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AUTHORS: E. Janić Hajnal, J. Kos, L. Pezo, B. Radić, A. Malachová, R. Krska, M. Sulyok

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PRESENCE OF *ALTERNARIA* TOXINS IN MAIZE FROM REPUBLIC OF SERBIA DURING 2016-2017

Elizabet **Janić Hajnal**[1*], Jovana **Kos**[1], Lato **Pezo**[2], Bojana **Radić**[1], Alexandra **Malachová**[3], Rudolf **Krska**[3], Michael **Sulyok**[3]

[1]University of Novi Sad, Institute of Food Technology in Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Republic of Serbia

[2]Institute of General and Physical Chemistry, University of Belgrade, Studentski Trg 12–16, 11000 Belgrade, Republic of Serbia

[3]Department IFA-Tulln, University of Natural Resources and Life Sciences Vienna (BOKU), Konrad Lorenz Str. 20, 3430 Tulln, Austria

Correspondence

Elizabet Janić Hajnal, University of Novi Sad, Institute of Food Technology in Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Republic of Serbia

Email:elizabet.janich@fins.uns.ac.rs

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ABSTRACT

The aim of this study was to apply modern analytical tools, liquid chromatography-tandem mass spectrometric method (LC-MS/MS), for identification and quantitation of *Alternaria* metabolites in maize samples. Maize samples were collected from the main maize-producing regions (Bačka, Banat and Srem) of the Republic of Serbia during two harvest seasons (2016 and 2017). The most commonly detected *Alternaria* toxin in maize samples from both years was tenuazonic acid. On the other hand, determined mean concentrations of quantified tenuazonic acid were significantly higher in maize samples from Banat from both maize growing seasons in comparison to its concentrations in maize samples from Bačka and Srem. As a consequence of influence of microclimate conditions in investigated regions, the highest percentages of contaminated maize samples by *Alternaria* toxins, both 2016 and 2017, years were 48% and 47% respectively from Srem, whilst the lowest percentages of 29% and 23% respectively were found in Bačka.

Key words: *Alternaria* toxins, maize, Republic of Serbia, LC-MS/MS

Novelty impact statement

The main novelty of this study is achieved through increased knowledge and creation of one of the few database from this part of Europe regarding the frequencies of occurrence of “emerging” *Alternaria* toxins in maize, depending on weather conditions and some of the applied agrotechnical measures during a two-years period.

1. INTRODUCTION

Food and feed quality is very important for human and animal nutrition. The secondary metabolites of various fungi are frequent contaminants of food and feed, mainly cereals. These toxins can cause physiological, biochemical and pathological changes in both humans and animals. Therefore, they are considered as a major threat to food and feed safety (Stanicu et al., 2015).

Alternaria is one of the most common fungal genera with the ability to produce a wide spectrum of secondary metabolites. The most common *Alternaria* species include *Alternaria* (*A.*) *alternata*, *A. tenuissima*, *A. radicina*, *A. arborescens*, *A. infectoria*, *A. brassicae* and *A. brassicicola* (Escriva et al., 2017). While the most common *Alternaria* toxins belong to 5 different classes according to their structure: dibenzo-α-pyrones which include alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT); perylene quinones which include altertoxins I, II, III (ATX-I, ATX-II, ATX-III); tenuazonic acid (TeA); miscellaneous structures such as the cyclic tetrapeptide tentoxin (TEN), and *Alternaria alternata* f. sp. lycopersici toxins (AAL-toxins) (EFSA, 2011). *Alternaria* species and their toxins are widespread in both humid and semi-arid regions and have been isolated from a wide range of food and feed. Primarily, *Alternaria* is the principal contaminating fungi in cereals (mainly in wheat, sorghum and barley), fruits, vegetables, oilseeds, beverages, silage, feed and feed ingredients (Escriva et al., 2017).

Based on their effect on plants, *Alternaria* toxins are divided into two categories: non-host-specific and host-specific (Thomma, 2003). Some non-host-specific toxins such as AOH, AME, TeA and ATX are described to induce harmful effects in animals, including teratogenic and fetotoxic effects. On the other hand, the animal toxicity of host-specific toxins such as AAL-toxins has not been fully examined (Thomma, 2003; Barkai-Golan, 2008). However, due to the lack of comprehensive data available regarding toxicological effects and occurrence of *Alternaria* toxins, they are referred as “emerging” mycotoxins. Hence, due to their possible harmful effects, *Alternaria* toxins are of concern for public health (EFSA, 2011). However, currently there are no regulations on *Alternaria* toxins in food and feed in Europe or in other regions of the world, except for a limit of 500 µg/kg for TeA in sorghum/millet-based infant food suggested by the Bavarian Health and Food Safety Authority (Rychlik et al., 2016).

Toxin production depends on the fungal strain, the growing substrate, and the environmental conditions, the two most important of which are temperature and humidity (Vaquera et al., 2016). In general, the optimum temperature for *Alternaria* growth is between 22 and 28° C, which enables the fungi to grow well at room temperature in different climatic regions. However, *Alternaria* can also grow at low temperatures, and its minimal developmental temperature is -3° C (Barkai-Golan, 2008). After infection, *Alternaria* species require relatively high humidity, which is why *Alternaria* rots are more common in fruits and vegetables than in cereals and seeds, which are generally stored at low humidity. However, the growth of *Alternaria* species may be favored in cereals harvested during wet weather and stored in humid conditions, which make the *Alternaria* genus responsible for the spoilage of several commodities during transport and storage, even if they are refrigerated (EFSA, 2011). Therefore, knowledge of the influence of environmental conditions on fungal growth and toxin biosynthesis can be important in predicting food and feed contamination with *Alternaria* toxins (Vaquera et al., 2016).

