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ORIGINAL ARTICLE

Contamination of wheat grain with microscopic fungi and their metabolites in Poland in 2006–2009

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Abstract

Microscopic fungi are microorganisms commonly found in cereal products. Pathogens of cereals colonising kernels are responsible, among other things, for deterioration of the technological value of grain. However, the greatest threat is posed by mycotoxins produced by toxin-forming strains of these microorganisms. The aim of the present study was to determine the level of contamination with microscopic fungi and mycotoxins from the group of trichothecenes in wheat grain from Poland in a 4-year cycle. In the period 2006–2009, studies were conducted on the content of fungal metabolites (ergosterol [ERG] and type A and B trichothecenes) and the content of microscopic fungi expressed in colony-forming units (CFU) in wheat grain. A total of 129 grain samples were examined. Analysed wheat samples had similar contents of both the investigated fungal metabolites and levels of microscopic fungi. Contents of microscopic fungi were low. Concentration of ERG, on average, was 2.64 mg/kg, while in colony forming units this value ranged from 10^1 CFU/g to over 10^3 CFU/g. The total concentration of type A and B trichothecenes was also low and within the 4 years of the investigation did not exceed 0.062 mg/kg. Concentration of DON did not exceed 1,250 µg/kg, established as safe in grain for human consumption, in any of the tested samples. For the results collected in the years 2006–2009 and presented in this paper, correlations were calculated between the amount of mycoflora and analysed metabolites in 3 possible combinations: 0.7096 for ERG/total toxin concentration, 0.6086 for ERG/log CFU/g, and 0.4016 for the concentration of total toxins/log CFU/g. Highly significant correlations between the content of trichothecenes and the concentration of ERG indicate that the level of this metabolite is closely related to the content of mycotoxins in grain.

Key words

CFU, ergosterol, trichothecenes, wheat

INTRODUCTION

Cereals are crops with the longest history of cultivation and the products of their milling constitute the staple diet not only for humans, but also animals. They are significant sources of energy, contain large amounts of complex carbohydrates and low value protein, and supply vitamin B and minerals. Cereal grain contains approximately 2% fat, comprising mainly essential saturated fatty acids and tocopherols [1]. They are most typically consumed in the processed form. It is assumed that a balanced daily diet should contain 5 servings of wholemeal cereal products [2]. Due to the considerable amounts of cereal products consumed by humans, it is justified to ensure thorough quality control of both raw materials and cereal products. The risk of their exposure to microbiological contamination is high and possible at any time, starting from the vegetation period of the crop, through harvest and processing, up to storage and transport of the final product [3].

Microorganisms commonly found in cereal products include microscopic fungi, cereal pathogens colonising kernels and responsible, e.g. for the deterioration of the technological value of grain [4]. The greatest hazard is posed by mycotoxins produced by microscopic fungi. They are secondary metabolites exhibiting toxic action towards both humans and animals. The problem of mycotoxin contamination in food raw materials and final products is connected with documented poisonings of humans and animals caused by consumption of mouldy food or feed. Thus, the monitoring of their contents in cereals and their milling products is extremely important in the context of toughening of legal regulations concerning the level of health protection standards and protection of consumer interests. Apart from national legal regulations concerning food products, specifying threshold values for toxins produced by fungi from the genus *Fusarium*, the Directive of the European Commission (EC) No. 856/2005 of 1 June 2005, must also be mentioned here, which – in view of Poland being an EU member state – is an act of superior legal power (Commission Regulation, 2005, 2006). In contrast to national regulations, the EU directives referring to mycotoxins also include admissible values for freshly-harvested grain, which is to prevent cereal with high contamination levels being introduced into the food chain.

Mycotoxin content in grain is inseparably connected with the presence of fungal biomass, and its presence may indicate the occurrence of a fungus and, indirectly, products of its metabolism.

Direct microbiological methods are the primary methods to determine the amount of fungal biomass. They are mostly based on the microscopic analysis of the tested material or counting the number of colony forming units (CFU) after inoculation on respective microbiological media. A definite advantage of these methods results from the possibility to

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identify individual fungal species, but they also reflect only viable fungal biomass. Chemical methods determining concentrations of specific fungal markers in order to identify the level of contamination with microscopic fungi in the tested material include analyses of contents of chitin, ATP [5] and ergosterol [6]. Among them, ergosterol (ERG), a specific fungal marker, needs to be concentrated on. Thus, when determining the level of mycoflora as ERG concentration, it may be inferred with high probability that there is grain contamination with mycotoxins, or even try to estimate their concentration. Numerous studies may serve as examples in this respect, where the correlation coefficient determined between one of the most common toxins in cereal grain, i.e. deoxynivalenol (DON), or total contents of trichothecenes [7], and the concentration of ergosterol is high. The content of ERG is also significantly correlated with the concentration of another mycotoxin highly frequently found in cereal grain – ochratoxin A, and with the number of colony-forming units of microscopic fungi [8].

Objective. The aim of the presented study was to analyse the content of *Fusarium* toxins, for which in 2006 the European Union implemented a directive on legal regulations concerning the content of deoxynivalenol (DON), as well as quantitative and qualitative assessment of contamination with microscopic fungi using conventional microbiological methods and a chemical method to determine the concentration of ergosterol in samples of wheat grain analysed in a 4-year cycle. An additional aspect in this study was to determine the correlation between the concentration of trichothecenes in tested grain and the number of CFU and concentration of ERG.

