FUNGAL AND MYCOTOXIN CONTAMINATION OF MAIZE HYBRIDS IN DIFFERENT MATURITY GROUPS

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Abstract: In the present study, the frequency of toxigenic fungi and occurrence of aflatoxin B_1 (AFB_1), deoxynivalenol (DON) and total fumonisins (FBs) in the kernels of six maize hybrids from different FAO maturity groups (ZP 341, ZP 427, ZP 434, ZP 560, ZP 606, ZP 666) in three localities (Belosavci, Lađevci and Divci) in Serbia, during the harvest in 2013, was investigated. Using standard mycological tests of maize kernels, the presence of potentially toxigenic fungi species from the genera *Aspergillus, Fusarium* and *Penicillium* was found. In the studied localities, species *Fusarium verticillioides* was the most frequently isolated from the most hybrids, with a maximum frequency of 30%, while the presence of *Aspergillus* spp. ranged from 0 to 16%, and *Penicillium* spp. from 0 to 20%. By applying Immunoadsorbent enzymatic assay (ELISA) the concentrations of AFB_1, DON and FBs were determined in maize. The differences between tested hybrids in the level of mycotoxins in kernels were statistically significant ($P \leq 0.01$) for DON and FBs, but not for the content of AFB_1. Also, the interaction between the hybrid and location was significant ($P \leq 0.01$) for the level of DON and FBs, while there was no statistical significance for the level of AFB_1. Maximum values of AFB_1, DON and FBs level were 1.02 µg kg^{-1} (ZP 427), 12 µg kg^{-1} (ZP 341) and 1528.56 µg kg^{-1} (ZP 427). The concentrations of the tested mycotoxins in kernels did not exceed the maximum allowed limits stipulated by Serbian regulations *(Službeni glasnik RS, 2014)*. Given that agro-ecological conditions in Serbia are favourable for the occurrence of toxigenic fungi and their mycotoxins, it is necessary to exert the control of maize kernels annually, in harvest and postharvest periods.

Key words: toxigenic fungi, mycotoxins, maize hybrids

Introduction

Maize is one of the economically most important cultivated plants in Serbia and around the world and is the main energy source for animal feed. Toxigenic fungal species can develop in maize crops in the field and during storage and contaminate food and/or feed with mycotoxins that exhibit toxic effects in animals and humans *(Biagi, 2009)*. The most important fungal species and mycotoxins in maize are: *Aspergillus flavus* and aflatoxins, *Fusarium verticillioides* and *F. proliferatum* and fumonisins, *F. graminearum* and trichothecenes and zearalenone *(Chulze, 2010)*. Aflatoxin is a problem in many food stuffs, but it is the primary problem in maize. This is because the maize is infected yet in the field which is in relation to external environmental conditions. Contamination of maize kernels depends on the co-existence of sensitivity of hybrids and environmental conditions suitable for fungal infection, development and toxinogenesis *(Blandino et al., 2009)*.

The aflatoxins are a group of closely related, highly toxic, mutagenic and carcinogenic compounds produced primarily by *A. flavus* and *A. parasiticus*. Nearly all strains of *A. parasiticus* are toxigenic, and synthesis of aflatoxins in *A. flavus* varies considerably between strains *(Resanović, 2000)*. Aflatoxin B_1 was the first mycotoxin isolated in feed causing death of 100,000 turkeys in 1960 in England *(Kuhn and Ghannoum, 2003)*. Aflatoxins have been globally established in different types of animal feed, and their amount varies depending on numerous factors. There is a big difference in the frequency of contamination of animal feed in some years. The occurrence of aflatoxin B_1 (AFB_1) in feed for cattle may impair the safety of milk and dairy products, because AFB_1 in food for cattle is transmitted into milk as aflatoxin M_1 (AFM_1). Therefore, many countries have legally permissible limits for AFB_1 in feed and AFM_1 in milk *(Driehuis et al., 2008)*.

Fumonisins are the group of structurally related mycotoxins, which were first isolated from cultures of *F. verticillioides*, one of the most common type of fungus that contaminate maize. Fumonisins are grouped into four series: A, B, C and P. The most widespread and directly related to the pathogenic effects are fumonisins B (FB_1, FB_2, FB_3, FB_4) and A (FA, FA_1 i FA_2) series *(Meronuck and Concibido, 1996)*.

From the group of trichothecenes, deoxynivalenol (DON) is the most common in animal feed (between 20 and 100%) *(Driehuis et al., 2008)*.

