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Fumonisins and Related *Fusarium* Species in Pre-harvest Maize Ear Rot in Poland

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Two *Fusarium* species were identified in mouldy maize ears with the highest frequency during 2005–2014 in 7 seasons: *F. subglutinans* (3.1–42.0%) and *F. verticillioides* (44.1–70.3%). Two other species were also found but with lower frequency: *F. graminearum* (1.0–13.0%) and *F. poae* (1–45.7%). In 2005 fumonisin FB_1, and in 2013 and 2014 three fumonisins (FBs) – FB_1, FB_2 and FB_3 – were identified in harvest samples. The *Fusarium*-damaged kernel (FDK) fraction contained almost the totality of mycotoxins (90.0–95.0%), while healthy looking kernels (HLK) contained only below 5.0 to 10.0%. Kernels naturally infected by *F. verticillioides* and *F. proliferatum* contained (in mg kg^{-1}) up to 710.00 of fumonisin B_1, up to 209.72 of fumonisin B_2 and up to 35.72 of fumonisin B_3.

Keywords: mycotoxins, maize ear rot, *Fusarium* species, fumonisins

Introduction

Maize (*Zea mays* L.) is a crop widely grown around the world. Among the main concerns are: maize kernel rot, ear rot, seedling blight, stalk rot, reduction in crop yield worldwide and also grain contamination with mycotoxins (Abbas et al. 2006; Arino et al. 2007). *Fusarium* species are characterized by exceptional intraspecies and interspecies variability with respect to morphological, physiological, toxigenic and genetic properties (Tancic et al. 2012). An important part of an effective crop protection strategy is monitoring the *Fusarium* species associated with maize as well as with the specific environment. Temperature may be one factor that determines the extent of invasion of the stalk and ear by *Fusarium* spp. by affecting both plant and fungal growth. *Fusarium verticillioides* is more common in regions with hot growing conditions, especially before or during pollination (Pascale et al. 2002; Murillo-Williams and Munkvold 2008). The most important secondary metabolites produced by *F. verticillioides* include group B fumonisins (fumonisin B_1, B_2, B_3).

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Discovery of fumonisins in 1988 contributed to finding significance of fumonisin B_1 and derivatives as frequent contaminants of maize grain. Fumonisin B_1 was discovered in *F. verticillioides* cultures and caused field outbreaks of leukoencephalomalacia in horses and porcine pulmonary oedema in swine, and was found to be hepatotoxic and hepatocarcinogenic to rats (Bezuidenhout et al. 1988; Gelderblom et al. 1988).

The fumonisin content in kernels depends on the origin of isolates, on susceptibility of the host plant and the geographic region (Czembor et al. 2015). Fumonisin production in maize in Poland was restricted to two species, *F. verticillioides* and *F. proliferatum* (Waśkiewicz et al. 2010; Stępień et al. 2011). In commercially available, processed corn products for human consumption (ground corn grain, corn meal, grits, polenta, semolina, cornflakes and sweet corn) contamination with these toxins typically does not exceed 1000 mg kg^{-1}, although in some countries it is higher.

Undesirable effects of FBs in animal and human organisms result from the similar chemical structure of fumonisin B_1 compared to sphingosine and sphinganine – substrates of ceramide synthetase, being a key enzyme in the biosynthesis of sphingolipids. FB_1 as a specific inhibitor of this enzyme also inhibits the formation of sphingolipids, leading to a reduction of their contents in eukaryotic cells as well as in serum, kidneys, the liver and urine (Direito et al. 2009). Inhibition of sphingosine biosynthesis and increase in sphinganine concentration is the most sensitive indicator of exposure to fumonisins. Disruption of sphingolipid metabolism probably also explains the important biological effects caused by these toxins, e.g. disorders in the cell cycle, an increase in oxidative stress, and cell apoptosis followed by necrosis (Domijan et al. 2008). Based on numerous data concerning contamination of agricultural products with fumonisins and the diseases they cause, the International Agency for Research on Cancer (IARC) in 2002 classified fumonisin B_1 among substances probably carcinogenic to humans (class 2B) (IARC 2002). Moreover, in 2007 the Regulation of the EC Commission no. 1126/2007 updated the highest admissible concentrations for the two most important fumonisins, B_1 and B_2, found in corn and its processed products (European Commission Regulation EC 2007).

