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NATURAL TOXIGENIC FUNGAL AND MYCOTOXIN OCCURRENCE IN MAIZE HYBRIDS

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Abstract: The objective of the present study was to investigate the susceptibility of maize hybrids to the natural occurrence of toxigenic fungal species, in particular toxigenic *Aspergillus* and *Fusarium* species, and mycotoxins (aflatoxin B_1 (AFB_1), deoxynivalenol (DON) and total fumonisins B_1, B_2 and B_3 (FBs)). Grain samples of six commercial maize hybrids (MAS 34.B – FAO 300, MAS 40.F, MAS 48.L, KWS Konfites and ZP 427 – FAO 400, and MAS 56.A – FAO 500) were collected at harvest in 2018. A total of seven fungal genera, *Acremonium*, *Alternaria*, *Epicoccum*, *Fusarium*, *Nigrospora*, *Penicillium* and *Rhizopus*, were identified of which only species from the genus *Fusarium* were present on maize grains of all hybrids tested. The incidence of *Fusarium* spp. was higher in the hybrids MAS 48.L (54.6%), and MAS 56.A (53.3%), compared to MAS 40.F (37.3%), KWS Konfites (28%), MAS 34.B (22.6%) and ZP 427 (12%) hybrids. Among the identified *Fusarium* species (*F. graminearum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides*), *F. proliferatum* was present in all hybrids, ranging from 9.3% (ZP 427) to 30.7% (MAS 48.L), whereas *F. subglutinans* was present in two hybrids, MAS 40.F (16%) and MAS 56.A (9.3%). The incidence of *F. graminearum* ranged from 0% (KWS Konfites) to 9.3% (MAS 34.B), while the incidence of *F. verticillioides* ranged from 0% (MAS 34.B and ZP 427) to 21.3% (MAS 48.L). In the samples, *Aspergillus* species were not identified. The effect of maize hybrids was significant on the level of mycotoxins. MAS 34.B hybrid had a statistically significantly higher levels of AFB_1 and DON than other hybrids. The FBs level was the highest in the hybrid MAS 34.B (1202 μg kg^{-1}) and the lowest in the hybrid KWS Konfites (88.33 μg kg^{-1}). However, the FBs level did not differ between hybrids MAS 34.B, MAS 40.F, and MAS 56.L, MAS 40.F, MAS 48.L, and MAS 56.A, and KWS Konfites and ZP 427. In all hybrids, AFB_1, DON, and FBs levels were below the maximum permissible levels stipulated by the legislation of the European Union and the Republic of Serbia in unprocessed maize.

The agro-ecological conditions in 2018 favored the development of *Fusarium* species on maize grains of the hybrids tested, especially fumonisin producing species.

Key words: toxigenic fungal species, mycotoxins, maize hybrids

Introduction

Maize is the main cereal crop in Serbia, grown on about one million hectares (*Statistical Yearbook of the Republic of Serbia, 2019*). It is used for human and animal nutrition and industrial processing. However, under stress abiotic and biotic factors, the maize grains can be infected by mycotoxigenic fungi. The most common mycotoxigenic fungi isolated from harvested and stored maize grains belong species from the genera *Fusarium*, *Aspergillus* and *Penicillium* (*Krnjaja et al., 2015*). These fungal species produce secondary metabolites (mycotoxins) which cause adverse effects on animal and human health, and economic losses. In particular, species from genera *Aspergillus* and *Fusarium*, causers of ear rot, cause serious risk of mycotoxin accumulation in maize (*Masiello et al., 2019*). Recently, aflatoxins, which are secondary metabolites produced by *Aspergillus flavus* Link and *A. parasiticus* Speare have been isolated in high levels on the maize grains in Serbia (*Kos et al., 2018; Obradović et al., 2018*). Also, mycotoxins such as type B trichothecenes (deoxynivalenol) and zearalenone produced primarily of *Fusarium graminearum* Schwabe and fumonisins produced mainly of *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsush.) Nirenberg have been detected on maize grains in Serbia (*Jajić et al., 2008; Krnjaja et al., 2015; Obradović et al., 2018; Jakšić et al., 2019*).