Due to the high presence of *Alternaria* species and their toxins in food and feed, there has been a growing interest in scientific research on this fungal genus (Patriarca, 2016). Half of the recent publications were focused on fruits, vegetables, and their products, while cereals and cereal products represented 38% (Escriva et al., 2017). Currently, there are relatively scarce occurrence data on *Alternaria* toxins in food products and cereals from the Republic of Serbia, and the majority of the studies have been conducted in wheat (Janić Hajnal et al., 2015; Janić Hajnal et al., 2016; Janić Hajnal et al., 2019), while there has been only one study in maize (Janić Hajnal et al., 2020). Considering that the Republic of Serbia is one of the biggest maize producers and exporters in Europe, with an annual production of 7.3 million tonnes and export of 3.1 million tonnes in 2019 (FAO, 2019), growing maize in the Republic of Serbia is a traditional and very important part for the national agriculture.

Consequently, the present study was undertaken with the aim to determine the presence of seven different *Alternaria* metabolites in maize samples collected from the main maize producing regions (Bačka, Banat, and Srem) in Northern Serbia during the 2016 and 2017 growing seasons. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used for the detection and quantification of *Alternaria* toxins in maize samples. In addition, the impact of weather conditions during maize growing seasons, as well as, the impact of the preceding crops were investigated on the frequency and level of *Alternaria* toxins (TeA, AOH, AME, TEN, altersetin (ALS), infectopyron and macrosporin A) in maize samples.

2. MATERIALS AND METHODS

2.1 Samples collection

A total of four hundred and fifty-eight (n=458) maize samples were collected from the field trials from the agricultural advisory services from the Banat, Bačka and Srem regions. In total, 277 and 181 maize samples were collected in 2016 and 2017 years, respectively. The agricultural advisory services are under the auspices of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia**.** The crops were managed according to standard agricultural procedures and good professional practice. The distribution of the number of maize samples in different years and regions of Northern Serbia were as follows: from Bačka 73 (2016) and 82 (2017); from Banat 127 (2016) and 48 (2017); from Srem 77 (2016) and 51 (2017). Sampling was performed according to the EU requirements (European Commission 2006) in order to overcome the irregular distribution of mycotoxins. Aggregate maize samples (approximately 10-15 kg) were transported to the laboratory of the Institute of Food Technology in Novi Sad. Homogenisation of these aggregate samples were performed using a Nauta mixer (model 19387, Nauta patenten, Netherlands) and after that, quartered to get 500-1000 g of a laboratory sample. The laboratory samples were ground to a 1 mm particle size using a laboratory mill (KnifetecTM 1095 mill, Foss, Hoganas, Sweden), and were stored prior to analysis at -20°C in the freezer. In 2018, a total of 458 subsamples (30 g each) were placed in marked sealable plastic storage bags and transported into a cooler to the Department for Agrobiotechnology (IFA-Tulln, Austria), where they were analysed.

2.2 Sample preparation and LC-MS/MS analysis

Sample preparation and analyses of *Alternaria* toxins were performed according to the multi-mycotoxins LC-MS/MS method published by Sulyok et al. (2020), without any modifications, as well as with the same equipment, chemicals and reagents. The limits of detection (LOD) were determined following the EURACHEM guide (Magnusson and Örnemark, 2014). The performance characteristics (LOD and Apparent Recovery with relative standard deviation (RSD)) of the applied analytical method for *Alternaria* toxins are presented in Table 1.

Table 1

Quantification of examined fungal metabolites was performed using an external calibration based on serial dilutions of a multi-analyte stock solution. The quantified concentrations of *Alternaria* toxins were corrected for the apparent recovery.

2.3 Weather conditions

All meteorological data were provided from the Republic Hydrometeorological Service of Serbia (http://www.hidmet.gov.rs/). The monthly average air temperatures and sum of precipitation were taken for each region for the entire period of maize growing seasons (April-September) in 2016 and 2017. Furthermore, the humidity conditions were estimated on the basis of the standardized precipitation indexes (SPI-6 and SPI-3) for each region. SPI-6 and SPI-3 are indexes which characterize meteorological drought on a range of timescales. SPI-6 represents the period of six months, from April to September, while SPI-3 characterized summer period, from June to August. Data on monthly average air temperatures and sum of precipitation of each year by regions in comparison with the long-term annual data (Figure 1), as well as the SPI-6 and the SPI-3 were used to characterise the differences between the years of investigation.