The aim of the study was to examine the level of fumonisin accumulation in maize kernel samples with pre-harvest maize ear rot in Poland collected in locations within the most intensive maize growing regions and related *Fusarium* species in seasons between 2005 and 2014.

Materials and Methods

Fungi isolation and identification

Maize ear samples (total number 1001) were collected in October of 2005–2009 and 2013–2014 in three locations of the most intensive maize growing fields in Central East, Central West and Southern West Poland. Ears with significant ear rot were scored for *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. Then to identify *Fusarium* species, surface mycelium and a small piece of kernel from each ear

were placed on agar plates in duplicate with low nutrient SNA medium (Nirenberg 1981; Kwaśna et al. 1991). After preliminary identification conidia from each culture were transferred both to potato dextrose agar and synthetic SNA low nutrient agar. The identification of *Fusarium* species was made according to Nelson et al. (1983), Kwaśna et al. (1991) and Leslie and Summerell (2006) manuals.

Kernels of ears were manually separated into two fractions: *Fusarium*-damaged kernels (FDK) and healthy looking kernels (HLK – symptomless kernels). Then kernels of the FDK fraction of samples colonized by the species *F. verticillioides* and *F. proliferatum* were subjected to chemical analysis of mycotoxins using methodologies described below.

Molecular analyses: DNA extraction, primers and PCR conditions

To confirm the morphological identification of the *Fusarium* strains isolated from maize, genomic DNA extraction was done using a CTAB-based method (Mulé et al. 2004; Stępień et al. 2011). A partial sequence of the *tef1-alpha* gene was amplified using the Ef728M/Tef1R primer combination according to our previous paper (Błaszczyk et al. 2005; Stępień et al. 2011).

DNA sequencing, analysis and comparison to NCBI GenBank sequences

PCR-amplified DNA fragments for sequence analysis were purified with exonuclease I [Epicentre, Madison, WI, USA] and shrimp alkaline phosphatase [Promega, Madison, WI, USA] using the following program: 30 min at 37 °C, followed by 15 min at 80 °C. Both strands were labelled using the BigDyeTerminator 3.1 kit [Applied Biosystems, Foster City, CA, USA], according to Błaszczyk et al. (2005) and the manufacturer's instructions. To remove the remains of the reagents, labelled fragments were precipitated with ethanol. Sequence reading was performed using Applied Biosystems equipment.

Sequences obtained were compared to the NCBI GenBank-deposited sequences to confirm the correct morphological species identification using the BLASTn algorithm (MEGABLAST). Our own collection of *Fusarium* strains originating from different host species was included for comparative analysis.

Mycotoxin analyses

The content of fumonisin B_1 was examined in corn kernels in 2005, and three fumonisins (B_1, B_2 and B_3) were identified in maize ear samples in seasons 2013–2014.

Chemicals and reagents

Standards: Fumonisin B_1, B_2 and B_3 were provided by Sigma-Aldrich (Steinheim, Germany).

Solvents: Acetonitrile and methanol (HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany).

Reagents: Glacial acetic acid, 2-mercaptoethanol, *o*-phthaldialdehyde, *o*-phosphoric acid, sodium tetraborate, sodium dihydrophosphate, dipotassium phosphate, potassium chloride and paper filter (Whatman 4) were provided by Sigma-Aldrich (Steinheim, Germany). Water (HPLC grade) was obtained from MilliQ system (Millipore, Billerica, MA, USA).

Sample preparation, extraction and HPLC analysis

The procedure of extraction and purification of FBs was reported in detail previously (Waśkiewicz et al. 2012). Purified FBs were quantitatively determined by the HPLC/FLD method. The fumonisin B_1, B_2, and B_3 standard (5 µl) or extracts (20 µl) were derivatized with 20 or 80 µl of the *o*-phthaldialdehyde (OPA) reagent. After 3 min, the reaction mixture (10 µl) was injected onto an HPLC column. Methanol sodium dihydrogen phosphate (0.1 M in water) solution (77:23, v/v) adjusted to pH 3.35 with *o*-phosphoric acid, after filtration through a 0.45 µm Waters HV membrane, was used as the mobile phase with a flow rate of 0.6 ml min^{-1}. A Waters 2695 apparatus (Waters Division of Millipore, Milford, MA, USA), with a C-18 Nova Pak column (3.9 × 150 mm) and a Waters 2475 fluorescence detector (λ_{Ex} = 335 nm and λ_{Em} = 440 nm) were used in the metabolite quantitative determination. The limits of detection (LOD) were 1.0 ng g^{-1} for FB_1, FB_2, and FB_3. The limit of quantification (LOQ) was calculated as three times the LOD. Positive results (on the basis of retention time) were confirmed by HPLC analysis and compared with the relevant calibration curve (correlation coefficients for FB_1, FB_2, and FB_3 were 0.9967, 0.9983, and 0.9966, respectively). The high correlation coefficients (R^2) of the calibration curves show good linearity of the method in the range of 0–100 ng g^{-1}. Samples in which the content of fumonisin went beyond the curve were diluted. Recoveries for FB_1, FB_2, and FB_3 were 93, 96, and 87% respectively, which were measured in triplicate by extracting the mycotoxins from blank samples spiked with 1.0–100 ng g^{-1} of the compound. The relative standard deviations were less than 8%.