Farm animals are sensitive to higher mycotoxin levels. Among the four main aflatoxins (B_1, B_2, G_1, and G_2), aflatoxin B_1 (AFB_1) is the most toxic and causes liver damage in animals. The toxic effects of deoxynivalenol (DON) on pigs are feed rejection, vomiting, reproductive, and neurological disorders *(Biagi, 2009; Reddy et al., 2017)*. Leukoencephalomalacia of horses and porcine pulmonary edema of pigs are diseases caused by fumonisins B_1, B_2 and B_3 (FBs) *(Dohnal et al., 2010; Leggieri et al., 2015)*.

Maize grains can be contaminated with toxigenic fungi and their mycotoxins before and after harvesting. High temperatures and levels of humidity from silking to maturity stages are favourable conditions for fungal ear colonization by *Fusarium* species and synthesize of *Fusarium* mycotoxins (*Logrieco et al., 2002*), while drier growing seasons suitable for the growth of *Aspergillus* spp. and aflatoxin accumulation in maize crops (*Giorni et al., 2019*). The occurrence of mycotoxins in maize grains is also dependent on hybrid susceptibility (*Blandino et al., 2017*).

The control strategy for the prevention of fungal and mycotoxin contamination of maize grains includes pre- and post-harvest measures. The most important preventive measures in the pre-harvest time are good agricultural crop practices and the utilization of tolerant maize hybrids (*Blandino and Reyneri, 2008*). The drying grains to below 15% moisture content, insect control, the application of detoxification methods are the most common post-harvest measures in storages (*Di Gregorio et al., 2014; Kumar and Kalita, 2017*).

Since the selection and sowing of the tolerant and less susceptibility maize hybrids as one of the success measures in reduce fungal contaminants, the research purpose was to determine the natural occurrence of toxigenic fungal species, in particular, toxigenic *Aspergillus*, and *Fusarium* species, and mycotoxins, AFB_1, DON, and FBs, in five foreign and one domestic maize hybrids in agro-ecological climate conditions in Serbia.

Materials and Methods

Fungal and mycotoxin contamination of maize grains was evaluated in six commercial hybrids. Four French hybrids (MAS 34.B - FAO 300, MAS 40.F and MAS 48.L - FAO 400, and MAS 56.A - FAO 500), one German hybrid (KWS Konfites - FAO 400) and one Serbian hybrid (ZP 427 - FAO 400) were investigated.

Hybrids were grown in 2018 in the experimental field of the Institute for Animal Husbandry, Belgrade-Zemun. The sowing and harvesting date of the hybrids was consistent with the FAO maturity groups. The plot size was 440 m x 50.4 m, sub-plot was 440 m x 8.4 m. Each maize hybrid sown in 12 rows, with a 0.7 m inter-row spacing. Crop densities were in accordance with manufacturers recommendations.

Maize grain samples were collected at harvest time. The sub-plot divided into three parts. A total of 30 ears were randomly taken per each hybrid (sub-plot), 10 ears from each part, then put in the paper bags and transferred to the laboratory. The maize grains of 10 ears (sub-sample) manually removed. A total of 18 maize grain sub-samples, each sub-sample approximately of 500 g weight, were kept at 4°C until analyses.