Figure 1

2.4 Statistical analysis

Box plots were employed to visualize the distribution comparison of the data belonging to the seven groups of parameters. All data were processed statistically using the software package STATISTICA 14.0.0.15 (StatSoft Inc., Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

Based on several published studies from around the world (Abia et al. 2013; Shephard et al. 2013; Abdallah et al. 2017; Blandino et al. 2017; Oliveira et al. 2017; Getachew et al. 2018; Oyeka et al. 2019; Janić Hajnal et al. 2020; Tebele et al. 2020; Topi et al. 2019), maize can also be contaminated with *Alternaria* toxins. Taking into account the fact that maize is the most important cereal crop produced in the Republic of Serbia and represents a very important agricultural crop intended for export, as well as the fact that *Alternaria* toxins may implicate health concerns for humans and animals (EFSA, 2011) this study was focused on the determination of the presence of *Alternaria* metabolites in maize samples from the main maize-producing regions (Bačka, Banat and Srem) of the Republic of Serbia during 2016 and 2017 harvest seasons. Besides the influence of weather conditions during the maize growing seasons, additionally the influence of preceding crops on the frequency and concentration levels of the examined *Alternaria* metabolites in maize samples were considered. Since the weather conditions (especially air temperature and amount of precipitation) during maize growing season represent factors with the strongest influence on the occurrence of mycotoxins in maize in general (Medina et al. 2015), for the better interpretation of the obtained results in this study, detailed analysis of weather conditions parameters was conducted.

3.1 Weather conditions analysis

In general, the climate of the northern province of the Republic of Serbia (Vojvodina), can be described as moderate-continental with more or less pronounced local characteristics. The weather condition during maize growing season in 2016 (April-September) on the territory of Bačka, Banat and Srem was warmer (Figures 1 a,b) with a slightly higher amount of precipitation compared to the long-term average values (1981-2010). The deviation of mean daily temperatures during the maize growing season had positive values of 1.0 ° C, 1.2 °C, and

1.6 ° C in Srem, Bačka and Banat, respectively. The sum of precipitation during maize growing season in Banat was higher (+ 84 mm) in comparison to other investigated regions, as well as to the long-term average values (1981-2010). Only in Srem, the sum of precipitation was lower (-9 mm) in regards to the long-term average values (1981-2010). However, the SPI-6 for this period (April-September 2016) shows normal humidity conditions in Bačka, Banat and Srem. On the other hand, according to the SPI-3, in the summer period of 90 days (June – August 2016), in investigated regions, from moderate to severe drought has been reported. Maize growing season in 2017 (April-September) was warmer and drier compared to 2016, as well as to average conditions. The deviation of mean daily temperatures (Figure 1a) compared to the long-term average values (1981-2010) varied in following order: 1.4 ° C in Bačka, 1.5 °C in Srem and 2.0 °C in Banat. From April to September (Figure 1b), sum of precipitation in investigated regions was lower, which was about 20% less than the average values. Contrary to the maize growing season in 2016, in 2017 in Banat the sum of precipitation was lower (-93 mm) in comparison to the long-term average values (1981-2010), as well as in comparison to its values in other investigated regions. The SPI-6 determined for maize growing season (April-September 2017) showed normal humidity conditions in Bačka and Srem, while a moderate drought prevailed in most part of Banat. However, in part of Banat, extreme drought has been reported according to humidity conditions in this region, estimated on the basis of SPI-3 determined for summer period of 90 days (June-August 2017). On the other hand, a severe and moderate drought occurred in Bačka and Srem region, respectively, during the summer months in 2017.

3.2 Occurrence of *Alternaria* toxins during the period 2016 – 2017

Among 458 analysed maize samples the following *Alternaria* metabolites were detected: TeA, AOH, AME, ALS, TEN, infectopyron and macrosporin A. In total, 38% of examined maize samples were contaminated with listed *Alternaria* metabolites. The overall average incidence of *Alternaria* metabolites in the investigated two-year period was as follows: 30% of maize samples contained TeA, 9% AOH, 12% AME, 9% ALS and 1% TEN, infectopyron and macrosporin A (each). The frequency and the level of each metabolite varied by production years, as well as by the regions (Figure 2).

Figure 2

Namely, in 2016 production year among 277 analysed maize samples 34% were contaminated with TeA, 8% with AOH, 14% with AME and 13% with ALS, while none of the analysed maize samples were contaminated with TEN, infectopyron and macrosporin A. On the other hand, in 2017 maize growing season, 25% of maize samples were contaminated with TeA, 9% with AOH, 7% with AME and 6% with ALS. Additionally, TEN (2%), infectopyron and macrosporin A (3% of each) were detected in maize samples from investigated regions. It was found that in 2016 maize growing season, 39% of the examined maize samples was contaminated with at least one *Alternaria* metabolite, while a slightly smaller proportion (36%) of examined maize samples was contaminated during 2017 production year. In 2016 maize growing season the share of contaminated maize samples by regions was as follows: 41% of maize samples from Banat, 29% from Bačka and 48% from Srem were contaminated. The share of contaminated maize samples, with *Alternaria* toxins, by regions in 2017 production year was as follows: 46%, 23% and 47% from Banat, Bačka and Srem, respectively. The prevalence of 6 quantified, among 7 examined *Alternaria* metabolites are shown in the Figure 2. Figure 2 show the summary statistics results by box plots including values of mean concentrations from positive samples only, by regions and maize growing seasons, as well as the preceding crops in order to have an insight whether the weather conditions by regions and the preceding crops have an influence on the prevalence of *Alternaria* metabolites. Infectopyron, which was detected and/or quantified only in 5 maize samples in 2017 maize growing season is not shown in the box plot of the Figure 2. This metabolite was detected only in 8% of 48 analysed maize samples from Banat in 2017 and it was quantified only in 2% of 51 anlysed maize samples from Srem in 2017. The quantified concentration of infectopyron was 58 µg/kg.