Statistical analysis

Arithmetic means and medians of mycotoxin concentrations as well as the frequency of toxin occurrence and infection level (percentage of infected kernels per sample) were calculated using Microsoft Excel.

Recovery rates were estimated in triplicate by extracting mycotoxins from blank samples spiked with 1.0–00 ng g^{-1} of the compounds. The limits of detection (LOD) corresponded to the concentration that gave a signal-to-noise ratio of 3:1. Precision of the method was evaluated as the relative standard deviation (RSD) of replicate (n = 6) measurements of blank samples spiked with mycotoxins at 10 ng g^{-1}.

Results

Fusarium *species infecting pre-harvest maize ears*

Two species were identified as dominating in pre-harvest *Fusarium*-infected maize ear samples examined in the 2005–2014 season: *F. subglutinans* and *F. verticillioides*, representing 3.1–43.1% and 44.1–70.3%, respectively, of the total number of isolates. It is shown in Table 1 that prevailing species in harvest seasons of 2005–2014 varied over the years, depending on agroecological conditions. *F. subglutinans* frequency was high in seasons 2005, 2007 and 2009, while *F. verticillioides* was found at a higher frequency in all seasons of studies. Frequency of the species *F. graminearum* was 1–13% and of *F. poae* 1–45.7%. The species *F. proliferatum* was identified only in two seasons (2006, 2014), with frequency of about 2%. Apart from the five species mentioned above, the following species were identified: *F. avenaceum*, *F. culmorum*, *F. sporitrichioides* and species not listed in Table 1 with low frequency: *F. tricinctum* and *F. equiseti.*

Identification of 24 *Fusarium verticillioides* isolates was confirmed using diagnostic sequences of the *tef1alpha* gene and species-specific primers. Sequences of the *tef1alpha* gene were compared with the GenBank nucleotide sequence database and showed 99–100% homology to type cultures sequences. Both molecular methods gave the same results and differentiated isolates belonging to the closely related species *F. proliferatum*, *F. subglutinans* and *F. verticillioides* as well as other *Fusarium* species.

Comparing the two years 2013 and 2014, there was found a decrease in the percentage of FDK fraction but a slight increase of the frequency of *Fusarium verticillioides* occur-

Table 1. Fusarium species isolated from maize with ear rot or kernel rot symptoms in seven seasons between 2005–2014 in Poland

	Percentage of *Fusarium* species isolates							
	F. aven	*F. prolif*	*F. cul*	*F. gram*	*F. poae*	*F. sub*	*F. spor*	*F. vert*
2005	0	0	3.0	1.0	1.0	42.0	1.0	46.0
2006	0.3	2.5	0.3	0.3	0	19.0	0	68.0
2007	0	0	0	7.8	0	43.1	0	46.6
2008	0	0	0	4.5	0	15.3	0	70.3
2009	0.9	0	0.9	10.3	0	30.2	0	53.5
2013	0	0	0	7.1	45.7	3.1	0	44.1
2014	0	2.0	0	13.2	14.0	26.3	0	46.5

F. aven – Fusarium avenaceum (Fries) Saccardo
F. prolif – Fusarium proliferatum (Matsushima) Nirenberg
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)

rence. Such a significant decrease in the fumonisins content in 2014 could also be associated with the co-occurrence of the other *Fusarium* species *F. graminearum* and *F. poae*. In 38.2% of the samples collected in 2013 there were isolated at least two *Fusarium* species, out of which in 23.8% there was *F. graminearum*. In the next year of studies presence of two *Fusarium* species was found in 46.4% of total samples, and *Fusarium graminearum* participation increased to 34.5%. The change of *Fusarium* species may be related to the decrease in fumonisin content and a significant increase of content of other toxins: zearalenone, deoxynivalenol, nivalenol, moniliformin and beauvericin (paper in preparation).

