doi: 10.17221/119/2016-PSE

Mycotoxins and related *Fusarium* species in preharvest maize ear rot in Poland

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ABSTRACT

This work presents a survey on mycotoxins (seasons 2013 and 2014) and *Fusarium* species (seasons from 1985 to 2014) in maize ear rot in Poland. Twelve mycotoxins were identified in maize kernel samples exhibiting symptoms of *Fusarium* ear rot or rotten kernels at the harvest in two locations in Poland during the seasons 2013 and 2014. This is the first complex survey on the co-occurrence of four *Fusarium* mycotoxin groups in maize kernels: the group of the mycohormone zearalenone; the group of trichothecenes – deoxynivalenol and nivalenol; the group of fumonisins; and the group of cyclic hexadepsipeptides – beauvericin and enniatins; and in addition, moniliformin. Four *Fusarium* species were identified in preharvest maize ear rot in the 2013 and 2014 harvests namely: *F. graminearum*, *F. poae*, *F. subglutinans* and *F. verticillioides.* Since 1985, eleven *Fusarium* species have been identified in 13 investigation seasons. Apart from those mentioned above, *F. avenaceum*, *F. cerealis*, *F. culmorum* and *F. sporotrichioides* were observed with irregular frequencies, and three species, i.e. *F. proliferatum*, *F. tricinctum* and *F. equiseti*, were identified sporadically. A significant increase of *F. verticillioides* frequency and a decrease of *F. subglutinans* frequency and changes of mycotoxin profile have been observed in the two decades since 1995.

Keywords: ascomycota; *Zea mays* L.; pathogenic species; infection; toxic metabolites

Maize ear rot is a disease found worldwide, induced by several toxigenic *Fusarium* species, causing accumulation of several groups of mycotoxins in kernels. The prevailing pathogenic species can vary over the years depending on various factors such as the continent and region, agroecological conditions (Bottalico and Perrone 2002), insect damage (Lew et al. 1991), other stress factors and susceptibility of cultivars (hybrids) to infection by *Fusarium* species and to mycotoxin accumulation in kernels (Pascale et al. 2002).

This research on mycotoxins contaminating maize kernels and toxigenic species in Poland has been conducted for 28 years since 1985, in 13 crop seasons. *Fusarium subglutinans* was found as the prevailing species from 1985 until 1991, and it was accompanied by seven other species. Since 1995 the frequency of *F. verticillioides* has been significantly higher in most years, and this species replaced *F. subglutinans*, whose frequency decreased, in particular in the seasons of 1993 and 2006. *F. poae* has been found recently as a species infecting maize ears and was a species frequently isolated from mouldy maize ears in several crop seasons, with a frequency of up to 20%. Trichothecenes, i.e. nivalenol (NIV), fusarenon X, diacetoxyscirpenol and monoacetoxyscirpenol, were commonly produced by *F. poae* isolates under laboratory conditions as well as being detected in field sample. Maize ear kernels infected by *F. poae* contained significant amounts of beauvericin and enniatins (Chełkowski et al. 1994, Chełkowski 1998, Thrane et al. 2004).

The aim of this study was to examine the natural preharvest co-occurrence of twelve mycotoxins in selected mouldy maize ears affected by *Fusarium* species in the seasons of 2013 and 2014, and to

Supported by the Polish National Science Centre, Project No. 2014/15/B/NZ9/02169.

doi: 10.17221/119/2016-PSE

compare changes in the profile of mycotoxins and *Fusarium* species in Poland after 28 years of surveys. The attention was focused both on zearalenone (ZEA), deoxynivalenol (DON) and nivalenol (NIV) and on beauvericin (BEA), enniatins (Enns), fumonisins (FBs) and moniliformin (MON) to provide more recent results on their co-occurrence in maize kernels.

MATERIAL AND METHODS

Fungal isolation and identification. Maize ear samples were collected in October 2013 (80 maize ear samples) and 2014 (100 maize ear samples) in two locations in maize fields in two main growing areas in Poland (16°56'E, 50°58'N and 52°48'N, 16°83'E). Ears with significant ear rot symptoms were scored for the *Fusarium* ear rot rating (1–100% kernels mouldy, discoloured and shrunken) and placed in separate paper bags, transported to the laboratory and dried at room temperature in the same way as in previous studies. Then to identify *Fusarium* species surface mycelium and small pieces of kernels from each ear were placed in duplicate on agar plates with a low nutrient SNA medium (Nirenberg 1981, Kwaśna et al. 1991). After preliminary identification conidia from each culture were transferred to both potato dextrose agar and synthetic SNA low nutrient agar. *Fusarium* species were identified according to Kwaśna et al. (1991) and Leslie and Summerell (2006).

