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Influence of the cultivar and nitrogen fertilisation level on the mycotoxin contamination in winter wheat

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RESEARCH ARTICLE

Abstract

Toxigenic *Fusarium* spp. are common pathogens of wheat and other cereals worldwide resulting in severe reductions of grain yield. Mycotoxins – secondary metabolites produced by a range of widespread fungi, including *Fusarium* – are capable of causing diseases in plants, animals and humans. Two research hypotheses were verified in this work, namely: (1) various wheat cultivars exhibit substantially varied resistance to *Fusarium* mycotoxins; and (2) large doses of nitrogen fertilisers that facilitate the development of dense cereal fields also create favourable conditions for the biosynthesis of *Fusarium* mycotoxins. The two nitrogen fertilisation levels tested in this work were 120 and 200 kg N/ha. The mycotoxin levels in several wheat cultivars grown during 2013-2015 in the Osiny Station, which belongs to the Kępa-Puławy Experimental Station in Poland, were investigated. The analysed mycotoxins included not only those commonly found in wheat but also deoxynivalenol/zearalenone derivatives and enniatins, which, even if more rarely described in the literature, may also pose a threat to food safety. Three groups were identified from the perspective of wheat susceptibility to mycotoxins biosynthesis, namely: (1) relatively resistant Astoria and Fidelius cultivars; (2) medium-susceptibility Oxal, Kepler, Forkida, and KWS Dacanto cultivars; and (3) Bamberka, Kampana and Meister cultivars, which, compared to the others, accumulated most of the mycotoxins. It was found that the majority of the investigated mycotoxins accumulated in wheat significantly more at the 200 kg N/ha nitrogen fertilising level than at the 120 kg/ha level. A preliminary statistical analysis of the acquired data revealed some correlation between the mycotoxin concentrations and both the wheat cultivar and the nitrogen fertilisation level.

Keywords: *Fusarium* mycotoxins, nitrogen fertilisation, winter wheat, cultivar, accumulation of mycotoxins

1. Introduction

By volume of produced cereals, common wheat (*Triticum aestivum* L.) is worldwide the third plant right after maize and rice. With 11.6 million tonnes produced in 2014 Poland was the 15th largest wheat producer in the world and the 4th largest in EU (CSO, 2016). Wheat is mainly used in bakery and confectionery, but also in fodder fed to numerous species of bred animals. Healthy grain without any contaminants is needed in any food/feed application. While cereal plants are generally susceptible to diseases caused by *Fusarium* fungi, wheat is especially susceptible to *Fusarium* head blight (FHB), a disease very frequently caused by *Fusarium graminearum*, but also by *Fusarium culmorum* and *Fusarium avenaceum*. Symptoms appear within the plant growth period (Wegulo *et al.*, 2015). Grain infected with *Fusarium* is of low quality, yield may drop by even 30% (Hajdukowski *et al.*, 2005). Incidence of various fungi species is strongly correlated with climate, first of all with average air temperature and relative humidity. *F. graminearum* live mainly in warm and moist regions of North America, East Europe, Australia, and south China, while *F. culmorum* prefer somewhat cooler regions, e.g. West Europe (Parry *et al.*, 1995). This is not, however, a fixed rule; *F. culmorum*, *F. avanaceum* and *Fusarium poae* have been sometimes identified as the cause of FHB

in Poland (Lenc *et al.*, 2015). Metabolites synthesised by *Fusarium* and other species of fungi are commonly referred to as mycotoxins. Mycotoxins are capable of causing disease and death in plants, animals and humans, as demonstrated by their carcinogenic, mutagenic and teratogenic (toxic for reproduction) properties (Goliński *et al.*, 2009). The most widely recognised mycotoxins produced by *Fusarium* fungi include substances belonging to the group of trichothecenes A and B (deoxynivalenol (DON), nivalenol and the T2/HT-2 toxins), fumonisins, and zearalenone (ZEA); however, the significance of enniatins (ENNs) and beauvercin has been noted increasingly more frequently in recent years (Ferrigo *et al.*, 2016). These phytotoxic compounds belonging to the hexa-depsipeptides act as antibiotics and insecticides and are produced by various *Fusarium* fungi species, such as *Fusarium proliferatum*, *Fusarium subglutianans*, *Fusarium oxysporum*, *F. poae*, and *F. avanaceum* (Chrpová *et al.*, 2016).

Mycotoxins cause numerous unfavourable morphological/physiological changes in plants, such as reduced enzyme activity, disordered mitotic divisions of meristematic cells, restrained biosynthesis of proteins/DNA/RNA, and damaged mitochondria functions (Packa, 2005). During long-term evolution processes, plants have developed certain mechanisms that decrease the negative effects caused by xenobiotics, and masking the mycotoxins is one of them. The term masked mycotoxins, first introduced by Gareis *et al.* (1990), denotes molecules of mycotoxins bound (coupled) to certain enzymes produced by plants. Such coupled forms of mycotoxins are not toxic for plants. Rather, the enzymes in question, more often, are dedicated to plant detoxification processes, i.e. the neutralisation of xenobiotics. The majority of data acquired thus far on masked mycotoxins apply to deoxynivalenol-3-glucoside (DON-3G), which was first isolated in 2005 from naturally polluted wheat and maize (Berthiller *et al.*, 2005) and is, by far, the most frequently masked mycotoxin found in wheat (Zhang and Wang, 2014).

Developing wheat cultivars that are resistant to FHB is a high priority objective in numerous wheat breeding programmes worldwide (Zhu *et al.*, 2016). However, environmental factors also play a critical role in the pathogenesis. For example, high temperature and humidity during the wheat blossoming phase strongly favour the development of head blight (Wegulo, 2012). Accordingly, various crop production techniques and cultivation strategies have been devised to control FHB on wheat, such as the application of foliar sprayed fungicides, crop rotation, etc. However, their effectiveness is generally regarded as limited (Berthiller *et al.*, 2005; Góral *et al.*, 2015; Mikos-Szymańska and Podolska, 2013).

