

THE OCCURRENCE OF MYCOTOXINS IN SWEET MAIZE HYBRIDS

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The objective of the present study was to investigate the susceptibility of maize hybrids to the natural mycotoxins contamination: aflatoxin total (AFLA), deoxynivalenol (DON), zearalenon (ZEA) and fumonisins (FB). Hybrids were grown during two production years (2019 and 2020) in two locations. Mycotoxin contamination of maize grains was evaluated in five sweet maize hybrids. Contamination level of investigated hybrids of first cluster was mainly by below average values of ZEA (all equal to zero) and DON. Hybrids PK4 (S) 2020, PK6 (MS) 2020 and PK4 (MS) 2020 had below average values for AFLA, while remaining treatments of this cluster have mostly elevated values of this mycotoxin. Contamination level of investigated hybrids of second cluster mostly had increased values of mycotoxins ZEA and DON and below average values of AFLA. Samples did not contain fumonisins. Mycotoxin contamination were significantly affected by hybrids and years. We have established that DON and ZEA levels were influenced by the environmental conditions. There were no significant effects of location on the level of AFLA in the sweet maize hybrids. The variation in the properties of mycotoxin content (DON) was significantly influenced by hybrids, and there was no significance of hybrids x location interaction. Differences were more expressed for the content of ZEA and AFLA compared to the content of DON. Hybrid PK1 had the lowest content of DON, while it had the highest content of ZEA. Mycotoxin analyses showed that in all tested hybrids, levels of AFLA, DON, ZEA and FBs were below the maximum permissible levels stipulated by the legislation of the European Union and the Republic of Serbia in maize intended for direct human consumption. These results confirmed that the susceptibility of hybrids is one of the important risks, in

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addition to climatic factors, for the appearance of toxigenic fungi and their mycotoxins. Genotype tolerance is very important as a preventive measure, which indicates that breeders have to pay attention to it in sweet maize breeding programs.

Key words: aflatoxins, deoxynivalenol, zearalenon, fumonisins

INTRODUCTION

Sweet maize is a mutation of maize in which a greater accumulation of sugars and water soluble polysaccharides provide a specific taste and texture (SRDIĆ *et al.*, 2011). One of the main purposes of cultivating sweet maize is its consumption directly as food. Sweet maize is harvested during the milk stage, and its use is limited by time, because fresh ears lose their quality due to a fast decay. The appropriate time of maize harvesting is significant (20-25 days after silking i.e. pollination) as much as the shelf life of fresh ears is limited to 2-5 days depending on the genotype and storage conditions (SRDIĆ *et al.*, 2019).

Mycotoxin contamination is the most important trade restriction for agricultural products that adversely affects the health and profit/earnings of small-holder farmers, local and international trade, and the global economy (LOGRIECO *et al.*, 2018). Furthermore, mycotoxins are very stable compounds that are accumulated in maize kernels in the field after fungal infections during the crop growing season. There is a possibility of their increase in the post-harvest period if the environment favours fungal activity. One of the major issues in contamination of maize is the infection caused by *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare, and the resulting occurrence of aflatoxins (AFLA). *Fusarium* species also infect maize and contaminate grains with mycotoxins, which including deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FBs), nivalenol (NIV), T-2 toxin (T2), and HT-2 toxin (HT2). The co-occurrence of AFs and FBs is common in maize (CAMARDO LEGGIERI *et al.*, 2015). According to the reports of the Food and Agriculture Organization of the United Nations, approximately 25% of the cereals produced in the world are contaminated by mycotoxins (REDDY *et al.*, 2010). A range of toxic effects has been associated with the length of exposure to mycotoxins, which may provoke negative effects on health of humans and many animal species (ESKOLA *et al.*, 2018). Since there are risks of mycotoxin contamination, many countries have regulations for maximum acceptable tolerance limits of mycotoxins in food and feed. The maximum concentrations of the main class of mycotoxins in agricultural food and feed products, as well as in their commodities, are regulated in Europe (EU). Among the AFs, aflatoxin B1 (AFB1) is the most toxic and the most potent (LUONGO *et al.*, 2014). According to the International Agency for Research on Cancer (IARC), AFB1 has been classified as the human carcinogen, Class 1 (IARC, 1993). In accordance with the Official Gazette of the Republic of Serbia, issue 76/19 and the EU Regulations, in maize, intended for human direct consumption, the maximum permissible levels of AFLA, DON, ZEN and FBs in 10 µg kg⁻¹ are 0,75 µg g⁻¹, 75 µg kg⁻¹ and 1 µg g⁻¹, respectively.