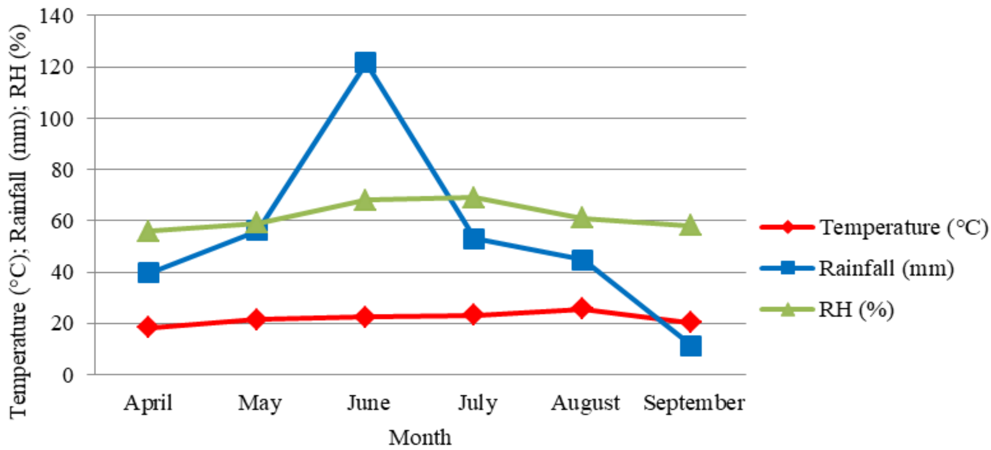
The moisture content of maize sub-samples was determined using OHAUS MB35 (USA) moisture analyser. In mycological analyses, maize grains were disinfected in 1% NaOCl (sodium hypochlorite) for a few minutes, rinsed in distilled water, and dried on the filter paper. Per each hybrid, 300 grains were plated on potato dextrose salt agar, 100 grains per sub-sample, 5 grains per plate (*Krnjaja et al., 2019*). After14 days of keeping plates on the room temperature, fungal species were identified using fungal keys of Leslie and *Summerell (2006)* and *Watanabe (2002)*. The incidence of fungal species on maize grains was calculated according to *Lević et al. (2012)*.

Before the mycotoxicological analysis, ground maize sub-samples dried during 72 h at 60°C. Enzyme-Linked Immunosorbent Assay (ELISA) for determining AFB_1, DON, and FBs levels was applied according to the manufacturer's instructions Celer Tecna® ELISA kits in three repetitions. The limit of detection for AFB_1. DON and FBs were 1 μg kg^{-1}, 40 μg kg^{-1} and 750 μg kg^{-1}, respectively.

Effect of different maize hybrids on the mycotoxin levels was determined by one-way analysis of variance (One-Way ANOVA) using SPSS software (IBM, Statistic 20). Comparing means values with significant differences at $P \leq 0.05$ was done using the Tukey's test. The Pearson correlation coefficient was used in correlation analyses.

Results and Discussion

In a Belgrade area, according to the meteorological data of the Republic Hydro Meteorological Services of Serbia, in 2018, the mean monthly temperatures (> 20°C), total monthly rainfall (> 40 mm) and mean monthly relative humidity (RH) (> 60%) at the flowering stage (July) and milk stage (August) were suitable for fungal maize colonization (Graphic 1).



Graphic 1. Mean monthly temperatures, total monthly rainfall and mean monthly relative humidity (RH) in Belgrade area from April to September in 2018

The average moisture content of maize grain sub-samples was the highest in the hybrids MAS 34.B and MAS 56.A (16.4%), followed by hybrids MAS 48.L

(14.8%), ZP 427 (14.3%), KWS Konfites (13.9%) and MAS 40.F (12.5%) (data not presented).