The impact of nitrogen fertilisation on the biosynthesis of mycotoxins using *Fusarium* spp. fungi is uncertain. Reid *et al.* (2001) studied FHB in maize cultivated on soil fertilised at three nitrogen levels, namely, 0, 100 and 200 kg N/ha, and observed less intense FHB at 100 kg N/ha than at 0 kg N/ha but more intense FHB at 200 kg N/ha. Champeil *et al.* (2004) observed increased vulnerability of wheat cultivated on soil fertilised with nitrogen to infestations of *F. avenaceum* and *Microdochium nivale* fungi. Martin *et al.* (1991) observed significantly more intense FHB in ears of wheat and barley cultivated on soil fertilised during the seedbed phase with ammonium nitrate at a level of 70 kg N/ha and then with additional doses of 50 kg N/ha at the beginning of the shoot into culm phase and the swelling of the sheathing leaf phase. Czaban *et al.* (2015) reported that the higher the dose of nitrogen used in the initial phase of wheat plant growth, the greater the probability of the colonisation of wheat grain by *Fusarium* spp. Lemmens *et al.* (2005) observed an increased concentration of DON in grains of wheat cultivated on soil additionally fertilised with nitrogen. Bernhoft *et al.* (2012) observed more intense infestation of *F. graminearum* at more intense levels of mineral fertilisation. Some researchers (Bernhoft *et al.*, 2012; Champeil *et al.*, 2004) have further suggested that nitrogen fertilisation may impair plant cell wall structures and negatively affect plant chemical composition, causing them to be more vulnerable to fungi infestation. Excess nitrogen results in bushier plants and in fields that are more crowded and humid, factors that favour *Fusarium* infestation and mycotoxin contamination.

Influence of cultivar and nitrogen fertilisation level on accumulation of mycotoxins in cereals was studied only by few researchers. In view of that the principal objective of this work was to study the dependence of the amount of *Fusarium* mycotoxins and their derivatives in winter wheat on: (1) wheat cultivars; and (2) the fertilisation of the wheat cultivation area with nitrogen. If the mycotoxin level in wheat grain is found to be dependent on the level of fertilisation with nitrogen of the wheat cultivation area, the recognised crop production techniques may be appended with suggestions aimed to control mycotoxin contamination.

2. Materials and methods

Field experiments

Wheat was cultivated during the two seasons: 2013-2015 on the Osiny Farm, which belonged to the Kępa-Puławy Experimental Station owned by the State Research Institute of Soil Science and Plant Cultivation in Puławy, Poland (51°27'53'N 22°03'52'E). Two nitrogen fertilisation doses, 120 and 200 kg N/ha, were applied. The nine studied cultivars of winter wheat included Astoria, Meister, Oxal, Kampana, KWS Dacanto, Bamberka, Fidelius, Forkida, and Kepler. Winter wheat was sown on October 4, 2013, and on September 29, 2014 (optimal dates). A two-factorial

field experiment was set up using the split-plot method in three blocks (repetitions). The area of the harvested land plot was 30 m^2. Crop density was 450 germinable seeds/m^2. Wheat was sown on class IIb soil belonging to a very good rye soil complex. Before the onset of the experiment the soil was analysed down to a depth of 30 cm. The soil pH was 6.23 pH/KCl, and the soil fertility indicators were 26.3 mg of P_2O_5, 20.5 mg of K_2O and 6.8 mg of Mg per 100 g of soil. Rape seed was used as the pre-crop. Pre-sowing practices, pre-sowing ploughing, and mineral fertilisation (NPK) were accomplished before sowing. A 350 kg/ha dose of the Polifoska 6-NPK (6 20 30) fertiliser was applied, which was equivalent to 21 kg of N, 70 kg of P_2O_5, and 105 kg of K_2O per ha. Nitrogen in the planned total dose of 200 or 120 kg/ha was applied in divided doses, specifically, 40% at the start of vegetation, 30% at the shooting stage, and 30% at the earing stage. Experimental land plots were chemically protected against pests. Weed control consisted of 1.0 l/ha of 675 SL + 0.6 l/ha of Medax Top + 1.5 l/ha of Capalo. Pathogen control consisted of 1.0 l/ha of Tilt Turbo + 0.3 l/ha of Fury 100 EW at the flag leaf stage, and 2.0 l/ha of Adaxar Plus after blossoming. The crop was harvested at full maturity on July 28, 2014, and on August 4, 2015. From the crop harvested each season, 54 grain samples were sampled, i.e. 9 wheat cultivars ×2 nitrogen fertilisation levels ×3 repetitions. The basic characteristic features of the studied wheat cultivars are provided in Table 1. Their resistance to FHB was assessed by Research Centre For Cultivar Testing in Słupia Wielka (Poland) in the years 2009-2013. To that end plants in various grain ripening phases (from early dough to hard dough, BBCH 8/83-87) were visually inspected on 15 experimental land plots not treated with any crop protection chemicals. The inspected plants were rated against 9-grade scale (1 = total ear infestation, 9 = no signs of any infestation) in line with the methodology proposed by European and Mediterranean Plant Protection Organization (EPPO, 2012).

After harvest, grain samples (each of 500 g) were milled in the MLU-202 laboratory mill (Bühler GmbH, Uzwil, Switzerand). The milled grain was stored at -10 °C.