From the Figure 2 it could be noticed that significant differences exist in the prevalence of the 6 quantified *Alternaria* metabolites between investigated years, regions and in some cases depending on the preceding crops. First of all, the most commonly detected *Alternaria* toxins in maize samples from both years was TeA. Although there is no statistically significant difference in its mean concentration by production years in general (599 ± 1287 µg/kg in 2016 and 339± 490 µg/kg in 2017), significant differences could be noted in its mean concentration by regions within and between production years (Figure 2a). Namely, the mean concentration of TeA in maize samples from Banat in 2016 (952±1695 µg/kg) was the highest and significantly diferent in comparison to its content in contaminated maize samples from Bačka (173±175 µg/kg) and Srem (224±198 µg/kg). In addition, in the maize samples from Banat the widest range of TeA content (LOD – 9542 µg/kg in 2016 and LOD – 2283µg/kg in 2017) was observed in relation to its range in maize samples from Bačka (LOD – 716 µg/kg in 2016 and LOD – 1832 µg/kg in 2017) and Srem (LOD – 983 µg/kg in 2016 and LOD – 839 µg/kg in 2017) from both maize growing seasons. A similar trend was observed in 2017. The recorded mean content of TeA and the share of contaminated maize samples by regions were as follows: 511± 2283 µg/kg in 31% in Banat, 389±569 µg/kg in 23% in Bačka and 175±220 µg/kg in 44% in Srem. It is well known that the weather conditions during maize growing season, primarily air temperature and amount of precipitation, had the strongest influence on the growth and development of fungi and consequent synthesis of fungal metabolites (Zinedine and Akhdari, 2021). Thus, obtained differences in the prevalence as well as in concentration levels of TeA could be explained by different weather conditions recorded in the two investigated years by regions. From the Figure 1 it could be noticed that in summer period in 2016 in Banat a significantly higher amount of sum of precipitation (285 mm) was recorded in comparison to the other two investigated regions, especially in June 2016. Contrary, in the summer period in 2017 the sum of precipitation was lower in all regions, especially in Banat in July, in comparison to the long-term average values (1981-2010). Further, in the same period in 2016 in Banat the highest deviation of mean daily temperature (1.4 °C) was observed in comparison to Bačka and Srem. The same case was recorded in the summer period in 2017 since again in Banat the highest deviation of mean daily temperature of 3.1 °C was observed. On the other hand, from the Figure 2 it could be seen that only in Banat wheat was used as a preceding crop in 2016 maize growing season, while in 2017 season, soybean was used as a preceding crop. In Bačka and Srem as a preceding crop for maize cultivation soybean and sunflower were mainly used in both investigated years. Since *Alternaria* toxins are the main contaminants of small grain cereals (Ostry, 2008; Patriarca, et al., 2007; Logrieco et al, 2009), it can be noted that wheat as a preceding crop in Banat in interaction with the micro-climatic conditions in 2016 contributed to the determined highest concentration of TeA in this region. Further, higher sum of precipitation (Figure 1b) and moderate to severe drought conditions, estimated on the basis of SPI-3, for a summer period of 90 days (June – August) also contributed to higher TeA synthesis, resulting in higher frequency and higher quantified concentrations of this *Alternaria* toxin. Comparing these findings with the only available published study from the Republic of Serbia, which investigated *Alternaria* metabolites (Janić Hajnal et al., 2020, Part 2) in maize samples in a four-year period (2012-2015), it can be noted that in our study during both investigated years higher mean concentrations and higher maximum values of TeA content and a lower percentage of contaminated maize samples were recorded. Namely, the highest share of contaminated maize samples (67%), the highest mean concentration of TeA (230 ±114 µg/kg), as well as the highest maximum value (503 µg/kg) of its content was recorded in 2012 which was characterized as extreme drought year. In comparison with the data published so far on the prevalence of *Alternaria* metabolites, it can be noted that TeA, in addition to maize samples from Serbia, also occurred in maize samples from Albania, South Africa, and Nigeria (Shephard et al. 2013; Abdallah et al. 2017; Oliveira et al. 2017; Getachew et al. 2018; Oyeka et al. 2019; Topi et al. 2019; Tebele et al. 2020). It should be noted that in comparison with published findings (Oyeka et al. 2019; Topi et al. 2019; Tebele et al. 2020), the highest mean concentration of TeA was recorded from maize samples from the Republic of Serbia, while the share of contaminated samples with this *Alternaria* metabolite is approximately at the same level. Among 458 maize samples 9% were contaminated with AOH during the two-year period. The quantified mean amount of AOH was 6.5±8.9 µg/kg with a range of its concentration from 0.16 to 33 µg/kg. The most frequently contaminated maize samples from investigated regions, were from Srem in 2016 (16%), while the highest mean concentration of AOH (14±12 µg/kg) was determined in maize samples from Banat in 7% of contaminated samples (Figure 2b). Conversely, in 2017 the highest proportion (15%) of contaminated samples by AOH was recorded in Banat, while the highest mean concentration of AOH (9.4±15 µg/kg) was obtained in maize from Srem. The recorded frequency of prevalence and the mean concentration of AOH in this study are similar to previously published findings from the Republic of Serbia (Janić Hajnal et al., 2020, Part 2). Based on this study and previously published data (Abia et al. 2013; Abdallah et al. 2017; Blandino et al. 2017; Oliveira et al. 2017; Getachew et al. 2018; Oyeka et al. 2019; Topi et al. 2019; Tebele et al. 2020), it can be noted that AOH rarely contaminated maize, while the recorded mean concentration of AOH was the highest in maize samples from the Republic of Serbia. So far, AOH occurred only in Nigeria (Oyeka et al. 2019) and Egypt (Abdallah et al. 2017) with a frequency above 90%, but the recorded mean content of AOH is quite low. The second most common *Alternaria* toxins in maize samples from Serbia was AME and this toxin contaminated 12% of analysed maize samples. Its content ranged from LOD to 14 μg/kg and the established mean concentration was 1.9 ± 3.1 μg/kg. The distribution of contaminated maize samples with AME by regions was as follows: 23%, 13% and 8% from Srem, Banat and Bačka in 2016, and 17%, 6% and 2% from Banat, Srem and Bačka in 2017, respectively. The Figure 2c indicated that the highest mean content of 5.1 ± 8 μg/kg, as well as the highest range of AME content (LOD - 14 μg/kg) were determined in maize samples from Srem in 2017. AME more often and with higher concentrations contaminated maize samples in Serbia in comparison with so far published data (Abia et al. 2013; Abdallah et al. 2017; Blandino et al. 2017; Oliveira et al. 2017; Getachew et al. 2018; Oyeka et al. 2019; Topi et al. 2019; Tebele et al. 2020; Janić Hajnal et al. 2020, Part 2).