Mycotoxins accumulated in maize ears

Amounts of *Fusarium* mycotoxins in FDK and HLK fractions in previous seasons indicated that the FDK fraction accounted for almost the totality (up to >95%) of fumonisins in the isolate inoculated by *F. verticillioides* (or *F. proliferatum*) as well as in naturally infected ears. This fraction of kernels was visibly mouldy and covered with mycelium (Pascale et al. 1999 and 2002). The HLK fraction in our previous studies contained very low amounts of mycotoxins. Therefore, the authors decided that mycotoxin analysis would be carried out only in the fraction with disease symptoms (FDK).

The average content of the FDK fraction of maize kernels in different years (2005–2014) was in the range from 24.81% in 2009 to 43.81% in 2013. In spite of climate

Table 2. Fumonisins content in pre-harvest maize ear rot in Poland, colonized by *F. verticilliodes* and *F. proliferatum*

Year	Positive samples [%]	Min	Max	Average
FB_1 [mg kg^{-1}]				
2005	100.0	0.150	710.00	119.25
2013	61.8	0.045	525.92	99.19
2014	98.2	0.008	103.21	3.19
FB_2 [mg kg^{-1}]				
2005	–	–	–	–
2013	58.2	0.003	209.72	25.85
2014	53.6	0.002	31.85	1.33
FB_3 [mg kg^{-1}]				
2005	–	–	–	–
2013	41.8	0.004	35.72	7.07
2014	17.9	0.002	7.24	1.10

changes and the use of different maize varieties over the years, the average percentage of fractions with disease symptoms was rather constant. However, an increase of samples with the maximum content of FDK fraction from 77.6 (2005) to 100% (2013, 2014) was found in several samples.

The percentage of FDK fraction of the total mass of grains does not always indicate the presence of high amounts of mycotoxins. For example, a sample with *F. verticillioides* as the dominating species contained very high amounts of FB_1 (525.92 mg kg^{-1}), FB_2 (209.72 mg kg^{-1}) and FB_3 (37.54 mg kg^{-1}), but only 6.6% of kernels were found in the FDK fraction.

In 2005 the fumonisin B_1 content reached an average of 119.25 and a maximum of 710 mg kg^{-1} (Table 2). Maize samples examined in the 2013 season contained: fumonisins B_1, B_2 and B_3 up to 525.92, 209.72 and 37.54 mg kg^{-1}, respectively. Samples examined in 2014 contained significantly lower amounts of fumonisins B_1 with a maximum at 103.21 mg kg^{-1}, and it was difficult to explain those results (Table 2).

Discussion

The frequency of *F. verticillioides* from the 1995 season was significantly higher in most years and this species replaced *F. subglutinans*, whose frequency decreased, in particular in the 2006 and 2008 seasons. The same shift of both species was also found in other countries of central Europe (Adler et al. 2002). Ears of the 2013 and 2014 harvest were colonized frequently (>38% and 46% of samples, respectively) with two species: *F. poae* + *F. verticillioides* or *F. verticillioides* + *F. graminearum.* Maize ear colonization by the two mentioned *Fusarium* species was significantly influenced by insect damage caused by such pests as the European corn borer (ECB) *Ostrinia nubilalis* (Lew et al. 1991; Munkvold et al. 1997). We did not find the European corn borer in collected and examined ears before the 2014 harvest. On the other hand, in 2014 a high percentage of ear samples exhibiting kernel rot were injured by *Ostrinia nubilalis* larvae (paper in preparation). *Aspergillus flavus* in examined samples of maize ears was not found.

It should be emphasized that *F. verticillioides* may be a primary causal agent of disease, a secondary invader or an endophyte and systemically colonizes kernels. The fungus infects the emerging maize seedlings, the maturing plant and the new kernel. This species was also frequently recovered from healthy maize seeds (Pamphile and Azevedo 2002). Another endophyte of maize, *Acremonium zeae*, was found to be a producer of antibiotics inhibitory to *F. verticillioides* and *Aspergillus flavus* (Wicklow et al. 2005). Interaction of *F. verticillioides* species with maize plant and with other fungi including pathogens *F. graminearum* and *F. poae*, with endophytes such as *Acremonium zeae* and hyperparasites such as *Trichoderma*, is very complex and may influence final contamination of kernels with fumonisins.