Molecular analyses: DNA extraction, primers and PCR conditions. To confirm the morphological identification of the *Fusarium* strains genomic DNA extraction from fungal mycelium was performed using the CTAB-based method (Stępień et al. 2011). A partial sequence of the *tef1-alpha* gene was amplified using the Ef728M/Tef1R primer combination according to Parry and Nicholson (1996), Mulè et al. (2004), and Błaszczyk et al. (2005).

DNA sequencing, analysis and comparison to NCBI GenBank sequences. Sequence reading of the *tef1-alpha* gene was performed using the Applied Biosystems equipment. Sequences were compared to the NCBI GenBank-deposited sequences to confirm the correct morphological species identification using the BLASTn algorithm (Megablast). The Department's collection of *Fusarium* strains (the *Fusarium* strains culture collection of the Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland) originating from different host species was included for comparative analysis.

Mycotoxin analyses

Chemicals and reagents. Mycotoxin standards (ZEA, DON, NIV, MON, FBs, Enn A, A1, B and B1, BEA) and all chemicals were supplied by Sigma-Aldrich (Steinheim, Germany). Water of high-performance liquid chromatography (HPLC) grade from our own Millipore water purification system was used for analyses.

Sample preparation, extraction and HPLC analysis. Zearalenone, deoxynivalenol, nivalenol and moniliformin analyses were performed according to the method described by Tomczak et al. (2002) and Goliński et al. (2010). The procedure of extraction, purification and detection of FBs was reported by Waśkiewicz et al. (2010). Beauvericin and enniatins were extracted, identified and quantified by HPLC as described by Jestoi et al. (2004).

Statistical analysis. Arithmetic means and medians of mycotoxins concentrations as well as the frequency of toxin occurrence and infection level (percentage of infected kernels per sample) were calculated using Microsoft Excel (Redmond, USA).

RESULTS AND DISCUSSION

***Fusarium* species infecting preharvest maize ears**. Four *Fusarium* species were identified in preharvest infected maize ears samples examined in the 2013 and 2014 seasons: *F. graminearum*, *F. poae*, *F. subglutinans* and *F. verticillioides*. The morphological identification was completely confirmed by the PCR method. Dominant species in harvest seasons from 1985 to 2014 varied over the years, depending on agroecological conditions and changes of cultivars produced (Table 1). *F. subglutinans* was the dominant species in the seasons from 1985 to 1991, and up to 8 species listed in Table 1 were found in this period in maize ears. Apart from the four species mentioned above, the following species were identified in the first decade: *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. sporotrichioides* and species detected with a low frequency: *F. proliferatum*, *F. tricinctum* and

doi: 10.17221/119/2016-PSE

Table 1. *Fusarium* species isolated from maize with ear rot or kernel rot symptoms in seasons between 1985–2014 in Poland

	Number of samples	Percentage of *Fusarium* species isolates							
		F. aven	*F. cer*	*F. cul*	*F. gram*	*F. poae*	*F. sub*	*F. spor*	*F. vert*
1985	106	7.0	8.0	3.0	5.0	1.5	59.5	5.0	5.0
1986	80	14.0	3.0	8.0	11.0	0	45.0	5.0	9.5
1987	70	40.0	0	0	0	2.0	36.0	6.0	6.0
1988	70	0	5.0	0	7.0	0	85.0	0	3.0
1989	90	1.0	0	1.0	0	7.0	86.0	1.0	3.0
1990	50	0.5	1.0	8.0	8.0	18.0	59.0	1.0	3.0
1991	50	0	4.0	12.0	16.0	9.0	56.0	3.0	3.0
1993	40	73.7	0	2.6	0	7.8	7.8	2.6	0
1995	50	0	0	0	0	0	65.0	0	35.0
1999	48	0	0	0	0	20.0	60.0	3.0	5.7
2006	35	0	0	0	0	0	22.9	0	77.1
2013	80	0	0	0	7.1	45.7	3.1	0	44.1
2014	100	0	0	0	13.2	14.0	26.3	0	46.5