ALS was quantified in 10% of maize samples, among 458 analysed maize samples. ALS was quantified for the first time from the Republic of Serbia, with the following range and recorded mean concentration: LOD – 458 μg/kg and 21±70 μg/kg, respectively. The share of contaminated maize samples with ALS by regions was as follows: Banat 16% and 13%, Bačka 8% and 2% and Srem 14% and 6% in 2016 and 2017, respectively.The highest share of contaminated maize samples (16%) and the widest range of quantified ALS (LOD – 458 μg/kg) has been established in maize samples from Banat in 2016 maize growing season (Figure 2d). From the Figure 2d it could be noted, that the highest mean concentration of ALS (86 ± 110 μg/kg) was recorded in maize samples from Bačka in 2017. The relevant data on the occurrence of ALS in maize are unavailable, both from Serbia and other countries, hence the results of our study cannot be compared to any other results. Therefore, the obtained results in this study will contribute to the creation of a database on ALS, ie. knowledge about the distribution of this *Alternaria* toxin in maize.

Based on the obtained results, it can be noticed that TEN rarely occurred in maize samples from Republic of Serbia (Figure 2e). This *Alternaria* metabolite was detected in only 2% of the examined maize samples from Bačka and Srem in 2017 with concentration range from 0.35 to 2.7 µg/kg. According to published data by Janić Hajnal et al. (2020, Part 2), TEN was present in maize samples from each production year (2012-2015), with a low frequency (0% - 10%) and very low level of its mean content (0.1±0.1 µg/kg – 0.3±0.2 µg/kg).

The second *Alternaria* metabolite which was for the first time detected in the maize samples from the Republic of Serbia was macrosporin A. As can be seen from the Figure 2f macrosporin A was not detected in maize samples from 2016. Further, in 2017 (Figure 2f) macrosporin A was detected in only 3% of maize sampels among 181 analysed samples. The share of contaminated samples with macrosporin A in Srem and Bačka in 2017 was 8% and 2%, respectively and its content ranged from 0.22 to 0.65 µg/kg. According to published data by Getachew et al. (2018) and Oliveira et al. (2017) macrosporin A was present with relatively low frequency and low mean content in maize samples from Brazil and Ethiopia respectively. On the other hand, macrosporin A occurred in maize with high frequency, but with relatively low mean content in maize in Nigeria (Oyeka et al., 2019) and Egypt (Abdallah et al., 2017).

3.3 Co-occurrence of investigated *Alternaria* metabolites

The percentage of the co-occurrence of investigated *Alternaria* toxins in maize samples, collected from different regions in the period 2016-2017, are shown in the Figure 3.

Figure 3

As can be seen (Figure 3), in maize samples collected from both maize growing seasons, among contaminated samples, the highest percentage of contaminated samples with one of the detected *Alternaria* toxins was from Bačka (76% in 2016 and 84% in 2017). Further, the highest percentage (25%) of two detected *Alternaria* toxins per sample was observed in samples from Srem in 2017 production year, while from 2016 maize growing season the highest percentage (17%) of co-occurrence of two *Alternaria* toxins in maize samples was observed in Banat. The highest percentage (17%) of co-occurrence of three different *Alternaria* toxins was noted in the samples from Banat in 2016, while none of the maize samples from Bačka in 2017 maize growing season was contaminated with three *Alternaria* toxins at the same time (Figure 3). Co-occurrence of four investigated *Alternaria* toxins was detected in the maize samples from all investigated regions in both years, while the highest percentage (17%) was detected in Banat (2016), followed by Srem (14% in 2016) and Banat (14% in 2017). The findings of this study indicated that TeA, AOH, AME and ALT always co-occurred in case of contamination with four *Alternaria* toxins in the maize samples from Serbia.