Analytical standards

Ready-to-use mycotoxin analytical standards purchased from the Romer Labs (Tulln, Austria) company included DON-3G (50 µg/ml); HT-2 toxin (HT-2), T-2 toxin (T-2), DON, ZEA (100 µg/ml each); and a-zearalenol (α-ZOL), β-zearalenol (β-ZOL) (10 µg/ml each). Depending on solubility, some were dissolved in acetonitrile and others in a mixture of acetonitrile and water. Analytical standards of 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), enniatin (ENN) A, ENN-A1, ENN-B, and ENN-B1 purchased from the Sigma Aldrich company (St. Louis, MO, USA) were solved in-house in acetonitrile, according to the recommendations given in their certificates. The basic concentration was 100 µg/ml. All standards were stored in amber glass vials at approximately -20 °C.

A mixture of the above listed standards was prepared before the analyses commenced. To that end, some specific volumes of individual standard solutions were transferred to a 5 ml volumetric flask. The flask was filled to the appropriate mark with LC-MS grade acetonitrile, thoroughly mixed, and then transferred to an amber glass vial and stored at approximately -20 °C. The mixture served both to run the analyses and to validate the analytical method. Final concentrations were as follows: (1) 2 µg/ml of DON-3G, 3/15-ADON, ZEA, DON; and (2) 0.2 µg/ml of α/β-ZOL, HT-2, T-2, ENN-A, ENN-A1, ENN-B, ENN-B1.

^{13}C-labelled internal standards of ZEA and HT-2 (25 µg/ml) were added to all analysed samples. Other reagents included HPLC-grade and LC/MS-grade methanol, HPLC-grade and LC/MS-grade acetonitrile (Rathburn Chemicals Ltd, Walkerburn, UK), LC/MS-purity water (Merck, Darmstadt,

Table 1. Basic characteristic features of the studied wheat cultivars.

Wheat cultivar	Supplied by	Resistance to head blight[1]	Average plant height (cm)	Number of days after January 1 to reach the waxy stage
Astoria	Poznańska Hodowla Roślin	7.8	95	150
Meister	RAGT Semences Polska	8.1	88	154
Oxal	RAGT Semences Polska	8.2	88	154
Kampana	DANKO Hodowla Roślin	7.1	73	152
KWS Dacanto	KWS Lochow Polska	7.5	85	154
Bamberka	Hodowla Roślin Strzelce	7.7	89	153
Fidelius	DANKO Hodowla Roślin	7.9	90	148
Forkida	DANKO Hodowla Roślin	7.5	96	151
Kepler	Limagrain Central Europe Societe	8.0	81	154

[1] Resistance scale from 1 (very susceptible) to 9 (very resistant).

Germany), LC/MS-grade formic acid, dihydrate sodium citrate, hexahydrate dibasic sodium citrate, silica-bound PSA, C-18 silica gel, LC/MS-grade ammonium formate (Sigma Aldrich), HPLC-grade formic acid, analytical grade magnesium sulphate (POCH, Gliwice, Polska), and inert alumina (Merck). Analytes were extracted using HPLC-grade water produced in-house by a Hydrolab (Straszyn, województwo pomorskie, Poland) demineraliser.

Instruments

An Acquity H-class liquid chromatograph coupled with an LCQ Premiere XE mass spectrometer with a time-of-flight analyser (UHPLC-TOF-HRMS, Waters, Milford, MA, USA) was used to determine mycotoxins. Analytes were separated by a combination of a pre-column and a 2.1×100 mm UPLC C18 Cortecs 1.6 µm chromatographic column (Waters). Both constituents of the mobile phase, i.e. A: methanol and B: water, contained 0.2% formic acid, while the B phase also contained an additional 2 mM of ammonium formate. The applied chromatographic separation conditions and the mass spectrometer operational parameters/analyte ionisation conditions have been previously described (Bryła *et al.*, 2016).

Sample preparation technique

Two g of the milled sample, 10 µl of the ^{13}C-labelled ZEA internal standard, and 10 µl of the ^{13}C-labelled HT2 internal standard were transferred to a falcon tube. Two ml of water and 10 ml of 10% formic acid in acetonitrile were added to the tube, and its contents were extracted for three minutes in a homogeniser. Next, 2 g of anhydrous magnesium sulphate, 0.5 g of sodium chloride, 0.5 g of hexahydrate dibasic sodium citrate and 0.25 g of dihydrate sodium citrate were added. Directly after the reagents were added, the tube was vigorously shaken and centrifuged for ten minutes at 10,730×*g*. Acetonitrile with the extract was transferred to a 15 ml falcon tube and washed with 5 ml of hexane to eliminate the lipid fraction. The de-fatted extract was then deep frozen for 24 hours at approximately -30 °C. Three hours before the end of the freezing period, 5 ml cartridges with glass wool positioned in the bottom of the cartridge were placed inside the freezer. Deep frozen supernatant was filtered through the wool into a 15 ml centrifuge-type falcon tube. One g of anhydrous magnesium sulphate, 0.25 g of C-18 silica gel, 0.25 g of inert alumina and 0.4 g of PSA were added. The extract was vigorously shaken and centrifuged for five minutes at 10,730×*g*. Then, 2.5 ml of supernatant were transferred to a reaction vial and evaporated inside a heating block flushed by a stream of nitrogen. The residues were dissolved in 300 µl of methanol, supplemented with 200 µl of HPLC-grade water, and ultrasound-mixed. The extract was filtered through a 0.2 µm mesh syringe nylon filter into autosampler vials.

Method validation

To limit the matrix effects, which may significantly decrease the sensitivity of the used instrument, mycotoxin standards dissolved in matrix-like blank samples were used to validate the analytical method. Matrix-like blank samples were prepared in the manner as the real samples except the starting grain was assured to be mycotoxin-free. All analytical results were related to peaks of internal standards. Method recoveries for individual matrices were measured at three fortification levels, specifically, 200, 250 and 300 µg/kg, for DON, ZEA, and 3/15-ADON; 20, 25 and 30 µg/kg for ENNs; 50, 63 and 75 µg/kg for DON-3G; 40, 50 and 60 µg/kg for β-ZOL; and 63, 75 and 100 µg/kg for the HT-2 and T-2. The measured recovery rates were considered to recalculate analytical results.