F. aven – *Fusarium avenaceum* (Fries) Saccardo; *F. cer* – *Fusarium cerealis* (Cooke) Saccardo; *F. cul* – *Fusarium culmorum* (W.G. Smith) Saccardo; *F. gram* – *Fusarium graminearum* Schwabe; *F. poae* – *Fusarium poae* (Peck) Wollenw.; *F. sub* – *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas; *F. spor* – *Fusarium sporotrichioides* Sherbakoff; *F. vert* – *Fusarium verticillioides* (Saccardo) Nirenberg (= *F. moniliforme* Sheldon). Rarely occurring species: *F. equiseti* (Corda) Saccardo; *F. proliferatum* (Matsushima) Nirenberg; *F. tricinctum* (Corda) Saccardo

F. equiseti (not listed in Table 1) (Chełkowski et al. 1992, Chelkowski and Lew 1992, Lew et al. 1996).

F. proliferatum – an important toxigenic species in Southern Europe and in the USA – was found rarely in Poland, only in one season (1986) with a frequency below 3% of total *Fusarium* species. This species, of rather minor importance in Poland, is capable of producing the same toxic metabolites as two other species: *F. subglutinans* and *F. verticillioides* (Chełkowski et al. 1992, Chełkowski 1998, Logrieco et al. 1998, Pascale et al. 2002).

F. poae was detected with a high frequency of up to 46% in preharvest infected maize ears in the seasons of 1990–1991, 1999 and 2013–2014 (Table 1). This species is known for its low to medium aggressiveness, but on the other hand it is highly toxigenic and able to produce a significant number of mycotoxins: group A and B trichothecenes, beauvericin, enniatins and fusarin C (Chełkowski et al. 1994, Dinolfo and Stenglein 2014).

The frequency of *F. verticillioides* from the season of 1995 was significantly higher in most years and this species replaced *F. subglutinans*, which frequency decreased, particularly in the seasons of 1993, 2006 and 2013. The same shift of both species was found also in other countries of central Europe (Adler et al. 2002). Maize ear colonization by the above-mentioned two *Fusarium* species is significantly influenced by insect damage caused by such pests as the European corn borer (ECB) *Ostrinia nubilalis* (Lew et al. 1991). The ECB was not found in the collected and examined ears of the 2013 harvest. In the 2014 season a high percentage (41%) of ears exhibiting kernel rot symptoms were injured by the larvae of *Ostrinia nubilalis* alive during harvest.

Cladosporium kernel rot was found in 32.5% of ears in 2013; however, only a few kernels (< 1%) were black coloured and colonized by *C. cladosporioides* as the dominant species. This species was isolated from 35 ears, with co-occurring *Alternaria alternata*, *Epicoccum nigrum* and *Nigrospora* spp. Contamination of maize ears with the above-mentioned species of fungi with black mycelium and

doi: 10.17221/119/2016-PSE

spores may be considered almost non-significant in the seasons of 2013 and 2014, and it was not significant in the previous seasons. In 2014, besides *Fusarium* species, most often *Trichoderma atroviride* (31.0%), *Rhizopus stolonifer* (7.0%) and *Mucor hiemalis* (4.0%) appeared.

Mycotoxins accumulated in maize ears. Twelve mycotoxins were identified in maize ear samples, colonized preharvest by *Fusarium* species in 2013 and 2014 seasons, namely: zearalenone, deoxynivalenol, nivalenol, fumonisins B_1, B_2 and B_3 as well as moniliformin, beauvericin and enniatins A, A_1, B and B_1. The FDK fraction (fraction with disease symptoms) in maize ear samples ranged from 2.6–100%.

The high percentage of the FDK fraction in the total mass of grains does not always indicate the presence of high amounts of mycotoxins. Maize ears with *F. verticillioides* as the dominant species contained very high amounts of FB_1, FB_2 and FB_3, but only 6.6% of kernels were classified as the FDK fraction. Samples with a 100% FDK fraction and *F. poae* as the dominant pathogen contained very low levels of FB_1 and enniatin A_1, respectively. Also other samples where *F. poae* was the dominant species, contained lower amounts of mycotoxins, regardless of the high percentage of the FDK fraction. This result is different from our previous results indicating significant amounts of beauvericin and enniatins accumulated in maize samples identified as affected by *F. poae* (Chełkowski et al. 2007).