The results of this two-year study indicated that about 60% of the total contaminated maize samples contained one *Alternaria* toxin, about 20% two, about 10% three and about 10% four *Alternaria* toxins at the same time.

CONCLUSION

Four and seven different *Alternaria* toxins were detected in maize samples collected in 2016 and 2017 years, respectively. The most common quantified *Alternaria* toxin, in maize samples from both years, was TeA, with the highest frequency of prevalence, as well as with the highest reported mean content. In 2017 production year, for the first time the following *Alternaria* metabolites were detected in maize from the Republic of Serbia: ALS and macrosporin A. Further, the findings in this study indicate that the micro-climate conditions in the investigated regions, as well as the interaction of micro-climate conditions and preceding crops had a great influence on the contamination frequency and determined concentrations of *Alternaria* toxins in maize. However, due to the fact that there are no previously published data related to the examination of influence of the preceding crops, as well as the interaction of weather conditions and preceding crops on the *Alternaria* metabolites, the future research should be aimed at determining the dependence of microclimatic conditions and the applied preceding crops on the occurrence of *Alternaria* toxins in maize. The findings of our previous researches regarding the contamination of cereals from the Republic of Serbia with *Alternaria* toxins, as well as the results of this study indicate the need for continuous monitoring of *Alternaria* metabolites in small grain cereals as well as in maize in order to upgrade the currently existing knowledge and information about these “emerging” mycotoxins.

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Conflict of interest

The authors declare no conflict of interest.

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Captions

Figure 1. Monthly average air temperature (a) and sum of precipitation (b) for the maize growing season (April-September) in 2016 and 2017 in comparison with to long-term average values (1981-2010).

Figure 2. Box plots of *Alternaria* toxins concentrations in maize samples depending on the preceding crops used, regions (Bačka, Banat and Srem) in Republic of Serbia and maize growing season (2016 and 2017).

Figure 3. Percentage of contaminated maize samples by regions per year with different number of *Alternaria* toxins

Table 1. Performance characteristics of the LC-MS/MS method for *Alternaria* toxins detected in the investigated maize samples.

Analyte	LOD (µg/kg)	Apparent Recovery	± RSD (%)
Alternariol	0.10	80.2	± 12.4
Alternariol monomethyl ether	0.15	106.7	± 11.8
Altersetin	1.08	189.8	± 10.4
Tenuazonic acid	30.0	82.7	± 50.9
Tentoxin	0.11	120.9	± 4.0
Infectopyron	13.0	92.0	± 13.2
Macrosporin A	0.13	91.0	± 12.1

LOD - limit of detection; RSD - relative standard deviation.

Figure 1.