The simplest approach to prevent pre-harvest contamination by fungi is to develop maize hybrids resistant to fungi that produce mycotoxins. Stakeholders are concerned because fungi decrease crop yield and quality of maize, and because they contaminate kernels with mycotoxins. When grown in environments that favour outbreaks of aflatoxin or fumonisin, there is no commercial maize hybrid that can avoid contamination. Commercial maize hybrids have been

developed with substantial resistance to *Fusarium graminearum* Schwabe. Breeders make an effort to develop hybrids with adequate tolerance/resistance to *A. flavus* and *Fusarium verticillioides* (Sacc.) Nirenberg, by applying conventional breeding methods, which has proven to be a difficult task. Sweet maize is particularly susceptible to fungal pathogens due to its high sugar content and thin pericarp. Contamination with mycotoxins in sweet maize is even more important because toxins are taken directly into human organism, not through the food chain.

The World Health Organization (WHO) has been classified some of these mycotoxins as human carcinogens. Mycotoxins are grouped according to their toxic activity under chronic conditions, into mutagenic, carcinogenic, or teratogenic mycotoxins. Aflatoxins that occur naturally are classified as human carcinogens (Group 1); ochratoxins and fumonisin as possible human carcinogens (Group 2B), whereas trichothecenes and zearaleone are not recognised as human carcinogens (Group 3) (IARC, 1993). Practically, all mycotoxins can cause one or more crucial health problems. Certain mycotoxins can defeat the immune system (BENNETT and KLICH, 2003), thereby exposing the consumer to serious health threats. In Serbia aflatoxins have been detected in high levels on the maize kernels, which are secondary metabolites produced by *A. flavus* and *A. parasiticus* (KOS *et al.*, 2018; NIKOLIĆ *et al.*, 2018; OBRADOVIĆ *et al.*, 2018). Fumonisin produced mainly of *F. verticillioides* and *F. proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg and deoxynivalenol and zearalenone produced primarily of *F. graminearum* have been isolated on maize kernels in Serbia (KRNJAJA *et al.*, 2015; JAKŠIĆ *et al.*, 2019; KRNJAJA *et al.*, 2020).

The United States is the largest producer of sweet maize in the world (more than 35% of world production), but lately it has become increasingly popular in Europe, and its consumption has been increasing over years in Serbia.

According to data obtained during the four-year period (2014-2018), approximately 450 thousand tons of maize were produced annually in the United States. It is followed by Mexico, Nigeria, Hungary, Indonesia, France and Peru, which had an annual production of over 400 thousand tons (www.fao.org). The Statistical Office of the Republic of Serbia does not keep records on sweet maize production, and FAOSTAT estimates that 15-20 thousand tons per year were produced for the period of the last 5 years (<http://www.fao.org/faostat/en/?#data/SC>). In Europe, the largest producers are Hungary and France with 31000 and 25600 ha, respectively. Total world production area of sweet maize is 350000 ha and only 20% is in the EU (HANSEN, 2019).

Sweet maize is a highly consumed fresh vegetable in many parts of the world and can serve as source of nutrition. This suggests the increased demand for its production worldwide. Sweet maize is an excellent source of health promoting phytochemicals, such as carotenoids, tocopherols and phenolic acids (DAS and SINGH, 2016). The primary biological role of β -carotene is to enable provitamine A activity (GRUNE *et al.*, 2010). For the stated reasons, an attempt to obtain food of high nutritional quality, which is at the same time safe for consumption has become a worldwide trend.