For all investigated analytes except DON-3G, the measured recovery rates did not exceed the 79 to 124% range (103 to 104% for DON, 91 to 115% for the HT-2, 79 to 107% for the T-2, and 87 to 124% for enniatins, with relative standard deviations of RSD = 4 to 7%, 5 to 11%, 8 to 15%, 4 to 17%, respectively). However, DON-3G recovery rates spanned the 47 the 64% range (RSD = 2 to 18%).

Limit of determination (LOD) was estimated at 25 µg/kg for DON, 30 µg/kg for DON-3G, 20 µg/kg for 3/15-ADON, 2 µg/kg for the HT-2 and β-ZOL, and 1 µg/kg for the remaining mycotoxins.

Statistical data analysis

The experiment was set up in the split-plot design in three replications. The results of laboratory analyses were statistically processed using the Statistica version 7.1 software package (Tulsa, OK, USA). The influence of two independent factors (variables) and their correlations were analysed using the ANOVA methodology. Nitrogen dose (two values) was the first factor, and cultivar (nine values) was the second factor. Differences between mean values were compared using the Tukey's range test. Statistical significance was declared at α=0.05. Relations between factors were investigated using the correlation tests procedure.

3. Results

The grain of the investigated cultivars of winter wheat contained *Fusarium* mycotoxins and their derivatives in concentrations that depended on cultivar and nitrogen fertilisation level. Out of 108 samples of wheat grain analysed in this work for concentrations of the 12 selected *Fusarium* mycotoxins. 96% of samples contained all 12 of mycotoxins, and the remaining 4% contained 11 out of the 12 mycotoxins (Table 2). The average/min/max concentrations, standard deviations, and coefficients of

Table 2. Mycotoxins found in wheat grain samples.

Mycotoxin/derivative[1]	% of positive samples (n=108)	Concentration (µg/kg)			Standard deviation	Coefficient of variation
		Min	Max	Average		
ZEA	100	1.0	48.0	16.2	14.9	92
β-ZOL	100	3.0	24.0	7.8	5.6	72
HT-2 toxin	100	2.0	57.0	7.7	14.6	189
T-2 toxin	96	1.0	24.0	2.8	4.8	171
DON	100	80.0	1,618.0	590.0	364.7	62
3-ADON	100	23.0	96.0	31.7	11.0	35
15-ADON	100	25.0	100.0	45.6	22.0	22
DON-3G	100	40.0	168.0	69.7	45.1	65
ENN-A	100	1.0	15.0	2.2	2.9	134
ENN-A1	100	1.0	20.0	6.7	4.9	73
ENN-B	100	37.0	144.0	83.3	34.3	41
ENN-B1	100	13.0	70.0	37.5	18.3	49

[1] 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON = deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; ENN-A = enniatin A; ENN-A1 = enniatin A1; ENN-B = enniatin B; ENN-B1 = enniatin B1; ZEA = zearalenone; β-ZOL = β-zearalenol.

variability for ZEA, β ZOL, HT-2/T-2, DON, 3/15-ADON, DON-3G, and ENN-A/A1/B/B1 mycotoxins found in wheat grain are displayed in Table 2. Mycotoxins were found in practically all samples, although generally at low levels, with the exception of DON (max 1,618, average 590 µg/kg). Relatively large CV values for the HT-2/T-2 and the ENN-A indicate substantial variability of contamination with those mycotoxins (2-57 µg/kg for the HT-2, 1-24 µg/kg for the T-2, 1-15 µg/kg for ENN-A); the variability was moderate for other mycotoxins. The average concentration of the β-ZOL masked mycotoxin was approximately 48% ZEA (the free form). Similarly, the DON-3G/DON masked-to-free form concentration ratio was 12%. ENN-B and ENN-B1 were the most abundant enniatins, and their shares of the total amount of all enniatins were 64 and 29%, respectively.

Toxin pairs, for which some statistically significant positive correlations were found in the tested wheat samples included: DON/DON-3G (r=0.68); DON/ENN-B (r=0.44); ZEA/β-ZOL (r=0.73); and HT-2/T-2 (r=0.82) (Table 3).

It was found that both, the year of experiment and the investigated cultivar, influenced mycotoxin concentration in wheat kernels. The concentrations of all mycotoxins were about 70% higher in 2014 compared to 2015 (Table 4). DON ranged from 176 to 1,580 µg/kg in 2014 and from 96 to 229 µg/kg in 2015. The concentration of DON-3G, 15-ADON and 3-ADON were lower than DON in all tested samples. In both years, the most severe contamination of DON (P<0.05) was observed in Bamberka, Forkida and Meister cv., whereas the least severe in Fidelius and Astoria. The four cultivars (Bamberka, Forkida, Meister and KWS Dacanto) had higher concentration of DON than the average concentration for all investigated cultivars. The concentration of DON-3G varied from 68 to 228 µg/kg in 2014 and from 96 to 229 µg/kg in 2015. In both years, the Bamberka cv. accumulated the highest amounts whereas, Astoria, Fidelius, Forkida and Kepler, the lowest. The concentration of 15-ADON and 3-ADON in all cultivars was much lower than DON, however the variations between the cultivars were noticeable regardless. The lowest contamination of 15-ADON were found in Astoria, Meister and Fidelius, but the highest in Forkida. The concentration of ZEA in both experimental years was low. The average concentration in 2014 equalled 25.9 and 6.5 µg/kg in 2015. In 2014, ZEA levels ranged from trace amounts in Astoria cv. to as much as 60.5 µg/kg in Kampana cv. However, in 2015, ZEA amounted to trace values for Astoria, Oxal and Kepler cv. and to as much as 23 µg/kg in Kampana cv. Average concentrations of HT-2 in both years were low and equalled 13.2 and 2.6 µg/kg, respectively. Additionally, we assessed the amounts of T-2 and found only trace amounts of it (4.4 µg/kg in 2014 and 1.2 µg/kg in 2015). Among the studied varieties, Meister accumulated the most of T-2.

