and Šabac).

Wheat samples were based on a few smaller samples collected from different parts of stored kernels one month after the harvest in 2005 and 2006. Thirty two kernels from each subsample (four subsamples per location) were incubated at water agar (WA) in order to analyse diversity of *Fusarium* species.

Ten maize ears with *Fusarium* symptoms, collected diagonally from each field in 22 sites of 10 locations during the 2006 and 2007, were taken as a basic sample. Ten kernels were taken as subsamples from each ear (100 kernels per sample) and they were incubated at potato dextrose agar (PDA) for diversity analyses of *Fusarium* species.

Fungal isolates

For the fungal identification, 128 wheat kernels (32 per replication) and 100 maize kernels (25 per replication) of each sample were incubated for seven days on water agar (WA) and potato dextrose agar (PDA), respectively. *Fusarium* species were identified according to LESLIE and SUMMERELL (2006). All isolated *Fusarium* cultures were refined to monosporous isolates, and 22 isolates of *F. verticillioides* and 20 isolates of *F. proliferatum* were selected for further toxicological investigations analyses.

Sample preparation for fumonisin assessment

Fifty grams of maize kernels were soaked in sterile distilled water to reach 40% of moisture. After 24 hours, maize kernels were autoclaved for 30 min. Sterilised maize kernels were inoculated with 3-5 plugs (5x5 mm) of monosporous *F. verticillioides* or *F. proliferatum* isolates previously cultivated for seven days on PDA at 25°C in the dark. Inoculated flasks were incubated for 2-3 weeks at 27°C in the dark, occasionally shaken to prevent sample conglomeration and unequal mycelial growth, which could reduce fumonisin production. After incubation, infected maize kernels were dried at 50°C for 3 days and milled to powder, which was kept in plastic bags at 4°C till further fumonisin extraction.

Toxicology assessment

The FB_1 production potential of tested isolates was assessed by two methods: High Performance Thin Layer Chromatography (HPTLC) according to LOGRIECO et al. (1995) and Enzyme-linked immunosorbent assay (ELISA). The HPTLC method had three phases: preparation of samples, fumonisin extraction and purification, and HPTLC fumonisin detection. For fumonisin extraction, 70% methanol was used. Powdered samples and methanol (1:10) were blended for 3 min (1300 rpm) and filtered through Wathman filter paper No 1. Filtrates (10 ml) were purified by Bonded Phase Sax Columns and appropriate solvents in Solid-Phase Extraction Manifold. Tubes with extracted fumonisin were then evaporated in the vacuum evaporator at 50°C till the appearance of a dry residue at the bottom of the tube. The fumonisin dry residue was resolved in 1 ml of methanol, and 5 µl of a sample was used for the further fumonisin detection. HPTLC plates 20x10 cm Kiesgel 60 F254 MERC with the appropriate solvent, development solution and FB_1 standard, were used for fumonisin detection in samples.

The ELISA method also encompassed three phases: sample preparation, ELISA assay procedure and ELISA fumonisin detection. The ELISA assay procedure was performed according to CELER FUMO (code MF101) Test Kit Manual, while the ELISA fumonisin detection was done at 450 nm (Labsystems MultiScan® MCC/340).

Statistical analyses

Basic statistic analyses (mean values, variability coefficient, correlation between HPTLC and ELISA data) were done using software Statistica 7.0 (StatSoft) and significant differences in mean values of the FB_1 production were ascertained by the Mann-Whitnev U test.

RESULTS AND DISCUSSION

Due to the application of two different methods for the quantitative analysis of FB_1 (HPTLC and ELISA), the positive correlation between these methods ($p<0.05$) was established by the statistical analysis. Coefficients of correlation between the HPTLC and ELISA were $r = 0.9633$ and $r = 0.9697$ for FB_1 production by *F. verticillioides* and *F. proliferatum*, respectively. The application of the ELISA method provided a greater reliability and preciseness, and the data obtained by ELISA were used for further analyses.