Making food safety a priority is the biggest trend in all processes of food production. Therefore, the main intention of the authors of this scientific paper has been to present the natural occurrence and levels of mycotoxins in five sweet maize hybrids under agro-ecological conditions of Serbia during two research years (2019 and 2020).

MATERIAL AND METHODS

Mycotoxin contamination of maize kernels was evaluated in five sweet maize hybrids:

- 1) PK 1 (S) and PK 1 (MS) - ZP 355su;
- 2) PK 3 (S) and PK 3 (MS) - ZP 477/4su;
- 3) PK 4 (S) and PK 4 (MS) - ZP 486/3su;
- 4) PK 5 (S) and PK 5 (MS) - ZP 493/2su;
- 5) PK 6 (S) and PK 6 (MS) - ZP 496/2su.

Hybrids were grown during two production years (2019 and 2020) in two locations: the experimental field of the Maize Research Institute, Zemun Polje, Belgrade-Zemun and the experimental field, Krnješevci. Maize kernel samples were collected at the milk stage of the endosperm development, 23-25 days after silking. The grain analysis was performed with 2 kg sub-samples drawn from the primary sample. The primary sample was obtained by mixing smaller samples, taken from several different places of one location, into one sample. The obtained quantity of the sample was divided into four portions and from each quarter 0.5 kg of grain was sampled. Samples were stored at -20°C until analysis.

Prior to the mycotoxicological analysis, all kernel samples were first dried for 72 h at 60°C and then grounded. The enzyme-linked immunosorbent assay (ELISA) for determining AFLA, DON, ZEA and FBs levels was applied according to the manufacturer's instructions (Celer Tecna® ELISA kits). The limits of detection (LOD) for AFLA, DON, ZEA and FBs were: 2 µg kg⁻¹; 0,04 µg g⁻¹; 10 µg kg⁻¹ and 0,75 µg g⁻¹, respectively.

Pericarp was removed manually. The thickness of wet kernel coats of sweet maize was measured by a calliper in three replicates.

Effects of different sweet maize genotypes on the mycotoxin levels were visualised with a clustergram. The Pearson's correlation coefficient was used in correlation analyses.

RESULTS AND DISCUSSION

Meteorological conditions in Serbia for two years (2019 and 2020), the mean monthly temperatures (>20°C), total monthly rainfall (>35 mm) and mean monthly relative humidity (RH) (>50%) at the flowering stage (June) and the milk stage (July) were suitable for fungal maize colonisation (Figure 1). Climate factors in both growing seasons were similar and favourable for the development of fungal species.

Studied similarities of certain treatments and the correlation of measured parameters were presented by the clustergram. Raw data has been standardised (adjusted to a zero mean and unit deviation). The Euclidean distance was used as a measure of the similarity of certain treatments, while the Pearson's correlation coefficient was used as a measure of the similarity of parameters. Ward's minimum variance method was used for treatments linkage. The colour bar is presented on the left side of the clustergram. The colours point out to the level of measured parameters (mycotoxins). The blue, white and burgundy colours indicate the below average, close to average and above average values, respectively, of mycotoxins in the treatment.

Two groups of treatments are clearly observable in the clustergram. The first group is consisted of treatments from PK4 (S) 2020 to PK5 (MS) 2020 (looking from the top to the bottom of the clustergram). These treatments are mainly characterised by below average values of ZEA (all equal to zero), as well as below average values of DON. Treatments PK4 (S) 2020,

PK6 (MS) 2020 and PK4 (MS) 2020 have below average values for AFLA, while remaining treatments of this cluster have mostly elevated values of this mycotoxin. The second cluster encompasses treatments from PK5 (MS) 2019 to PK1 (MS) 2019. These treatments mostly have elevated values of mycotoxins ZEA and DON and below average values of AFLA (Figure 2).