Similarly to T-2, trace amounts of ENN-A and ENN-A1 were found. However, ENN-B and ENN-B1 were accumulated at higher levels. The concentration of ENN-B in 2014, ranged from 174 (Meister) to 70.5 µg/kg (Fidelius) and from 71.2 (Forkida) to 10.5 µg/kg (Fidelius) in 2015. The concentration of ENN-B1 was lower and range from 83.3 (Forkida) to

Table 3. Coefficients of correlation (r) between concentrations of individual mycotoxins and fertilisation levels. Statistically significant correlations are given in bold.

Variable[1]	Nitrogen	ZEA	β-ZOL	HT-2 toxin	T-2 toxin	DON	3-ADON	15-ADON	DON-3G	ENN-A	ENN-A-1	ENN-B	ENN-B-1
Nitrogen	1.00												
Cultivar	0.00												
ZEA	-0.14	1.00											
β-ZOL	-0.08	**0.73**	1.00										
HT-2 toxin	0.06	0.26	0.27	1.00									
T-2 toxin	0.22	0.07	-0.00	**0.82**	1.00								
DON	0.17	0.31	0.23	0.18	0.19	1.00							
3-ADON	-0.20	0.21	0.18	-0.02	0.03	0.09	1.00						
15-ADON	0.13	0.25	**0.41**	0.27	0.05	**0.47**	0.27	1.00					
DON-3G	-0.09	0.16	0.03	-0.11	-0.07	**0.68**	0.12	0.21	1.00				
ENN-A	0.19	-0.08	-0.10	-0.06	-0.07	0.16	0.00	0.14	0.21	1.00			
ENN-A1	0.24	0.37	**0.40**	0.35	0.18	0.39	0.15	0.18	0.37	0.09	1.00		
ENN-B	**0.54**	0.33	0.33	**0.58**	**0.50**	**0.44**	0.04	0.32	0.06	0.24	**0.69**	1.00	
ENN-B1	**0.44**	0.37	**0.41**	**0.51**	**0.41**	0.15	0.22	0.32	0.14	0.20	**0.88**	**0.97**	1.00

[1] 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON = deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; ENN-A = enniatin A; ENN-A1 = enniatin A1; ENN-B = enniatin B; ENN-B1 = enniatin B1; ZEA = zearalenone; β-ZOL = β-zearalenol.

29.0 μg/kg (Fidelius) in 2014 and from 36 (Kampana) to 7.0 μg/kg (Fidelius) in 2015 (Table 4).

Acceptable maximum residue levels (MRL) of mycotoxins in wheat as specified by European Commission (EC No 1881/2006; EC, 2006) have been exceeded in only a few instances. For example, the 1,256 μg/kg average concentration of DON in Bamberka slightly exceeded the 1,250 μg/kg DON MRL for wheat. However, the ZEA MRL of 100 μg/kg was not exceeded in any of the tested samples.

Average concentrations of individual mycotoxins (μg/kg) found in nine investigated wheat cultivars cultivated on soil fertilised with two different doses of nitrogen are presented in Figure 1. Higher nitrogen fertilisation levels, i.e. 200 kg N/ha compared to the 120 kg N/ha reference dose, generally increased the concentration of DON, 15-ADON, all enniatins, and the T-2/HT-2, but generally decreased the concentration of ZEA, β-ZOL, and DON-3G. The most conspicuous positive correlation was observed for ENN-B (r=0.54) and ENN-B-1 (r=0.44) (Table 3). The effects of higher nitrogen fertilisation levels, i.e. 200 kg N/ha compared to 120 kg N/ha reference dose, on average concentrations of individual mycotoxins in winter wheat grain depended on the wheat cultivar (Table 5). The concentration of DON was higher in Astoria, Meister, KWS Dacanto, Bamberka, Forkida, and Kepler, but it was lower in Oxal and Fidelius. The concentration of ZEA was lower in Astoria, Meister, Oxal, KWS Dacanto, Bamberka, Fidelius, and Forkida. Increased nitrogen levels increased the concentration of the HT-2/T-2 only in Meister. In other words, increased nitrogen levels did not influence any other cultivar. Concentration of ENN-B and ENN-B1 was higher only in Meister, Kampana, KWS Dacanto, Bamberka, and Kepler.

4. Discussion and conclusions

On the average, mycotoxin level was higher in wheat samples grown during the 2014 vegetation season than in samples grown during the 2015 season. Different climatic factors during both seasons might be an obvious cause as it is well known that development, growth, and spread of *Fusarium* fungi – and in consequence the degree to which wheat plants cultivated on some given area become infected – strongly depend on temperatures/humidity across that area (Bryła *et al.*, 2016). However, variability of temperature prevailing in an ecosystem may change relative contribution of various species to the mix of *Fusarium* fungi colonising that ecosystem and in consequence may change relative contribution of various mycotoxins found in the tested grain (Doohan *et al.*, 2003). Nevertheless, development of FHB in wheat is in the first place correlated with temperatures and rainfall (Brennan *et al.*, 2003). Wind strength and sunshine intensity are other important factors influencing the rate of spread of *Fusarium* fungi (Doohan *et al.*, 2003).

Risk of FHB in wheat plants depends also on genetically determined resistance of the given wheat cultivar to *Fusarium* spp. New cultivars considered for common use are always checked from that point of view: DON level in seeds of that cultivar must be low (Zhang *et al.*, 2008). In our

Table 4. Average concentrations (µg/kg) of individual mycotoxins found in nine investigated wheat cultivars.