Table 1. FB_1 production by F. verticillioides isolates originating from wheat and maize grains

MRIZP Isolate Code	Origin of isolates			FB_1 analysis (ppm)	
	Host plant	Location	Year	HPTLC	ELISA
1280	Maize	B. Topola	2006	1200	934.80
1576	Maize	B. Topola	2007	1000	1271.30
611	Maize	Kikinda	2006	100	89.40
1583	Maize	Kikinda	2007	200	206.90
1618	Maize	Kovin	2007	1000	1277.90
1558	Maize	Novi Sad	2007	100	88.60
1594	Maize	Sombor	2007	1000	1300.60
1278	Maize	S. Mitrovica	2006	200	210.53
1624	Maize	S. Mitrovica	2007	400	450.41
1411	Maize	Kraljevo	2006	200	129.00
1654	Maize	Kraljevo	2007	1000	1143.00
1651	Maize	Loznica	2007	100	88.87
1320	Maize	Niš	2006	1000	1141.60
1634	Maize	Niš	2007	1000	1141.60
1379	Maize	Šabac	2006	200	197.20
1612	Maize	Šabac	2007	400	378.40
Average				568.75	628.13
881	Wheat	Novi Sad	2005	600	659.00
675	Wheat	Sombor	2005	600	578.90
894	Wheat	S. Mitrovica	2005	400	456.70
1006	Wheat	S. Mitrovica	2006	800	728.20
1221	Wheat	Kraljevo	2006	800	882.00
804	Wheat	Šabac	2005	800	873.50
Average				666.75	696.38
Total average				617.71	662.25

Based on the ELISA test all 22 investigated isolates of *F. verticillioides* had the ability to produce toxin FB_1, but in different concentrations (Table 1). In general, the FB_1 production by this fungus varied from 88.60 ppm (MRIZP 1558) to 1,300.6 ppm (MRIZP 1594) and averaged to 662.26 ppm. Isolates of *F. verticillioides* originating from maize in 2007 were recorded in five out of the ten sites and produced more FB_1 toxin than isolates from the same location in 2006. Exceptionally, isolates originating from the location of Niš produced the same amount of this mycotoxin (1141.60 ppm) during the period of two years.

F. verticillioides isolates tested in this study had an average or high FB_1 production potential. Moreover, the highest concentration obtained in this study was lower than highest concentrations obtained in other studies, such as 4,500 ppm (STĘPIEŃ *et al,* 2011), 4,000 ppm (STANKOVIĆ *et al*, 2008b) and 3,800 ppm (LOGRIECO *et al*, 2003). All produced FB_1 concentrations in this study were in the range of FB_1 production isolates tested by all above mentioned authors, and they were the closest to the FB_1 production potential of isolates originating from maize investigated in Croatia (CVETNIĆ *et al,* 2005). The similarity between fungal species presence in maize in Serbia and Croatia, and the similarity of their mycotoxin production abilities can be explained by the influence of the similar agroecological conditions in these two neighbouring countries. Isolates with the lowest FB_1 production in this study were within the range of FB_1 concentrations reported by PLATTNER *et al.* (1990).

According to the Mann-Whitnev U test, there were no significant differences between the FB_1 production mean values of the maize and wheat originating isolates of *F. verticillioides* (628.13 ppm and 696.38 ppm, respectively). However, intraspecies variability of FB_1 production potential by *F. verticillioides* was more expressed in maize than in wheat originating isolates (Figure 1). This fact was also confirmed by C_V values (variability coefficients), which were 81.5% and 23.9% for *F. verticillioides* isolates originating from maize and wheat, respectively. DESJARDINS *et al*. (1992) also concluded that the coefficient of variation for fumonisin production on maize kernels by *F. verticillioides* isolates was high.

Isolates of *F. proliferatum* produced FB_1 toxin in a range of 2.3 ppm (MRIZP 1322) to 1,246.00 ppm (MRIZP 1345) on the average 185.20 ppm (Table 2). In four cases, *F. proliferatum* isolates originating from maize biosyntetised a higher amount of the toxin in 2006 than the isolates originating from the same location in 2007.

F. proliferatum isolates tested in this research predominantly had the average FB_1 production potential, and a maximally produced concentration was lower than the highest concentrations reported by other authors - 1,670 ppm (NELSON *et al*, 1993), 2,250 ppm (LOGRIECO *et al,* 1995), 2,500 ppm (LOGRIECO *et al*, 2003), 1,820 – 2,419 ppm (STĘPIEŃ *et al*, 2011), but within the range of FB_1 concentrations produced by isolates tested by all above mentioned authors.