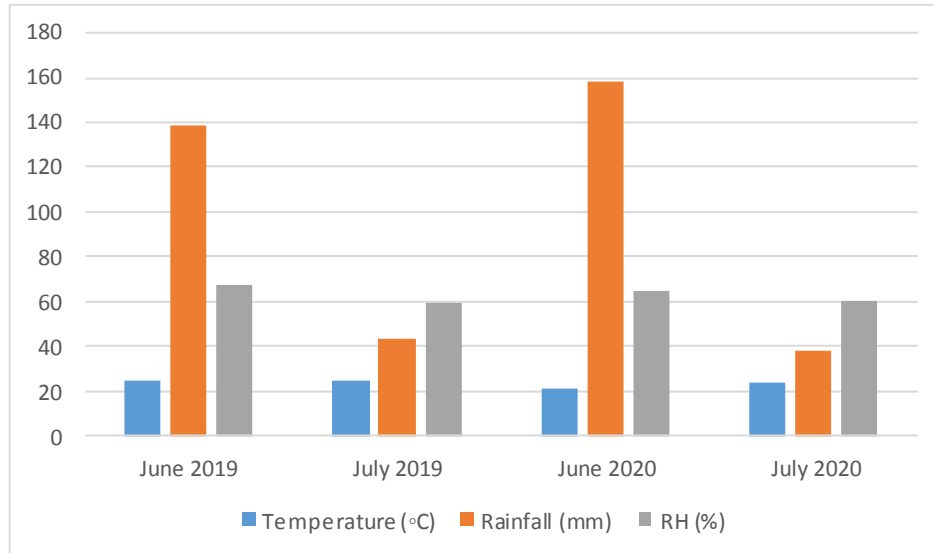


Figure 1. Mean monthly temperatures, total monthly rainfall and mean monthly relative humidity (RH) in Belgrade area during June and July in 2019 and 2020

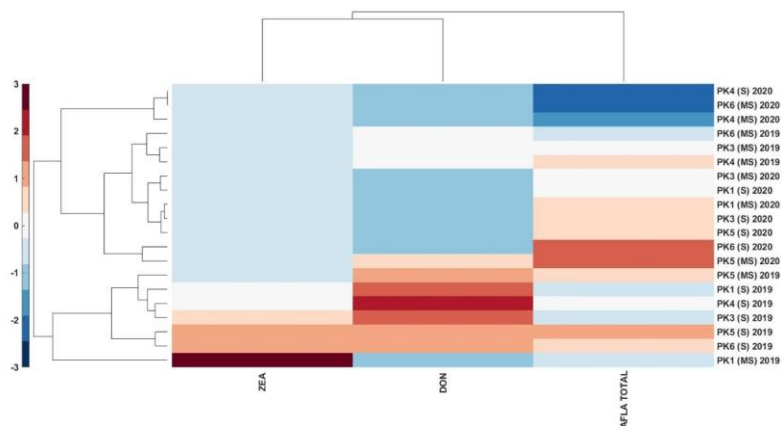


Figure 2. Clustergram shows similarity of treatments and correlations of parameters

Table 1. ANOVA for the mycotoxin contents in sweet maize hybrids

Source of variance	Degrees of Freedom	F value (DON)	F value (ZEA)	F value (AFLA)
Hybrids (Factor A)	4	4.2128*	22920.3936**	509.9274**
Locations (Factor B)	1	191.8599**	175310.2033**	0.0583
AxB	4	1.6947	22920.3936**	388.0981**
Years (Factor C)	1	31.8037**	349.9898**	185.3218**
AxC	4	9.6383**	56993.9664**	527.3331**
BxC	1	59.5549**	349.9898**	92.7337**
AxBxC	4	1.6727	56993.9664**	225.8950**

** – significant at the 0.01 level of probability; * – significant at the 0.05 level of probability; ns – not statistically significant

Differences among hybrids according to the contents of all three mycotoxins were significant (Table 2). Moreover, they were more pronounced for the content of ZEA and AFLA than for the content of DON. The hybrid PK1 had the lowest content of DON whereas it had the highest content of ZEA.