Mycotoxin[1]	Year	Average mycotoxin concentrations (µg/kg)[2]								
		Astoria	Meister	Oxal	Kampana	KWS Dacanto	Bamberka	Fidelius	Forkida	Kepler
ZEA	2014	6.0 d	37.4 b	12.0 c	60.5 a	10.8 c	36.7 b	16.0 c	42.3 b	12.0 c
	2015	2.0 d	6.6 bc	2.0 d	23.0 a	2.8 bc	8.0 b	4.0 bc	8.3 b	2.0 d
β-ZOL	2014	7.5 d	15.4 bc	9.0 cd	15.0 bc	8.0 d	18.0 ab	8.5 d	21.0 a	9.0 d
	2015	2.5 bc	3.8 bc	1.0 c	9.0 a	2.0 bc	4.0 b	2.5 bc	5.0 b	1.0 c
HT-2 toxin	2014	7.0 c	46.0 a	3.0 d	3.0 d	8.8 c	5.4 cd	3.0 c	36.2 b	6.0 cd
	2015	2.3 bc	6.0 a	1.0 c	2.0 bc	2.8 bc	3.2 b	1.0 c	4.2 a	1.0 c
T-2 toxin	2014	2.0 c	20.5 a	1.0 c	1.0 c	2.6 bc	2.4 bc	3.0 bc	5.5 b	1.5 c
	2015	1.0 c	2.5 a	1.0 c	1.0 c	1.0 c	1.0 c	1.0 c	1.5 b	1.0 c
DON	2014	651.0 e	1,070.0 c	888.0 cd	995 c	961 c	1,580.0 a	176.0 e	1,225.0 b	825.0 d
	2015	115.0 c	190.8 bc	160.0 bc	229.5 b	180.5 bc	894.0 a	96.0 c	225.0 b	125.0 c
3-ADON	2014	43.0 c	46.4 bc	62.5 a	41.0 bc	56.5 b	50.2 bc	38.5 c	76.8 a	47.5 bc
	2015	7.0 c	9.4 c	10.5 c	21.0 a	9.8 c	10.2 c	10.5 c	16.8 b	8.5 c
15-ADON	2014	40.5 c	57.5 c	100.0 ab	49.5 c	65.8 c	76.4 b	71.5 c	124.5 a	47.5 c
	2015	10.5 bc	9.5 c	19.5 b	16.5 bc	15.8 b	37.8 a	9.5 c	44.5 a	13.5 bc
DON-3G	2014	68.0 c	102.2 cb	173.0 b	120.0 bc	146.0 bc	228.0 a	70.0 c	71.5 c	70.0 c
	2015	12.0 c	18.2 c	33.0 ab	39.5 a	26.5 b	42.0 a	10.0 c	11.5 c	10.0 c
ENN-A	2014	1.0 d	1.0 d	1.0 d	1.5 b	14.5 a	3.1 b	3.0 bc	1.5 c	1.0 d
	2015	1.0 b	1.0 b	1.0 b	1.5 a	1.5 a	1.5 a	1.0 b	1.5 b	1.0 b
ENN-A1	2014	9.5 ab	10.0 ab	3.5 cb	8.5 b	11.7 ab	14.8 ab	5.5 cd	16.5 a	16.0 a
	2015	1.6 c	2.0 cb	1.5 c	4.5 a	3.0 b	2.8 bc	1.5 bc	2.5 bc	2.5 bc
ENN-B	2014	102.0 c	174.0 a	128.0 bc	125.3 bc	173.0 a	139.0 b	70.5 c	131.2 b	148.5 ab
	2015	18.0 cb	30.0 bc	21.5 cb	45.7 b	32.7 b	32.5 b	10.5 c	71.2 a	16.5 c
ENN-B1	2014	46.5 c	71.0 b	45.5 c	56.0 c	74.8 ab	72.8 ab	29.0 d	83.3 a	78.0 ab
	2015	8.5 cd	15.0 bc	7.5 cd	36.0 a	14.8 cd	12.8 cd	7.0 cd	23.3 b	14.0 bc

[1] 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON = deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; ENN-A = enniatin A; ENN-A1 = enniatin A1; ENN-B = enniatin B; ENN-B1 = enniatin B1; ZEA = zearalenone; β-ZOL = β-zearalenol.
[2] For each variable, means followed by the same letter are not significantly different at $P \leq 0.05$.

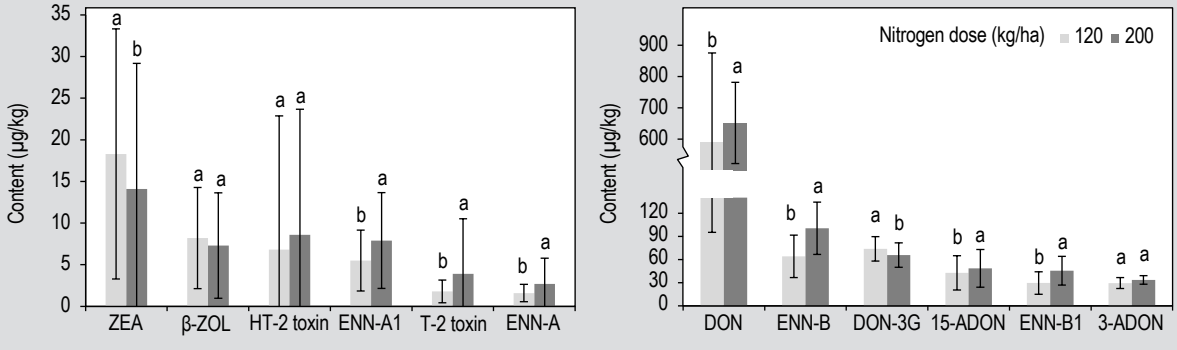

Figure 1. Average concentrations of individual mycotoxins (µg/kg) in nine investigated wheat cultivars depending on the level of nitrogen fertilisation (3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON = deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; ENN-A = enniatin A; ENN-A1 = enniatin A1; ENN-B = enniatin B; ENN-B1 = enniatin B1; ZEA = zearalenone; β-ZOL = β-zearalenol). Letters denote statistically homologous groups.