In animals and humans mycotoxins cause diseases called mycotoxicoses. Consumption of food and/or feed contaminated with mycotoxins can cause acute and chronic effects that may be teratogenic, carcinogenic, neurotoxic, estrogenic or immunosuppressive in humans and/or animals *(Binder et al., 2007)*.

Because of the potential risk and the inevitable occurrence of toxigenic fungi and their secondary metabolites (mycotoxins) in maize, as one of the most important and the most cultivated cereal in Serbia, the paper presents the results of mycological and mycotoxicological analysis of six maize hybrids obtained from three different locations in Serbia.

Materials and Methods

The incidence of potentially toxigenic fungi species and the natural occurrence of mycotoxins were studied in the samples of maize kernels of six hybrids from different FAO maturity groups (ZP 341, ZP 427, ZP 434, ZP 560, ZP 606, ZP 666), which were collected during the harvest in 2013 originating from three locations in Serbia: Belosavci (near Topola), Lađevci (near Kraljevo) and Divci (near Valjevo). Samples of each hybrid, taken from each location, were homogenized, so that the final sample was about 1 kg. Total of 18 samples were tested, 6 samples per site, 1 sample per hybrid. The samples were stored in a refrigerator at 4°C until analysis. Moisture content of the samples was determined using a laboratory moisture meter (OHAUS MB35, USA).

According to standard mycological methods toxigenic fungal species were isolated. For each sample 25 kernels of maize were analyzed, with four replications. Obtained colonies were purified in the procedure where monospore cultures were obtained and subsequently used to identify *Fusarium* species. Monospore cultures were subcultured on potato dextrose agar (PDA), medium with fragments of sterile carnation leaf (CLA) and the synthetic substrate (SNA). The cultures were incubated in the dark at 25±1°C, and on the CLA and SNA 12 hours in the combined light (fluorescent and ultraviolet light) and at 25±1°C, and 12 hours in the dark at 25±1°C. The characterization of these species was performed according to *Nelson et al. (1983)* and *Burgess et al. (1994).*

The incidence (I) of potentially toxigenic fungi was calculated according to the formula of *Lević et al. (2012):* I (%) = [Number of kernel samples in which a species occurred/Total number of kernel samples] x 100.

To test the presence of aflatoxin B_1 (AFB_1), deoxynivalenol (DON) and total fumonisins (FBs) samples were pulverized in a mill (IKA A11, Germany) to a fine powder. To 5 g of each sample was added 1 g of NaCl and homogenized in 25 ml of 70% methanol for 3 minutes in an orbital shaker (GFL 3015, Germany). Samples were filtered over Whatman 1 filter paper and the resulting filtrate was further analyzed using the competitive ELISA method according to the manufacturer's instructions Celer Tecna® ELISA kits. The limits of detection for AFB_1, DON and FBs were 1 $\mu g\ kg^{-1}$, 40 $\mu g\ kg^{-1}$ and 750 $\mu g\ kg^{-1}$, respectively. Each sample was analyzed in triplicate.

The results were analyzed using ANOVA with STATISTICA (StatSoft version 10). The level of significance was assessed at P ≤ 0.05 and P ≤ 0.01. The significance of the difference between the parameter mean values (means) was estimated using the F test at P ≤ 0.05 level.

Results

In the analyzed samples the kernel moisture content was from 11.21% (ZP 434) to 12.30% (ZP 427), with the average for all hybrids of 11.83% in the locality Belosavci, from 12.25% (ZP 666) to 13.24% (ZP 427) with an average for all hybrids of 12.63% in the locality Lađevci, and from 12.36 % (ZP 666) to 13.60% (ZP 341), with the average for all hybrids of 13.07% in the locality Divci.