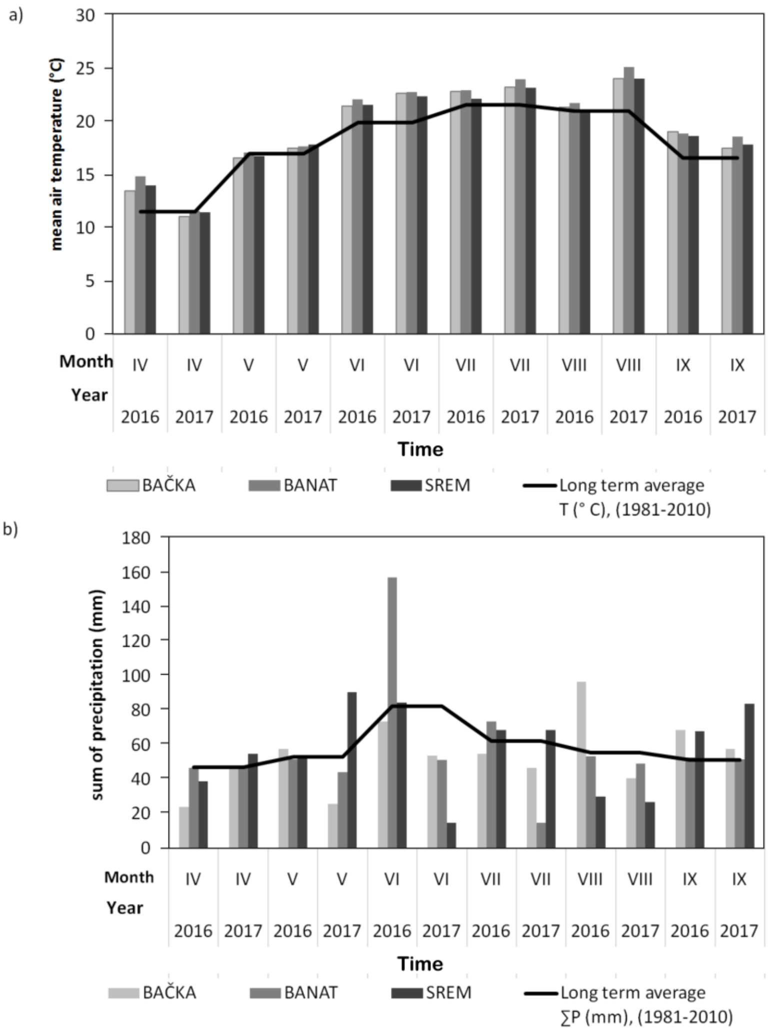

a)
30
25
20
15
10
5
0
mean air temperature (°C)
Month
IV IV V V VI VI VII VII VIII VIII IX IX
Year
2016 2017 2016 2017 2016 2017 2016 2017 2016 2017 2016 2017
Time
BAČKA
BANAT
SREM
Long term average T (° C), (1981-2010)
b)
180
160
140
120
100
80
60
40
20
0
sum of precipitation (mm)
Month
IV IV V V VI VI VII VII VIII VIII IX IX
Year
2016 2017 2016 2017 2016 2017 2016 2017 2016 2017 2016 2017
Time
BAČKA
BANAT
SREM
Long term average ∑P (mm), (1981-2010)

Figure 2.

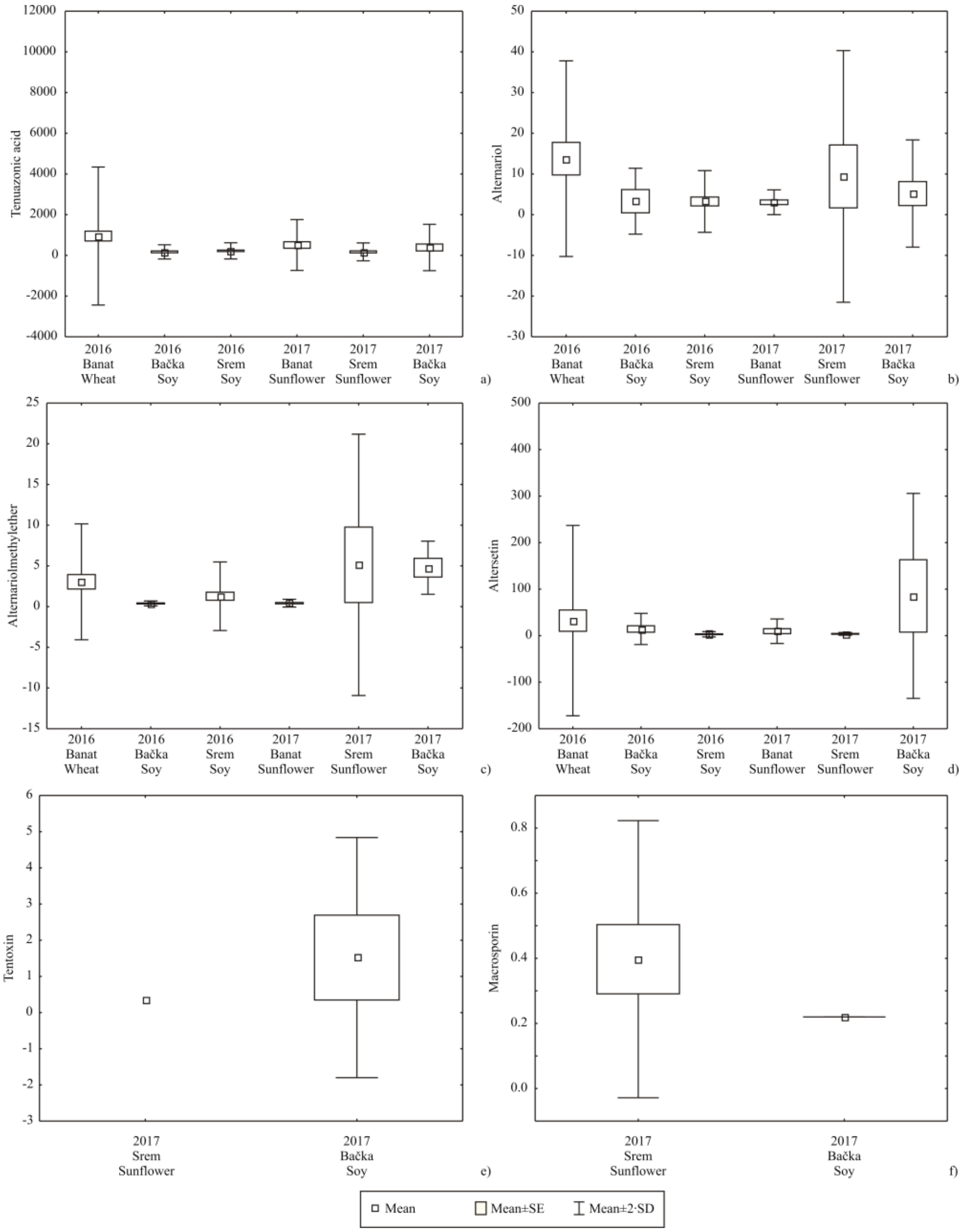
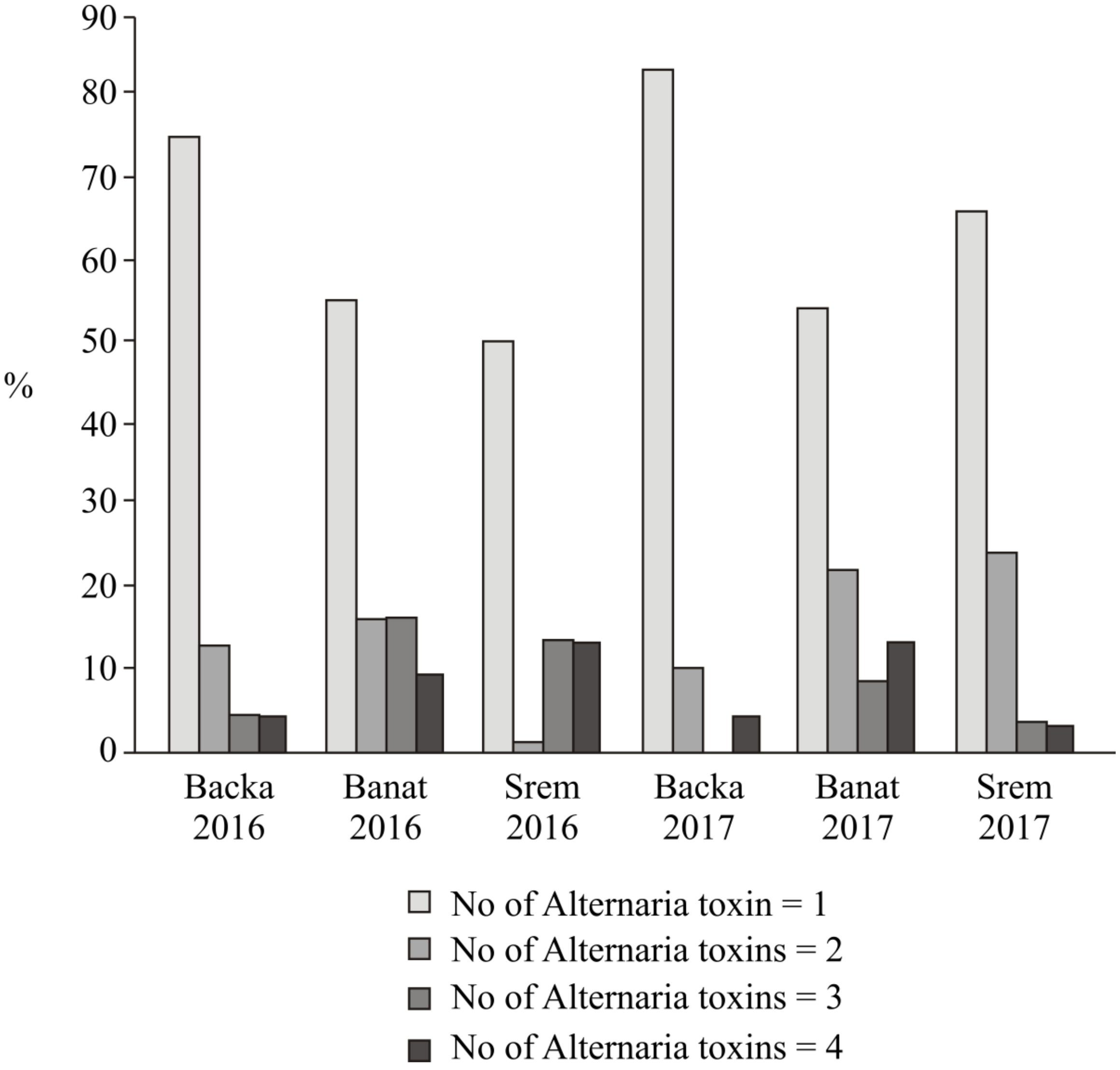