Table 2. The mean values of mycotoxins content for both years and both locations influenced by hybrids

	Zemun Polje		Krnješevci		Mean
	2019	2020	2019	2020	
DON ($\mu\text{g kg}^{-1}$)					
PK1	0.022	nd	nd	nd	0.005500c
PK3	0.024	nd	0.008	nd	0.008250abc
PK4	0.028	nd	0.007	nd	0.008875ab
PK5	0.016	nd	0.018	0.011	0.011370a
PK6	0.018	nd	0.007	nd	0.006250bc
ZEA ($\mu\text{g kg}^{-1}$)					
PK1	2.013	nd	43.689	nd	11.430a
PK3	9.546	nd	0.407	nd	2.488d
PK4	5.724	nd	1.536	nd	1.815e
PK5	nd	nd	nd	nd	3.595c
PK6	nd	nd	nd	nd	4.559b
AFLA TOTAL ($\mu\text{g kg}^{-1}$)					
PK1	1.538	2.551	1.844	2.865	2.200c
PK3	2.014	3.051	2.484	2.141	2.422b
PK4	2.188	nd	2.886	0.673	1.437d
PK5	3.463	3.277	2.744	4.504	3.497a
PK6	3.003	4.697	1.666	nd	2.341b

Means followed by the same letter within a column are not significantly different according to the Least Significant Difference Test ($P \leq 0.05$); nd – not detected;

Mycotoxin analyses showed that in all tested hybrids, levels of AFLA, DON, ZEA and FBs (Table 1) were below the maximum permissible levels stipulated by the legislation of the European Union and the Republic of Serbia in maize intended for the direct human consumption (OFFICIAL GAZETTE OF THE REPUBLIC OF SERBIA, 2019).

In 2020, the amount of ZEA was not detected, and in 2019 the mean content level ($9.5 \mu\text{g kg}^{-1}$) were low. Similar to our results, according to the EFSA (2011), the frequency of the ZEA occurrence in kernels of sweet maize (11%) for human consumption and the mean content level ($4.8 \mu\text{g kg}^{-1}$) were low in year 2011 in the European Union.

FBs was not detected in any of investigated hybrids. Similar to our results, CALDAS and SILVA (2007) did not detect FBs in any of the sweet maize analysed samples. According to the SCOOP (2003), both the prevalence and the level of FBs in the analysed sweet maize were low with 9% of positive samples and a mean level of $12.4 \mu\text{g kg}^{-1}$. In Taiwan, according to the results of TSENG and LIU (1997) the highest frequency (50%) of positive samples for the presence of FB1 was found in sweet maize. Half of them were on the average contaminated with $0.4 \mu\text{g kg}^{-1}$ of FB1 and $0.065 \mu\text{g kg}^{-1}$ of FB2. MARIN *et al.* (2013) were concluded that sweet maize had been less susceptible to *Fusarium* contamination than other maize varieties and had had low levels of FBs. Low contamination levels are expected in immature maize, which increase during the late maturity with a higher incidence of *F. verticillioides* (ALMEIDA *et al.*, 2002).

The thinnest pericarp of maize hybrids (data not presented) had a PK5 isolate (0.14 nm), in which the most DON (0.011370a) was synthesised. In contrast, the least DON (PK1-0.005500c; PK6-0.006250bc) was synthesised in the isolates with the thickest pericarp: PK1 (0.17 nm) and PK6 (0.17 nm).

The results of the Pearson's correlation coefficient indicated the existence of a statistically significant negative correlation between the mean values for mycotoxin production (DON and AFLA) and the pericarp thickness ($r=-0.99^{**}$; $r=-0.54^{**}$), respectively. It has established statistically very significant positive correlations between the mean values for mycotoxin production (ZEA) and pericarp thickness, for both years and both locations (Table 3).