Table 1. Incidence (%) of potentially toxigenic fungal species from *Aspergillus*, *Fusarium* and *Penicillium* genera in six tested maize hybrids samples from three locations

Parameters	*Aspergillus* spp.	*Fusarium graminearum*	*Fusarium subglutinans*	*Fusarium verticillioides*	*Penicillium* spp.
Location Belosavci					
ZP 341	6	0	0	0	0
ZP 427	8	0	0	30	4
ZP 434	16	0	2	2	20
ZP 560	10	0	2	20	14
ZP 606	8	0	0	30	12
ZP 666	8	0	0	30	6
Location Lađevci					
ZP 341	0	0	2	6	4
ZP 427	2	0	0	10	0
ZP 434	6	0	0	22	4
ZP 560	0	0	0	0	8
ZP 606	0	0	0	14	0
ZP 666	4	0	6	8	14
Location Divci					
ZP 341	0	0	10	8	10
ZP 427	0	0	6	6	10
ZP 434	0	0	4	14	0
ZP 560	0	10	2	6	16
ZP 606	0	4	0	18	12
ZP 666	0	0	12	16	0

Potentially toxigenic species of the genera *Aspergillus, Fusarium* and *Penicillium* were identified in the most hybrids at all locations, with the exception of the genus *Aspergillus* in locality Divci. *Aspergillus* spp. were isolated from all the hybrids from the site Belosavci with the incidence from 6% (ZP 341) to 16% (ZP 434). In the genus *Fusarium* three species were identified: *F. graminearum, F. verticillioides* and *F. subglutinans*. Among them species *F. verticillioides* was the

most commonly isolated in majority of studied hybrids from all three localities with the highest incidence of 30% in three hybrids (ZP 427, ZP 606 and ZP 666) in the locality Belosavci. Species *F. subglutinans* was the most frequently isolated from the majority of hybrids in the locality Divci with the highest incidence of 12% (ZP 666), as well as the species *F. graminearum* with the highest incidence of 10% (ZP 560). *Penicillium* spp. were isolated with the highest incidence of 20% in ZP 434 in the site Belosavci (Table 1). Representatives of the fungal genera *Alternaria, Rhizopus* and *Nigrospora* were isolated with an average incidence of 15%, 47.56% and 4%, respectively, from all the hybrids in all three considered test sites.

Table 2. Effect of hybrids and locations on AFB$_1$, DON and FBs levels

Factor	AFB$_1$ (μg kg^{-1})	DON (μg kg^{-1})	FBs (μg kg^{-1})
Hybrid effects (A)			
ZP 341	0.95	12[a]	1206[b]
ZP 427	1.02	nd	1528.56[a]
ZP 434	0.90	nd	853.22[c]
ZP 560	0.90	nd	595.67[d]
ZP 606	0.92	nd	657.56[d]
ZP 666	0.96	nd	957.11[c]
F test	ns	**	**
Locations effects (B)			
Belosavci	1.21[a]	nd	930.61[b]
Lađevci	0.85[b]	nd	1094[a]
Divci	0.77[b]	6[a]	874.44[b]
F test	**	**	**
Interactions (F test)			
AB	ns	**	**

nd – not detected; means followed by the same letter within a column are not significantly different by F Test at P≤0.05 level, ** - significant at the 0.01 level of probability, * - significant at the 0.05 level of probability, ns – not statistically significant

In mycotoxicological analysis it was found that the tested hybrids had statistically significant effect (P≤0.01) on the level of FBs and DON mycotoxins, but not on AFB$_1$ level (Table 2). The impact of location was highly statistically significant (P≤0.01) for the levels of all tested mycotoxins. However, there was no statistically significant difference between the tested hybrids in the level of AFB$_1$, while the hybrid ZP 341 statistically significantly differed from other hybrids in the level of DON. In this hybrid, 12 μg kg^{-1} of DON was recorded, while in other maize hybrids DON was not at all detected. The ZP 341 and ZP 427 hybrids were mutually and relative to other tested hybrids significantly different in the level of FBs, while between the hybrids ZP 434 and ZP 666, and ZP 560 and ZP 606 no statistically significant differences in the level of FBs were determined. The highest

level of FBs was 1528.56 μg kg^{-1} (ZP 427) and the lowest 595.67 (ZP 560). In regard to the studied locations, the level of AFB$_1$ was statistically significantly higher in the locality Belosavci, while between the other two sites, no statistically significant differences in the level of AFB$_1$ was established. Likewise, the level of DON was statistically significantly greater in the locality Divci, while on the other two sites DON was not identified. FBs level was significantly higher in the locality Lađevci compared to other test sites, among which there were no statistically significant differences in the level of FBs. *F* values for hybrid x location interaction were statistically highly significant (P≤0.01) for the levels of DON and FBs, and not significant for the level of AFB$_1$ (Table 2).

Maximum levels of studied mycotoxins did not exceed allowed limits stipulated by the Regulation on the amendments of the Regulation on maximum residue levels of plant protection products allowed in food and feedstuffs, and on food and feedstuffs for which the maximum residue levels of plant protection products are determined by *(Službeni glasnik RS, 2014)*.