Figure 1 presents a comparison of mycotoxin occurrence frequencies during two study years. Eight mycotoxins – ZEA, DON, NIV, FB_1, FB_2, FB_3, MON and BEA – were identified in 51.3% of samples examined in the season of 2013. DON and ZEA were present in the highest number of kernel samples with a similar frequency, up to 82% and 81%, respectively, and in most samples both toxins usually occurred together, although the ZEA:DON ratio varied greatly. Other mycotoxins relatively prevalent in corn grain are the fumonisins FB_1 and FB_2, as well as NIV, MON and BEA. Their frequency of occurrence ranged from 55–59%, the latter three compounds being present at a significantly lower level. The least often encountered mycotoxins in the analysed samples were enniatins, with their frequency ranging from 3.8–36%. Enniatin A was found only in 3.8% of examined samples at a level below 0.04 mg/kg.

The incidence of ZEA presence (100%) as well as fumonisin FB_1 (99.0%) significantly increased in 2014. Frequency of MON and BEA occurrence also increased to 92.1% and 78.6%, respectively. Enniatin A, which in 2013 appeared occasionally was present in 23.6% of the analysed samples in 2014. A significant decrease in the frequency of enniatin B_1 from 21.8% in 2013 to absence of the metabolite in 2014 was found.

The average and maximum mycotoxin contents of two study years (2013 and 2014) are summarized in Table 2. Our results showed that the same samples containing high levels of ZEA and DON were found in southern Poland. NIV content increased 56-fold in 2014 compared to 2013; the maximum toxin content was 368.2 mg/kg (FDK 100%), and in 13.5% of all samples it was higher than 10.0 mg/kg. Increase in NIV content was probably caused by both environmental factors (weather conditions)

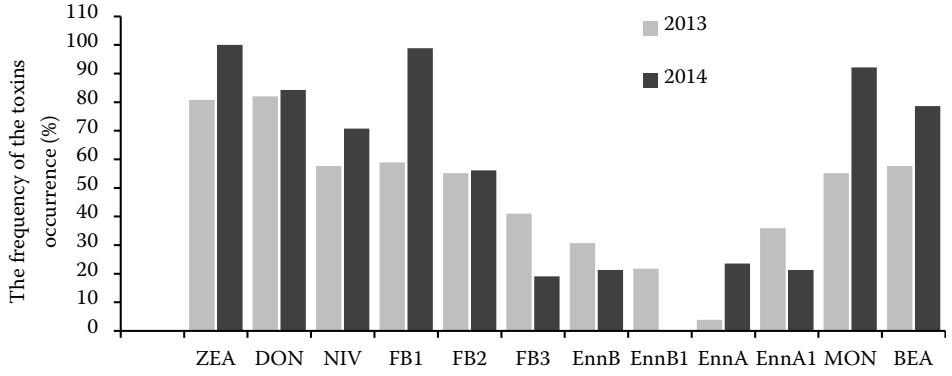

Figure 1. Frequency of mycotoxin occurrence during two years of studies: 2013 (78 samples) and 2014 (90 samples). ZEA – zearalenone; DON – deoxynivalenol; NIV – nivalenol; FBs – group of fumonisins; Enns – enniatins; MON – moniliformin; BEA – beauvericin

doi: 10.17221/119/2016-PSE

Table 2. Average and maximum content (mg/kg) of mycotoxins in *Fusarium* damaged kernels collected during 2013 (78 analysed samples) and 2014 (90 analysed samples) growing seasons

Toxin	2013		2014	
	average mycotoxin content	maximum mycotoxin content	average mycotoxin content	maximum mycotoxin content
ZEA	0.97	16.20	2.54	30.13
DON	6.15	134.36	90.69	1505.00
NIV	0.30	5.72	17.97	368.20
FB1	44.46	525.92	2.31	103.21
FB2	10.73	209.72	0.47	31.85
FB3	2.09	37.54	0.13	7.24
EnnB	2.36	11.53	9.42	194.10
EnnB1	0.93	64.71	nd	nd
EnnA	nd	nd	2.81	63.00
EnnA1	21.75	148.93	6.35	58.60
MON	0.29	3.10	5.29	118.50
BEA	4.33	256.50	43.67	445.50

ZEA – zearalenone; DON – deoxynivalenol; NIV – nivalenol; FBs – group of fumonisins; Enns – enniatins; MON – moniliformin; BEA – beauvericin

and mycological (increased incidence of *F. graminearum* occurrence).