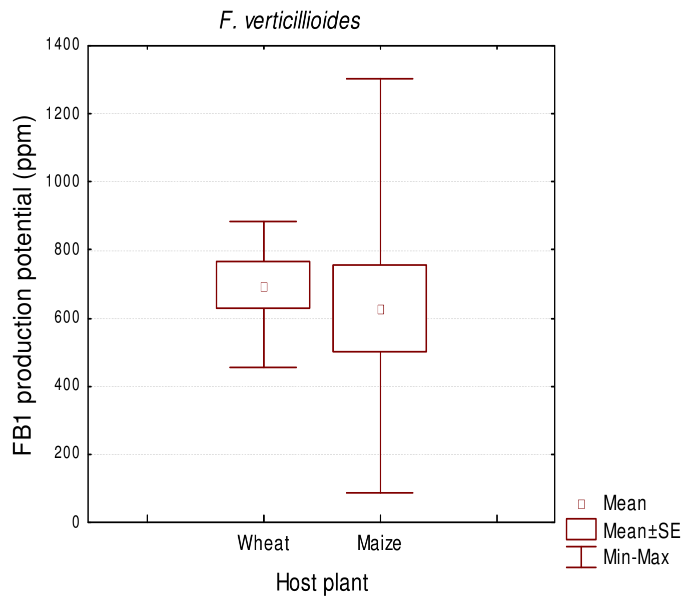

Figure 1. The *F. verticillioides* isolates variability in the FB_1 production according to the host plant origin

There were no significant differences between the FB_1 production mean values of the maize and wheat originating isolates of *F. proliferatum* based on the Mann-Whitnev U test (212.32 ppm and 158.07 ppm, respectively). Nevertheless, greater intraspecies variability in the FB_1 production was found between isolates of *F. proliferatum* originating from maize than those from wheat (Figure 2). These results are consistent with the C_V values, which amounted to 153.5% and 75.1% for *F. proliferatum* isolates originating from maize and wheat, respectively.

According to the Mann-Whitnev U test, there were no significant differences between FB_1 production mean values of maize and wheat originating isolates of both species, but higher variability coefficients values of maize originating isolates indicate that maize is a more suitable host plant for expression of diversity in the FB_1 production than wheat. In Brazil, researchers also detected maize as a more favourable host-plant during the analysing strains isolated from maize and sorghum in relation to their production of fumonisins (DA SILVA *et al,* 2006). In their research, maize originating strains demonstrated high levels of fumonisins, whereas the levels for sorghum originating strains were found to be low.

Table 2. FB_1 production by F. proliferatum isolates originating from wheat and maize grains

MRIZP Isolate Code	Origin of isolates			FB_1 analysis (ppm)	
	Host plant	Localition	Year	HPTLC	ELISA
1574	Maize	B. Topola	2007	100	109 .90
1584	Maize	Kikinda	2007	200	205 .80
1616	Maize	Kovin	2007	100	138.5
1345	Maize	Novi Sad	2006	1000	1246 .00
1555	Maize	Novi Sad	2007	200	141 .99
1275	Maize	Sombor	2006	200	111 .20
1598	Maize	Sombor	2007	100	109 .90
1653	Maize	Kraljevo	2007	trace	67 .80
1248	Maize	Loznica	2006	200	219 .50
1641	Maize	Loznica	2007	100	128 .10
1322	Maize	Niš	2006	trace	2 .37
1374	Maize	Šabac	2006	200	128 .50
1609	Maize	Šabac	2007	100	76 .80
Average				200.00	206.64
1432	Wheat	B. Topola	2006	trace	20 .01
721	Wheat	Kikinda	2005	200	118 .70
1336	Wheat	Kovin	2006	100	98 .30
847	Wheat	Šabac	2005	200	260 .20
674	Wheat	Sombor	2005	200	100 .20
899	Wheat	S. Mitrovica	2005	200	136 .31
1340	Wheat	Loznica	2006	400	372 .80
Average				216.67	158.07
Total average				208.34	185.20

Table 3 shows the frequency of *F. verticillioides* and *F. proliferatum* isolates depending on the host plant origin and the level of FB_1 toxin production. The largest number of *F. verticillioides* isolates originating from the maize produced medium (40.9% of isolates) to high (31.8% of isolates) levels of FB_1. The largest number of *F. proliferatum* isolates originating from both maize (55.0%) and wheat (30.0%) produced a medium level of the FB_1 toxin. No isolate of *F. proliferatum* originating from wheat produced high levels of toxin.

Figure 2. The *F. proliferatum* isolates variability in the FB_1 production according to the host plant origin

Table 3. Categorisation of tested isolates according to the origin and the level of the FB_1 production

Host Plant	Total no. of isolates per year			FB_1 production (ppm)*					
				No of isolates			% of isolates		
	2005	**2006**	**2007**	**Low** (<50)	**Medium** (50-500)	**High** (>500)	**Low** (<50)	**Medium** (50-500)	**High** (>500)
***F. verticillioides* isolates**									
Wheat	4	2	-	0	1	5	0.0	4.5	22.7
Maize	-	6	10	0	9	7	0.0	40.9	31.8
***F. proliferatum* isolates**									
Wheat	4	3	-	1	6	0	5.0	30.0	0.0
Maize	-	5	8	1	11	1	5.0	55.0	5.0

*Tested isolates were divided into three groups according to Nelson *et al*. (1993)

Assessment of interspecies variability accordingly the FB_1 production potential in this study indicated that in general *F. verticillioides* isolates had a greater potential for the FB_1 production than *F. proliferatum* isolates, which was also reported by other authors (ACUÑA *et al,* 2005; LOGRIECO *et al*, 2003).