Discussion

Meteorological data of the Hydro meteorological Service of the Republic Serbia for studied sites in the vicinity of Topola (Belosavci), Kraljevo (Lađevci) and Valjevo (Divci), where the maize hybrids were cultivated, are shown in Table 3. Considering all of the investigated localities, the average daily temperature from 21.9 to 22.8 ºC, relatively low precipitation (23.2 - 50.6 mm) and relative humidity from 60 to 62% in July 2013, were favorable for intensive development of toxigenic *F. verticillioides* species compared to other *Fusarium* spp., and significant production of fumonisins. Minor development of *Aspergillus* and *Penicillium* species as well as mycotoxins AFB$_1$ and DON was observed. Between hybrids, there were no statistically significant differences in the level of AFB$_1$, DON was detected only in hybrid ZP341, and the highest level of FBs was detected in hybrid ZP427. Certain statistically significant differences in the level of mycotoxins between the tested sites were recorded; however, the impact of sites on the content of mycotoxins was not consistent. Thus, in the locality of Belosavci the highest level of AFB$_1$ was found, in the locality Divci the highest level of DON, and in Lađevci the highest level of FBs (Table 2).

Similar to our results, in Northern Italy, *Balconi et al. (2014)* have found a larger number of maize kernels infected with *F. verticillioides* in July 2009 when higher average daily temperatures were recorded (> 20°C) than in July 2010 (<20°C), while the levels of fumonisins (FBs) were similar in both years. Also, in the examination of the fungal and mycotoxin contamination in Bt maize and non-Bt maize grown in Argentina (during 2002-2003 and 2003-2004 harvest seasons), *Barros et al. (2009)* have identified toxigenic species of the genera *Fusarium,*

Penicillium and *Aspergillus* in both Bt and non-Bt maize in seven localities tested. *Fusarium* species were the most common with average values from 58% (Bt maize) to 82% (non-Bt maize). Between *Fusarium* species, *F. verticillioides* was the most frequently isolated species (70% of isolates). According to the same authors the effect of location was very important to the level of fumonisins, the average DON levels did not differ significantly between the sites, and the presence of aflatoxin was not detected in both genotypes in both examined growing seasons. In Poland in three-year studies (2007-2009), that considered the impact of sensitivity between different hybrids (flint and dent hybrids) to the *Fusarium* species, *Wit et al. (2011)* have found that the dent hybrids showed a significantly higher level of infection. Similarly, in Italy *Blandino and Reyner (2008)* have found in the hybrids with harder kernels had less damaged kernels by insects and thus disease development and production of mycotoxins were reduced. In that case contamination of maize with *F. verticillioides* and fumonisins can be related with greater damage incidence by insects, primarily by European corn borer (*Ostrinia nubilalis* Hubn.). Further, *Blandino et al. (2009)* have determined a statistically significant effect of the year (P≤0.05) and agricultural measures (time of sowing, plant density and nitrogen fertilization) (P≤0.01) on the level of FBs in the kernel, while the impact of hybrids from two different FAO maturity groups (400 and 600) was not statistically significant for the level of FBs. During the maize harvest in 2003 in Mexico, *Reyes-Velázquez et al. (2011)* have investigated natural phenomena of *Fusarium* species and their mycotoxins and found that in seven hybrids dominant species was *F. verticillioides* with an incidence from 44 to 80%, but the level of fumonisins (FB_1 and FB_2) (up to 606 and 277 µg kg^{-1}, respectively) was low in all samples of maize.

Table 3. Mean daily temperature (T), total monthly rainfall and mean relative humidity (RH) from June to October 2013 in investigated locations Belosavci (A), Ladevci (B) and Divci (C)

Year 2013	Location								
	A			B			C		
Month	T (ºC)	Rainfall (mm)	RH (%)	T (ºC)	Rainfall (mm)	RH (%)	T (ºC)	Rainfall (mm)	RH (%)
June	19.8	85.4	72	20	96.1	71	20.5	63.9	69
July	21.9	50.6	62	22.5	23.2	60	22.8	44.1	61
August	23.1	50.1	60	23.8	23	58	23.7	21	60
September	16.2	49.6	69	16.5	48.6	68	16.6	54.8	72
October	13.6	41.7	73	13.6	50.7	75	13.5	52	78
Mean temperature (June-October)	18.9			19.2			19.4		
Total rainfall (June-October)		277.4			241.6			235.8	
Mean RH (June-October)			67.2			66.4			68