Table 3. Correlation between mean values of the mycotoxin concentrations and pericarp thickness

Mean values of the mycotoxin	Correlation coefficient (r)
	Pericarp thickness – all isolates, two years, two locations
DON	-0.99**
ZEA	0.59**
AFLA	-0.54**

** – statistically significant at $p < 0.01$

These results are consistent with the results of HOENISCH and DAVIS (1994) who also documented a correlation between a greater pericarp thickness and resistance to *F. verticillioides*. The thicker pericarp may inhibit the fungal growth as well as act as a barrier to insect feeding. SAMPIETRO *et al.* (2009) identified various properties of the pericarp and its wax layer as resistance factors. Sweet maize, which has been bred to have a thin pericarp, is extremely susceptible to both *F. graminearum* and *F. verticillioides* (REID *et al.*, 2000). Long

chain alkanes on the surface of maize silks have also been implicated in resistance to *F. graminearum* (MILLER *et al.* 2003).

CONCLUSIONS

The contents of mycotoxins AFLA, DON and ZEA were influenced by hybrids. These results confirmed that the susceptibility of hybrids was also one of the important risks, in addition to climate factors, for the appearance of toxigenic fungi and their mycotoxins. Genotype tolerance is very important as a preventive measure, which indicates that breeders have to pay attention to it in sweet maize breeding programmes. Breeders face huge challenges from climate changes to growing world population, for which efficient breeding strategies and new technologies are necessary. Together with rice and wheat, maize provides almost a half of all calories consumed around the world. The increase in the yield, quality and stress tolerance of this crop is of the essential importance for global food security. Given that sweet maize is vastly consumed in Serbia, it is of great importance that it is safe for consumption.

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POJAVA MIKOTOKSINA U HIBRIDIMA KUKURUZA ŠEĆERCA

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Izvod

Cilj ovog rada bio je da se prouči osetljivost hibrida kukuruza na prirodnu kontaminaciju mikotoksinima (aflatoksin B1 (AFB1), deoksnivalenol (DON), zearalenol (ZEA) i fumonizini (FB)). Hibridi su gajeni tokom dve proizvodne godine (2019. i 2020) u dve lokacije. Kontaminacija zrna kukuruza mikotoksinima ispitivana je na pet hibrida kukuruza šećerca. Nivo kontaminacije proučavanih hibrida prvog klastera bio je uglavnom ispod prosečnih vrednosti ZEA (sve vrednosti su bile nula) i DON. Vrednosti AFLA kod hibrida PK4 (S) 2020, PK6 (MS) 2020 i PK4 (MS) 2020 su bile ispod prosečne vrednosti, dok su vrednosti ovog mikotoksina bile više od prosečnih vrednosti za ostale tretmane ovog klastera. Nivoi kontaminacije proučavanih hibrida drugog klastera su uglavnom bili viši za mikotoksine ZEA i DON i niži za AFLA. Fumnonizni nisu utvrđeni u uzorcima. Na kontaminaciju mikotoksinima značajno su uticali hibridi i godine. Utvrđeno je da su uslovi sredine uticali na nivoe DON i ZEA. Lokacija nije značajno uticala na nivo AFLA kod hibrida kukuruza šećerca. Hibridi su značajno uticali na variranje sadržaja mikotoksina (DON), dok hibrid × lokacija interakcija nije bila značajna. Razlike su bile izraženije za sadržaj ZEA i ALFA nego za sadržaj DON. Najniži sadržaj DON utvrđen je kod hibrida PK1, kod koga je sadržaj ZEA bio najviši. Analize mikotoksina pokazuju da su nivoi AFLA, DON, ZEA i FB u svim ispitivanim hibridima bili ispod maksimalno dozvoljenih nivoa koji su propisani zakonima Evropske Unije i Republike Srbije za kukuruz koji je namenjen za direktnu ljudsku konzumaciju. Ovi rezultati potvrđuju da je osetljivost hibrida jedan od važnih rizika pored klimatskih faktora za pojavu toksigenih gljiva i njihovih mikotoksina. Tolerantnost genotipa je veoma važna preventivna mera, na koju oplemenjivači moraju da obrate pažnju u programima oplemenjivanja kukuruza šećerca.

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