Tenuazonic acid
12000
10000
8000
6000
4000
2000
0
-2000
-4000
2016 Banat Wheat
2016 Bačka Soy
2016 Srem Soy
2017 Banat Sunflower
2017 Srem Sunflower
2017 Bačka Soy
a)
Alternariol
50
40
30
20
10
0
-10
-20
-30
2016 Banat Wheat
2016 Bačka Soy
2016 Srem Soy
2017 Banat Sunflower
2017 Srem Sunflower
2017 Bačka Soy
b)
Alternariolmethylether
25
20
15
10
5
0
-5
-10
-15
2016 Banat Wheat
2016 Bačka Soy
2016 Srem Soy
2017 Banat Sunflower
2017 Srem Sunflower
2017 Bačka Soy
c)
Altersetin
500
400
300
200
100
0
-100
-200
2016 Banat Wheat
2016 Bačka Soy
2016 Srem Soy
2017 Banat Sunflower
2017 Srem Sunflower
2017 Bačka Soy
d)
Tentoxin
6
5
4
3
2
1
0
-1
-2
-3
2017 Srem Sunflower
2017 Bačka Soy
e)
Macrosporin
0.8
0.6
0.4
0.2
0.0
2017 Srem Sunflower
2017 Bačka Soy
f)
Mean
Mean±SE
Mean±2·SD

Figure 3.


%
90
80
70
60
50
40
30
20
10
0
Backa 2016
Banat 2016
Srem 2016
Backa 2017
Banat 2017
Srem 2017
No of Alternaria toxin = 1
No of Alternaria toxins = 2
No of Alternaria toxins = 3
No of Alternaria toxins = 4