Production of high quality maize kernels is of primary importance for livestock production because maize is the main component of animal feed. Summary of analysis of annual and multi-years results of the occurrence, the presence and frequency of potentially toxigenic fungi and their mycotoxins in feeds are important primarily due to the implementation of preventive measures, as well as increasing the awareness of the consequences of the harmful effects of these contaminants in the food chain. In order to prevent the biosynthesis of mycotoxins it is necessary to take measures that inhibit the development of fungi in the field. The most important preventive measure is cultivation of resistant genotypes. For this reason, continuous research of the sensitivity of domestic hybrids to toxigenic fungal species is required.

Conclusion

Potentially toxigenic species of fungi of the genera *Aspergillus, Fusarium* and *Penicillium* are identified in the present paper. However, in consideration of all of the test sites, *Fusarium* species were the most frequently isolated with *F. verticillioides* as the most common species in majority of tested hybrids. This points to the fact that environmental conditions in Serbia are very suitable for the development of fusariosis of maize ears and thus for production of fusariotoxins, in this case fumonisins. Low incidence of species *F. graminearum*, is reason why in most hybrids the presence of DON was not detected. In all hybrids and locations the presence of AFB_1 was detected, although the frequency of *Aspergillus* species was not high or was not observed in certain hybrids and not at all in the locality Divci. The interaction between genotype and location was not statistically significant for AFB_1 contamination, but it was statistically significant for DON and FBs contamination.

Although the detected concentrations of mycotoxins did not exceed the maximum residue levels determined by the Regulations of Republic of Serbia, it is necessary to carry out every year mycological and mycotoxicological analysis of maize in order to avoid potential adverse impacts from contaminants (fungi and mycotoxins) on human and animal health.

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Rezime

U radu je ispitivana učestalost potencijalno toksigenih vrsta gljiva i prirodna pojava aflatoksina B_1 (AFB$_1$), deoksinivalenola (DON) i ukupnih fumonizina (FBs) u zrnu šest hibrida kukuruza iz različitih FAO grupa zrenja (ZP 341, ZP 427, ZP 434, ZP 560, ZP 606, ZP 666) u tri lokaliteta (Belosavci, Lađevci i Divci) u Srbiji, tokom berbe u 2013. godini.

Primenom standardnih mikoloških ispitivanja zrna kukuruza ustanovljeno je prisustvo potencijalno toksigenih vrsta gljiva iz tri roda: *Aspergillus*, *Fusarium* i *Penicillium*. U ispitivanim lokalitetima, vrsta *Fusarium verticillioides* je bila najčešće izolovana kod većine hibrida, sa maksimalnom učestalošću od 30%, dok je prisustvo *Aspergillus* spp. bilo od 0 do 16%, a *Penicillium* spp. od 0 do 20%.

Primenom imunoadsorpcione enzimske metode (ELISA) određen je sadržaj AFB$_1$, DON i FBs u zrnu kukuruza. Razlike između ispitivanih hibrida u sadržaju mikotoksina u zrnu bile su statistički značajne (P≤0,01) za DON i FBs, ali ne i za sadržaj AFB$_1$. Isto tako, interakcija između hibrida i lokaliteta je bila značajna (P≤0,01) za sadržaj DON i FBs, dok nije bilo statističke značajnosti za sadržaj AFB$_1$. Maksimalne koncentracije AFB$_1$, DON i FBs bile su 1,02 µg kg^{-1} (ZP 427), 12 µg kg^{-1} (ZP 341) i 1528,56 µg kg^{-1} (ZP 427).

U zrnu kukuruza sadržaj ispitivanih mikotoksina nije premašio maksimalno dozvoljene vrednosti propisane Pravilnikom o maksimalno dozvoljenim količinama ostataka sredstava za zaštitu bilja u hrani i hrani za životinje i o hrani i hrani za životinje za koju se utvrđuju maksimalno dozvoljene količine ostataka sredstava za zaštitu bilja *(Službeni glasnik RS, 2014)*. S obzirom da su agroekološki uslovi u Srbiji povoljni za pojavu toksigenih gljiva i njihovih mikotoksina, neophodno je svake godine vršiti kontrolu zrna kukuruza, kako u žetvenom, tako i u postžetvenom periodu.

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