In the mycological analysis, the seven fungal genera were identified: *Acremonium*, *Alternaria*, *Epicoccum*, *Fusarium*, *Nigrospora*, *Penicillium* and *Rhizopus* (Table 1.) The genus *Fusarium* was present on grains of all maize hybrids. The high incidence of *Fusarium* spp., on the maize grains, was found in the hybrids MAS 48.L (54.6%) and MAS 56.A (53.3%), followed by hybrids MAS 40.F (37.3%), KWS Konfites (28%), MAS 34.B (22.6 %) and ZP 427 (12%). *Aspergillus* species were not identified in maize grain samples. Similar, *Tančić Živanov et al. (2019)* have identified 16 fungal genera, with the *Fusarium* species as the dominant pathogens in two Serbian commercial dent maize hybrids from FAO 600 maturity group, while *Aspergillus* and *Penicillium* species were established in low incidence. Also, in Italy, *Covarelli et al. (2011)* have found that the genus *Fusarium* was the most frequent on maize grains, followed by *Aspergillus* and *Penicillium* genera. Contrary, in Tunisia, the fungal genus *Aspergillus* was the predominant genus in the harvest maize grain samples in 2011 (*Jedidi et al., 2018*).

Table 1. The incidence (%) of fungal genera in maize hybrids tested in 2018

Maize hybrid	Fungal genus						
	Acremonium	*Alternaria*	*Epicoccum*	*Fusarium*	*Nigrospora*	*Penicillium*	*Rhizopus*
MAS 34.B	0	0	13.3	22.6	38.7	0	18.7
MAS 40.F	30.7	4	0	37.3	16	2.7	0
MAS 48.L	0	10.7	0	54.6	1.3	8	2.7
KWS Konfites	18.7	4	0	28	29.3	0	0
ZP 427	1.3	6.7	0	12	13.3	10.7	0
MAS 56.A	0	0	0	53.3	2.7	0	4

Among isolated *Fusarium* species, *F. proliferatum* was identified in all hybrids in range from 9.3% (ZP 427) to 30.7% (MAS 48.L) (Table 2). *F. subglutinans* was identified in MAS 40.F (16%) and MAS 56.A (9.3%) hybrids. The incidence of *F. graminearum* was ranged from 1.3% (MAS 48.L) to 9.3% (MAS 34.B), but not isolated in the KWS Konfites hybrid. The incidence of *F. verticillioides* was ranged from 1.3 (MAS 40.F) to 21.3% (MAS 48.L), but not isolated in the hybrids MAS 34.B and ZP 427. Sterile mycelia was isolated only in two hybrids MAS 34.B (6.7%) and KWS Konfites (2.7%), and from 0% (MAS 34.B) to 56% (ZP 427) grains were without mycelia (data not presented).

The incidence of *Fusarium* species on the maize grains was similar to the reported data of *De Curtis et al. (2011)* and *Covarelli et al. (2011)*. Analyzing the susceptibility of three maize hybrids to *Fusarium* and FBs contamination, in Southern Italy, *De Curtis et al. (2011)* have isolated *F. proliferatum* (up to 81.5%) and *F. verticillioides* (up to 26.5%) as the predominant fungal species in both investigated years (2005-2006). Then, *Covarelli et al. (2011)* were obtained that the *F. verticillioides* was the most predominant species isolated from the maize grains in 2006 (40.2%) and 2007 (65.2%) in central Italy, followed by *F. proliferatum* (up to 7.8%), *F. subglutinans* (up to 5.7%), while *F. graminearum* (2.6%), *F. culmorum* (0.9%), and *F. sporotrichioides* (0.9%) were isolated only in 2006. A recent study of *Tančić Živanov et al. (2019)* demonstrated that 11 *Fusarium* species were isolated from the grains of two commercial maize hybrids grown in Serbia, of which *F. verticilioides, F. graminearum*, and *F. proliferatum* were predominant.