Table 5. Influence of nitrogen dose on the concentration of individual mycotoxins in nine investigated winter wheat cultivars.

Mycotoxin[1]	N dose kg N/ha	Average mycotoxin concentrations (µg/kg) in wheat cultivar								
		Astoria	Meister	Oxal	Kampana	KWS Dacanto	Bamberka	Fidelius	Forkida	Kepler
ZEA	120	7.0 a	28.0 b	8.0 a	39.0 b	8.0 a	7.0 a	15.0 b	45.3 c	7.0 a
	200	1.0 a	16.0 b	6.0 a	44.0 c	5.7 a	37.0 b	5.0 a	5.3 a	7.0 a
β-ZOL	120	5.0 a	17.0 b	5.0 a	5.0 a	5.0 a	5.0 a	6.0 a	21.0 b	5.0 a
	200	5.0 a	6.0 a	5.0 a	13.0 ab	5.0 a	17.0 b	5.0 a	5.0 a	5.0 a
HT-2 toxin	120	4.0 a	2.0 a	2.0 a	2.0 a	7.0 a	2.0 a	2.0 a	38.3 b	2.0 a
	200	5.0 a	50.0 b	2.0 a	2.0 a	4.7 a	4.3 a	2.0 a	2.0 a	5.0 a
T-2 toxin	120	2.0 a	1.0 a	1.0 a	1.0 a	2.0 a	1.0 a	2.0 a	5.0 b	1.0 a
	200	1.0 a	22.0 c	1.0 a	1.0 a	1.7 a	2.3 ab	2.0 a	2.0 a	2.0 a
DON	120	187.0 b	416.0 c	633.0 c	1,019.0 d	432.0 c	896.0 d	190.0 b	675.0 c	305.0 b
	200	579.0 c	842.7 d	416.0 c	206.0 b	709.0 c	1,616.0 d	82.0 a	775.0 c	646.0 c
3-ADON	120	25.0 a	25.0 a	48.0 a	27.0 a	36.0 a	31.0 a	30.0 a	52.7 a	30.0 a
	200	25.0 a	31.0 a	25.0 a	35.0 a	29.0 a	29.3 a	25.0 a	41.0 a	25.0 a
15-ADON	120	25.0 a	25.0 a	49.0 b	40.0 a	25.0 a	37.0 a	56.0 b	91.3 c	36.0 a
	200	36.0 a	42.0 ab	70.0 c	25.0 a	56.7 b	78.7 c	25.0 a	78.0 c	25.0 a
DON-3G	120	40.0 a	58.0 a	166.0 c	122.0 b	40.0 a	115.0 b	40.0 a	43.0 a	40.0 a
	200	40.0 a	62.3 a	40.0 a	40.0 a	133.0 b	156.0 c	40.0 a	40.0 a	40.0 a
ENN-A	120	1.0 a	1.0 a	1.0 a	2.0 a	3.0 a	1.7 a	1.7 a	2.0 a	1.0 a
	200	1.0 a	1.0 a	1.0 a	1.0 a	13.0 b	3.0 a	2.3 a	1.0 a	1.0 a
ENN-A1	120	4.0 b	4.0 b	4.0 b	4.0 b	7.0 b	4.0 b	6.0 a	14.0 c	3.0 b
	200	7.0 b	8.0 b	1.0 a	9.0 b	8.3 b	13.7 c	1.0 a	5.0 b	18.0 c
ENN-B	120	53.0 a	62.0 a	68.0 a	50.0 a	83.0 b	51.0 a	42.0 a	113.3 c	55.0 a
	200	67.0 a	142.0 c	83.0 b	122.0 c	122.3 c	130.0 c	39.0 a	89.0 b	110.0 c
ENN-B1	120	23.0 b	26.0 b	26.0 b	21.0 b	36.0 b	22.0 b	21.0 b	67.7 c	24.0 b
	200	32.0 b	60.0 c	27.0 b	51.0 c	53.7 c	63.7 c	15.0 a	39.0 b	68.0 c

[1] 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; DON = deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; ENN-A = Enniatin A; ENN-A1 = Enniatin A1; ENN-B = Enniatin B; ENN-B1 = Enniatin B1; ZEA = zearalenone; β-ZOL = β-zearalenol.

tests, type and concentration of mycotoxins in the harvested grain depended on the wheat cultivar. In the majority of the wheat cultivars investigated in this study, larger nitrogen doses increased the concentration of certain mycotoxins, including DON/its derivatives (Astoria, Meister, KWS Dacanto, Bamberka, Forkida, and Kepler) and enniatins (Meister, Kampana, KWS Dacanto, Bamberka, and Kepler). No similar correlation was observed for other mycotoxins (ZEA, DON-3G, and the T2/HT-2). Similar differences are found in the literature. For example, Lori *et al.* (2009), Subedi *et al.* (2007a,b) and Yoshida *et al.* (2008) reported that FHB levels and mycotoxin content in spring wheat were similar regardless of application of ammonium nitrate (140 kg/ha). On the other hand, Yang *et al.* (2010) found a positive correlation between the nitrogen dose and the fraction of *Fusarium*-infested kernels, fungal biomass, mycotoxin concentration, and changes in cell proteome, and they observed more severe FHB in barley grown in soil fertilised with low doses of nitrogen than in barley grown in soil fertilised with high doses of nitrogen.