Recently we have focused attention on interaction of some *Trichoderma* species with toxigenic *Fusarium* species including *F. verticillioides*. *T. harzianum* was able to reduce growth of *F. verticillioides* and production of mycotoxins as well (paper in preparation). *Trichoderma atroviride* with high inhibitory activity against various *Fusarium* species

was isolated from maize ears from the 2005 harvest and with high frequency in 2014. Isolates of these species were able to significantly reduce growth and mycotoxin production of highly pathogenic *Fusarium* species (Gromadzka et al. 2009; Jeleń et al. 2013).

Fumonisins identified in maize samples represent well-recognized mycotoxins with proven toxicological and economic importance. Contamination of food commodities by fumonisins has become a serious food safety problem throughout the world. Consumers and farmers are more and more aware that the fumonisins constitute a real threat to human and animal health. In Italy, higher levels of fumonisin B_1 were often found (250.0 mg kg^{-1}) than those reported for other European countries (Bottalico 1998). Maize kernel samples infected by *F. verticillioides* examined in the second decade of experiments (seasons 1992–1993) accumulated fumonisins B_1 and B_2 in amounts up to 273.2 mg kg^{-1} and 102.6 mg kg^{-1}, respectively (Chełkowski et al. 1994). In Croatia fumonisin B_1 and B_2 occurred with the highest and mean concentrations of positive samples (FB_1+FB_2) at 11.66 and 0.65 mg kg^{-1}, respectively (Jurjevic et al. 1999). According to Demir et al. (2010), in Turkey fumonisin B_1 contamination ranged from 0.05 to 25.72 and B_2 from 0.05 to 5.7 mg kg^{-1}. In our studies FB_1 in the FDK fraction was detected at a much higher concentration of up to 710.0 mg kg^{-1} (Table 2). In this maize sample the percentage of the fraction with disease symptoms was very low (1.2%). However, during the studies also maize ears were completely colonized by the fungus (FDK 100%) at simultaneously a high content of toxins (FB_1 – 305.17 mg kg^{-1}). Czembor et al. (2015) examined the fumonisin B_1 occurrence during 2011–2012 seasons in Poland. All maize kernel samples contained FB_1, with average fumonisin B_1 contamination ranged from 0.005 to 1.19 mg kg^{-1}.

The problem of fumonisin-contaminated cereals is particularly important in Africa, where maize is the human staple food and is consumed without any processing. In Eastern and Southern Africa fumonisin B_1 was detected at concentrations ranging from 0.002 to 1.91 mg kg^{-1} (92.5% positive samples), while the sum of fumonisin ($B_1 + B_2 + B_3$) concentrations in the same samples ranged from 0.002 to 2.73 mg kg^{-1}. The results of frequent fumonisin occurrence at a high level combined with very high maize intake indicated that FB_s levels in maize from Africa regularly exceed the tolerable daily intake for fumonisins (Waalwijk et al. 2008). These are alarming quantities in particular taking into account the maximum recommended levels of this toxin according to the guidance values. The legislation in Europe (EC 2007) set a limit for fumonisins $B_1 + B_2$ of 1 mg kg^{-1} in maize intended for direct human consumption, and some samples the in literature exceed this limit.

There are promising results on lower accumulation of fumonisins in grains of transgenic Bt maize hybrids than in commercial varieties. Currently available commercial maize hybrids express Cry proteins in kernels and demonstrated low susceptibility to feeding by ECB. The use of Bt hybrids has been widely accepted in the United States but still has encountered opposition in Europe. Transgenic maize hybrids expressing Cry IA(b) protein in kernels can experience less *Fusarium* infection and insect feeding. Fumonisin accumulation in Bt hybrids was consistently lower than in non-transgenic hybrids (Munkvold et al. 1997, 1999; Ostry et al. 2010). Wu et al. (2004) estimated that Bt

corn saves farmers in the United States about $17 million annually through reduced fumonisin and deoxynivalenol damage alone. The benefits in mycotoxin reduction could be more significant in developing countries, particularly in regions where corn is a staple in the human diet.

The results showed that *F. verticillioides* was highly pathogenic to maize ears under field conditions. This species may contribute to significant contamination of maize with at least three toxic metabolites (fumonisins B_1, B_2 and B_3).

Prevention of *Fusarium* mycotoxin formation by proper agronomical 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 (GAP) to minimize the mycotoxin contamination of maize products.

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