Table 2. The incidence (%) of *Fusarium* species in maize hybrids tested in 2018

Maize hybrid	*Fusarium* spp.			
	F. graminearum	*F. proliferatum*	*F. subglutinans*	*F. verticillioides*
MAS 34.B	9.3	13.3	0	0
MAS 40.F	6.7	13.3	16	1.3
MAS 48.L	1.3	30.7	0	21.3
KWS Konfites	0	14.7	0	13.3
ZP 427	2.7	9.3	0	0
MAS 56.A	6.7	18.7	9.3	18.7

Levels of mycotoxins were significantly affected by hybrids (Table 3). The levels of AFB_1 and DON were significantly higher in the hybrid MAS 34.B compared to other hybrids. AFB_1 and DON levels found were below the detection limits in the hybrids KWS Konfites, ZP 427, and MAS 56.A, and KWS Konfites and MAS 56.A, respectively. There were no significant differences between hybrids MAS 34.B, MAS 40.F, and MAS 56.A, MAS 40.F, MAS 48.L, and MAS 56.A, and KWS Konfites, and ZP 427 for FBs levels. The highest FBs level was found in the hybrid MAS 34.B (1202 μg kg^{-1}), while the lowest FBs level was in the hybrid KWS Konfites (88.33 μg kg^{-1}).

Mycotoxin analyses showed that in the hybrids tested, the mean levels of AFB_1, DON and FBs did not exceed maximum limits of 5, 1750, and 4000 μg kg^{-1}, respectively, prescribed by European Commission (*EC, 2007; 2010*) and Serbian Regulation (*Službeni Glasnik RS, 2014*) for unprocessed maize.

Similar to our results, *Blandino et al. (2017)* have also established that FBs and DON levels in maize grains influenced by the type of the hybrids from FAO 500 and 600 maturity groups and environmental conditions. In addition, *Van Rensburg et al. (2016)* concluded that seven South African maize genotypes were differed in susceptibility to natural fungal and FBs contamination. *Leggieri et al. (2015)* found that there were no significant differences in the aflatoxins level between maize hybrids from different FAO maturity groups in two-year trials (2009-2011). Also, in Serbia, in 2013, *Krnjaja et al. (2016)* have demonstrated that there were no significant effects of hybrids and the interaction effect of hybrids and location on the level of AFB_1 in the maize hybrids from FAO 300, 400, 500, and 600 maturity groups.

Table 3. The effect of maize hybrids on the level of aflatoxin B_1 (AFB_1), deoxynivalenol (DON) and fumonisins (FBs)

Factor	AFB_1 (μg kg^{-1})	DON (μg kg^{-1})	FBs (μg kg^{-1})
MAS 34.B	1.52^{a}	64.67^{a}	1202.00^{a}
MAS 40.F	1.02^{b}	46.78^{b}	1181.89^{ab}
MAS 48.L	1.14^{b}	42.56^{b}	1088.78^{b}
KWS Konfites	< 1	< 40	88.33^{c}
ZP 427	< 1	40.89^{b}	115.33^{c}
MAS 56.A	< 1	< 40	1093.56^{ab}
F test	**	**	**
Mean	1.11	45.82	794.98

Means followed by the same letter within a column are not significantly different according to Tukey’s multiple comparison test ($P \leq 0.05$); ** - significant at the 0.01 level of probability, * - significant at the 0.05 level of probability, ns – not statistically significant

Using the Pearson correlation analyses, a statistically significant positive correlation ($P<0.05$) was established among the incidence of *F. verticillioides* and *F. proliferatum* ($r = 0.56$) (data not presented). No significant positive correlations were established among the incidence of *F. graminearum* and *F. subglutinans* ($r = 0.15$), the incidence of *F. verticilioides* and *F. proliferatum* and the FBs level ($r = 0.22$ and $r = 0.08$, respectively), and the incidence of *F. graminearum* and the DON level ($r = 0.40$). The incidence of *F. verticillioides* and *F. proliferatum* was in no significant positive correlations with the moisture grain content ($r = 0.22$ and $r = 0.24$, respectively). The coefficients of correlation indicate that FBs and DON levels were influenced by the incidence of their *Fusarium* producers. In the studies of *Balconi et al. (2014)*, it was also confirmed that the FBs level was depended on the incidence of *F. verticillioides*.