In the vast majority of our samples, DON was the major pollutant, with concentrations ranging from 136 to 1,256 µg/kg. Similar observations were made by Hajšlová *et al.* (2007), who also reported a statistically significant dependency of DON concentration on wheat cultivar ranging from 17 to 2,265 µg/kg. The rather strong positive correlation between DON and DON-3G (r=0.68) found in this study is in accordance with results published by Rasmussen *et al.* (2012), Lemmens *et al.* (2005), and Galaverna *et al.* (2009). The latter of these authors reported a DON-3G/DON concentration ratio ranging from 2 to 30%. In this work, the ratio ranged from 5.7% (Forkida) to 29.4% (Fidelius); however, on average it was equal to 13.5%. For comparison, ZEA in wheat was masked much more readily with the β-ZOL/ZEA ratio ranging from 22% (Kampana) to 125% (Astoria), with an average of 48%.

Mycotoxins found in the investigated nine cultivars of winter wheat included DON, ENN-B, DON-3G, 15-ADON, ENN-B-1, 3-ADON, ZEA, β-ZOL, HT-2, ENN-A-1, T-2, and ENN-A (ordered from high to low concentration). Concentrations varied depending on the cultivar and on the nitrogen fertilisation dose. While data on DON, ZEA, and the T2/HT-2 in wheat grain are relatively abundant in the literature, data on DON/ZEA derivatives and on enniatins are much less available. Stępień *et al.* (2013) found some enniatins in 95-100% (depending on the particular toxin) of the 21 tested samples of wheat naturally infested with *F. avanaceum.* ENN-B1 dominated the profile, its maximum concentration amounted to 28,520 μg/kg. Jestoi *et al.* (2004) found ENN-B/B1/A/A1 in 100/100/74/95% (respectively) of the 38 tested samples of cereals from Finland at levels varying from trace amounts up to 18,300 μg/kg, depending on the toxin. Uhlig *et al.* (2006) identified ENN-B in all tested samples of various cereals (73 oats, 75 barley, 80 wheat); the maximum level of 5,800 μg/kg was found in wheat. Other enniatins were identified in 25-94% of their samples, depending on the toxin. Depending on the vegetation season and the wheat cultivar, Covarelli *et al.* (2014) found ENN-B/ENN-A in 60-100/89-100%, respectively, of wheat samples from Italy; however, maximum concentrations were much lower (max. 335 μg/kg). Literature data published in recent years suggest positive correlation between DON and enniatins. Possible synergy in toxicological effects within digestive tract is still an open question (Chrpová *et al.*, 2016). Yoshinari *et al.* (2016) found simultaneously DON and some enniatins in 61/58% of imported/domestic wheat samples, respectively. Our data also suggest that contamination of wheat grain with enniatins may be correlated with contamination of the grain with DON; in fact a clear DON/ ENN-B correlation was found in our samples.

Both DON and ZEA may be synthesised by *F. graminearum* and *F. culmorum*, but environmental conditions optimal for both syntheses are different. For example, *F. graminearum* optimally synthesise DON in an acid environment (pH about 5.0) at a temperature about 24 °C, while ZEA is optimally synthesised at pH about 7.0 and a lower temperature about 18 °C (Wu *et al.*, 2017). The variability of the natural environment (rainfall, temperature) results in the frequent simultaneous occurrence of these toxins, where DON is detected at the highest concentrations (Chełkowski *et al.*, 2012; González-Osnaya and Farrés, 2011). In our studies, both DON and ZEA were found in all analysed samples, but no significant correlation was observed between their contents. The co-presence of DON and ZEA was also confirmed in agricultural (straw) and cereal products (Häggblom and Nordkvist, 2015; Nordkvist and Häggblom, 2014). Researchers are not unanimous in estimating the safety of food/feed polluted with different mycotoxins in view of the possible synergistic effects exerted by combinations of various mycotoxins on human/animal/plant organisms. Unfortunately, wheat is frequently contaminated simultaneously with several different mycotoxins, a situation that was confirmed by this study. Therefore, new wheat cultivars and new cultivation techniques capable of yielding better resistance to *Fusarium* fungi, i.e. capable of limiting contamination by mycotoxins, are constantly being sought.

Three cultivar groups can be identified based on their vulnerability to mycotoxins. In one group are the relatively resistant Astoria and Fidelius. DON was found to accumulate in greater quantities in Astoria than in Fidelius, while ZEA accumulated more in Fidelius than in Astoria. In a second group are those that exhibit medium vulnerability, namely, Oxal, Kepler, Forkida, and KWS Dacanto. Oxal's contamination with ZEA/its derivatives, the HT-2/T-2, and enniatins was comparable to the contamination of Astoria or Fidelius, but its contamination with DON/its derivatives was significantly higher. Similarly, Kepler's contamination with ZEA/its derivatives and the HT-2/T-2 was comparable to the contamination of Astoria or Fidelius, but its contamination with both DON/its derivatives and enniatins was significantly higher. Forkida's contamination with DON/its derivatives, ZEA/its derivatives, and the T-2/HT-2 was higher than the contamination of Kepler, KWS Dacanto, Oxal and Astoria. However, none of the levels of the several mycotoxins found in the cultivars of that group exceeded the legally acceptable mycotoxin MRLs in wheat specified by European Commission (EC No 1881/2006; EC, 2006). The third group consists of cultivars that are considered relatively vulnerable, namely, Bamberka, Kampana, and Meister. Bamberka exhibited the highest levels of contamination from DON/its derivatives, ZEA/its derivatives, and enniatins compared to the other cultivars.

A higher nitrogen dose, i.e. 200 kg N/ha compared to the 120 kg N/ha reference dose, generally increased the concentrations of the most mycotoxins with the exception only of ZEA, β-ZOL, and DON-3G. Our results confirmed relationships between level of fertilising the wheat cultivation area with nitrogen and type of cultivar and mycotoxin level in wheat grain. On this basis, the recognised crop production techniques might be appended with some suggestions aimed to keep mycotoxin contamination more under control. The studies suggests the continuation of work on the assessment of the effect of high dose of nitrogen on chemical composition, physical properties and morphological of the kernel and at the same time characterise these factors on the possibility of creating better conditions for mycelial growth and toxin formation.

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