The results showed that there was no sample in which all the analysed toxins were simultaneously present, or none was totally free of these compounds. In 2013 the maximum number of mycotoxins identified in one sample was nine (in 10.2% of maize ear samples). Eight and seven different toxins were found in 12.8% and 10.2% of maize ear samples, respectively. In other samples usually 4 to 6 compounds were identified; however, in one maize ear sample only one mycotoxin was present – DON. Comparing the obtained values with the maximum levels permitted by the EU Directives (EC, 2007) the limit for zearalenone was exceeded in 25.6% of samples in 2013 and 36.6% in 2014. As regards the DON and FBs contamination in 2013, the maximum toxins content was exceeded in 19.2% and 28.2% of the samples, respectively. In 2014, the acceptable range of the mycotoxins level was exceeded in 56.7% of samples in case of DON and 1.1% for fumonisins.

A significant shift of mycotoxins that accumulated in maize kernels before harvest was found in the seasons of our experiments from 1985 to 2013. Kernels infected by *F. subglutinans* in the first decade of our survey accumulated beauvericin up to 60 mg/kg and moniliformin up to 425 mg/kg (Lew et al. 1996). Moniliformin was found in maize kernels at the harvest of 2013 at a very low level up to 3 mg/kg, in most samples at 0.01 mg/kg. On the other hand, in the second decade of the survey kernels infected by *F. verticillioides* accumulated fumonisins B_1 and B_2 up to 273.2 mg/kg and up to 102.6 mg/kg, respectively (Lew et al. 1996), while in 2013 the content of fumonisins was two times higher.

Examination of interactions between *F. graminearum* and *F. verticillioides* showed that previous maize colonization by aggressive species *F. graminearum* in maize ears might promote subsequent infections by *F. verticillioides* (Picot et al. 2012), and it is probably true for other weak pathogens such as *F. subglutinans* and *F. poae*, according to the well-known rule that an aggressive pathogen facilitates later plant infection by a weak pathogen.

In the previous decade *F. poae* contributed to accumulation of a high number of mycotoxins, namely toxic hexadepsipeptides – both beauvericin up to 46.0 mg/kg and enniatins: enniatin A up to 37 mg/kg, enniatin B up to 46 mg/kg and enniatin B_1 up to 75 mg/kg (Chełkowski et al. 2007).

It is difficult to compare the obtained results in Poland with other European countries because mycotoxins occurrence depends on many factors: weather conditions, resistance of cultivars, agriculture practice. However a significant difference between seasons (2006 and 2007 harvest in Germany) was also observed by Goetz et al. (2010). DON and FB_1 maximum content (mg/kg) was reduced from 19, 57 to 16.25 in case of DON and from 20.69 to 0.05 for fumonisin. The ZEA contamination was increased from 0.86 in 2006 to 14.58 in 2007.

Both conducted studies and literature sources show that grain contamination with fungal metabolites is of prime concern. The health hazard posed by mycotoxins in general to humans and animals is now well recognized. Great concern has been shown in recent years concerning control of these toxins, and prevention of mycotoxin contamination in grain is the main objective of food and agricultural

doi: 10.17221/119/2016-PSE

industries throughout the world. Contamination of various cereal grains with *Fusarium* and mycotoxins is inevitable under the rapidly changing environmental conditions. While certain treatments have been found to reduce contamination with specific mycotoxins, no single method has been developed that is equally effective for a wide variety of mycotoxins (Ingle et al. 2010).

In conclusion, prevention of *Fusarium* mycotoxin formation by proper agronomic practices before and after maize seeding to avoid grain contamination is very important. As *Fusarium* mycotoxins are produced within the growing crop, it is important to understand how agricultural practices affect final mycotoxin contamination of grain. Such information could then be used to recommend guidelines on Good Agricultural Practice to minimize mycotoxin contamination of maize products.

Deoxynivalenol, zearalenone and fumonisins have so far been recognized as predominant *Fusarium* mycotoxins, present with a high frequency in maize grain samples. In view of the fact that lower amounts of ZEA may affect human and animal health, in the future more attention should be paid to the simultaneous accumulation of zearalenone, DON and fumonisins in grains. Apart from the above-mentioned three groups of mycotoxins, several other toxic *Fusarium* metabolites, i.e. moniliformin, beauvericin, enniatins, fusaproliferin, and trichothecenes of group A, are more and more frequently identified in FDK maize kernels. Also aflatoxins have been found to be a significant grain contaminant in some regions of the world.

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Received on February 17, 2016
Accepted on July 4, 2016

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