Both *F. verticillioides* and *F. proliferatum* isolates had a higher frequency in maize than in wheat kernels. Previous studies performed by LOGRIECO *et al.* (2003) indicated a greater variability of *Fusarium* species isolated from wheat than those isolated from maize kernels, which is in agreement with studies performed in Serbia (TANČIĆ, 2009; LEVIĆ *et al*, 2008; KRNJAJA *et al*, 2008; LEVIĆ *et al*, 2004). The diversity and incidence of *Fusarium* species can be influenced not only by abiotic environmental factors but also by a host-plant. It could be considered that the presence of *F. verticillioides* and *F. proliferatum* during two years of the *Fusarium* diversity analysis on wheat kernels was a result of the environmental conditions × host plants interaction.

In this study, the FB_1 potential production of two species was analysed under optimal conditions, which may not mean that these isolates would achieve the maximum of their genetic potential under the environmental conditions. DESJARDINS *et al.* (1992) started genetic examination of the fumonisin biosynthetic pathway and studied the heritability of fumonisin production in mating population A (*F. verticillioides*), and their results indicated that variation in fumonisin levels was largely due to analytical or environmental variables. Also, these authors proved that the group of seedlings treated with fumonisin-producing progeny was significantly different from control seedlings and from the group of seedlings treated with fumonisin-nonproducing progeny in seedling emergence and in shoot length. This indicated that fumonisin plays a role in virulence but also indicated that fumonisin production is not necessary or sufficient for virulence on maize seedlings. Furthermore, if we consider the fact that the isolate MRIZP 1594 in this research had a potential to produce 1,300.6 ppm $FB_{1,}$ the potential risk exists. The development of a molecular genetic analysis and genetic manipulation is a potentially powerful technique for identifying the fumonisin biosynthetic pathway and for determining the relationship between fumonisin production and the ability to cause animal and plant diseases.

CONCLUSION

Obtained results indicate that the origin of the isolates of *F. verticillioides* and *F. proliferatum* affect their potential for FB_1 biosynthesis. Namely, intraspecies variability for the mycotoxins production was greater in isolates originating from maize grain in comparison to isolates of these species from wheat grain. Interspecies variability according to the FB_1 production potential indicated that *F. verticillioides* isolates had a greater potential for the FB_1 production than *F. proliferatum* isolates. A year of the strains isolation had a distinct effect on the toxin production by *F. verticillioides* and *F. proliferatum* isolates.

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DIVERZITET *FUSARIUM VERTICILLIOIDES* I *F. PROLIFERATUM* IZOLATA U POTENCIJALU SINTEZE FUMONIZINA B_1 ZAVISNO OD POREKLA

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I z v o d

Vrste roda *Fusarium* karakteriše izuzetna intraspecijska i interspecijska varijabilnost u pogledu morfoloških, fizioloških i genetičkih svojstava. Proučavan je intraspecijski i interspecijski diverzitet izolata vrsta *Fusarium verticillioides* i *Fusarium proliferatum* u produkciji fumonizina B_1 zavisno od njihovog porekla sa zrna kukuruza i pšenice. Potencijal sinteze fumonizina B_1 kod 42 izolata je ispitivan HPTLC i ELISA metodom. Svih 22 izolata vrste *F. verticillioides* i 20 izolata vrste *F. proliferatum* su imali sposobnost stvaranja toksina fumonizina B_1. Potencijal za sintezu fumonizina je varirao od 88.60 ppm do 1,300.60 ppm kod izolata vrste *F. verticillioides* i od 2.37 ppm do 1,246.00 ppm kod *F. proliferatum* izolata. Prema Mann-Whitnev U testu nije bilo statistički značajnih razlika srednjih vrednosti sintetisanog FB_1 između *F. verticillioides* izolata poreklom sa kukuruza (628.13 ppm) i pšenice (696.38 ppm), kao ni kod *F. proliferatum* izolata poreklom sa kukuruza (212.32 ppm) i pšenice (158.07 ppm). Visoke vrednosti koeficijenta varijacije ukazuju da je varijabilnost u potencijalu sinteze fumonizina B_1 bila više izražena kod izolata poreklom sa kukuruza nego pšenice kod obe ispitivane vrste.

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