Conclusion

In this study, fungal species of the genus *Fusarium*, as economically important toxigenic species, were isolated from the grains of all maize hybrids, while *Aspergillus* species were not detected in any samples. Climatic factors in the growing season in 2018 were very favourable for the development of *Fusarium* species. Among *Fusarium* species, fumonisin producing species were dominant. The levels of mycotoxins, AFB_1, DON, and FBs, were influenced by hybrids.

These results confirmed that in addition to climatic factors (temperature, rainfall, and RH), the susceptibility of hybrids was also one of the important risks for the appearance of toxigenic fungi and their mycotoxins. Therefore, investigation of susceptibility of hybrids should also be the focus of further studies with an aim for advancing integrated pest management control in the maize production.

Prirodna pojava toksigenih gljiva i mikotoksina u hibridima kukuruza

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Rezime

Cilj rada je bio da se ispita osetljivost različitih hibrida kukuruza na prirodnu pojavu toksigenih vrsta gljiva, posebno toksigenih *Aspergillus* i *Fusarium* vrsta, i mikotoksina (aflatoksina B_1 (AFB_1), deoksinivalenola (DON) i ukupnih fumonizina B_1, B_2 i B_3 (FBs)). Uzorci zrna šest komercijalnih hibrida kukuruza (MAS 34.B – FAO 300, MAS 40.F, MAS 48.L, KWS Konfites i ZP 427 – FAO 400 i i MAS 56.A – FAO 500) sakupljeni su u vreme berbe 2018. godine.

Analizom mikobiota identifikovane su vrste iz sedam rodova, *Acremonium, Alternaria, Epicoccum, Fusarium, Nigrospora, Penicillium* i *Rhizopus*, od kojih su samo vrste iz roda *Fusarium* bile prisutne na zrnu kukuruza svih ispitivanih hibrida. Veća učestalost *Fusarium* spp. na zrnu kukuruza bila je kod hibrida MAS 48.L (54,6%) i MAS 56.A (53,3%) u poređenju sa hibridima MAS 40.F (37,3%), KWS Konfites (28%), MAS 34.B (22,6%) i ZP 427 (12%).

Među identifikovanim *Fusarium* vrstama (*F. graminearum*, *F. proliferatum, F. subglutinans* i *F. verticillioides*), vrsta *F. proliferatum* bila je prisutna kod svih ispitivanih hibrida u rangu od 9,3% (ZP 427) do 30,7% (MAS 48.L), dok je vrsta *F. subglutinans* bila prisutna kod dva hibrida MAS 40.F (16%) i MAS 56.A (9,3%). Učestalost *F. graminearum* je bila od 0% (KWS Konfites) do 9,3% (MAS 34.B), dok je učestalost *F. verticillioides* bila od 0% (MAS 34.B i ZP427) do 21,3% (MAS 48.L). U uzorcima zrna, *Aspergillus* vrste nisu bile identifikovane.

Hibridi kukuruza statistički su značajno uticali na sadržaj ispitivanih mikotoksina. Hibrid MAS 34.B imao je statistički značajno viši sadržaj AFB_1 i DON u odnosu na druge hibride. Sadržaj FBs bio je najviši kod hibrida MAS 34.B (1202 μg kg^{-1}), a najmanji kod hibrida KWS Konfites (88,33 μg kg^{-1}). Međutim, sadržaj FBs nije se razlikovao između hibrida MAS 34.B, MAS 40.F i MAS 56.A, MAS 40.F, MAS

48.L i MAS 56.A i KWS Konfites i ZP 427. Kod svih ispitivanih hibrida, sadržaji AFB_1, DON i FBs bili su ispod maksimalno dozvoljenih količina propisanih zakonskom regulativom Evropske Unije i Republike Srbije u neprerađenom kukuruzu.
Agroekološki uslovi u 2018. godini pogodovali su razvoju *Fusarium* vrsta na zrnu kukuruza ispitivanih hibrida, i to posebno fumonizin producenata.

Ključne reči: toksigene vrste gljiva, mikotoksini, hibridi kukuruza

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