MATERIALS AND METHOD

Wheat grain was collected in an identical manner from grain silos located at grain purchasing stations and in mills located in Poland from October to November during 2006–2009. A total of 129 samples were collected (35 samples in 2006, 31 samples 2007, 35 samples in 2008, and 28 samples in 2009), each of 5,000 g, in accordance with the Ordinance on the methods of sampling for selected foodstuffs to control the level of *Fusarium* toxins and criteria for analytical methods used in the determination of contents of *Fusarium* toxins (EU Journal of Law, L 191 of 30.04.2004, p. 1; EU Journal of Law, Polish Special Edition, Chapter 3, vol. 45, p. 200).

Analysis of trichothecenes. Grain samples were analysed for the presence of trichothecenes according to Perkowski *et al.* (2003) [9]. Sub-samples (10 g) were extracted with acetonitrile/water (82:18) and cleaned on a charcoal column (Celite 545/charcoal Draco G/60/activated alumina neutral 4:3:4 (w/w/w). Trichothecenes of group A (H-2 toxin, T-2 toxin, T-2 tetraol) were analysed as TFAA derivatives. To the dried sample, the amount of 100 µl trifluoroacetic acid anhydride was added. After 20 min the reacting substance was evaporated to dryness under nitrogen. The residue was dissolved in 500 µl of isooctane and 1 µl was injected onto a gas chromatograph-mass spectrometer. Trichothecenes of group B (DON, NIV, 3-AcDON, 15-AcDON) were analysed as TMS (trimethylsilylsilyl ether) derivatives. To the dried extract, the volume of 100 µl TMSI/TMCS (trimethylsilyl imidazole/trimethylchlorosilane, v/v 100/1) mixture was added. After 10 min 500 µl of isooctane were added and the reaction quenched with 1 ml of water. The isooctane layer was used for the analysis and 1 µl of the sample was injected on a GC/MS system. Analyses were run on a gas chromatograph (Hewlett Packard GC 6890) hyphenated to a mass spectrometer (Hewlett Packard 5972 A, Waldbronn, Germany), using an HP-5MS 0.25 mm × 30 m capillary column. The injection port temperature was 280 °C, the transfer line temperature was 280°C and the analyses performed with programmed temperatures, separately for group A and B trichothecenes. Group A trichothecenes were analysed using the following programmed temperatures: initial 80 °C held for 1 min, from 80 °C – 280 °C at 10 °C/min, and the final temperature maintained for 4 min. For group B trichothecenes, the initial temperature was 80 °C, held for 1 min, from 80 °C – 200 °C at 15 °C/min, held for 6 min, and from 200 °C – 280 °C at 10 °C/min, the final temperature being maintained for 3 min. The helium flow rate was held constant at 0.7 ml/min. Quantitative analysis was performed in the single ion monitoring (SIM) mode using the following ions for the detection of scirpentriol (STO): 456 and 555; T-2 tetraol: 455 and 568; T-2 triol: 455 and 569 and 374; HT-2: 455 and 327; T-2: 327 and 401; deoxynivalenol (DON): 103 and 512; 3-acetyldeoxynivalenol (3-AcDON): 117 and 482; 15-acetyldeoxynivalenol 15-AcDON: 193 and 482; nivalenol (NIV): 191 and 600. Qualitative analysis was performed in the SCAN mode (100–700 amu). Recovery rates for the analysed toxins were as follows: STO 82±5.3%; T-2 triol 79±5.1%; T-2 86±3.8%; T-2 tetraol 88±4.0%; HT-2 91±3.3%; DON 84±3.8%; 3-AcDON 78±4.8%; 15-AcDON 74±2.2% and NIV 81±3.8%. The limit of detection was 0.001 mg/kg.

Chemical analysis of ergosterol. Ergosterol was determined by high-performance liquid chromatography (HPLC) [10]. Samples containing 100 mg of ground grains were placed into 17-ml culture tubes, suspended in 2 ml of methanol, treated with 0.5 ml of 2 M aqueous sodium hydroxide, and tightly sealed. The culture tubes were then placed in 250-ml plastic bottles, tightly sealed and placed inside a microwave oven (Model AVM 401/1WH, Whirlpool, Sweden) operating at 2,450 MHz and 900 W maximum output. Samples were irradiated (370 W) for 20 s and after about 5 min for an additional 20 s. After 15 min, the contents of culture tubes were neutralized with 1 M aqueous hydrochloric acid, 2 ml MeOH were added and extraction with pentane (3×4 ml) was carried out within the culture tubes. The combined pentane extracts were evaporated to dryness in a stream of nitrogen.

Before analysis, samples were dissolved in 4 ml of MeOH, filtered through 13-mm syringe filters with a 0.5 mm pore diameter (Fluoropore Membrane Filters, Millipore, Ireland) and evaporated to dryness in a N_2 stream. The sample extract was dissolved in 1 ml of MeOH and 50 µl were analysed by HPLC. Separation was performed on a 150×3.9 mm Nova Pak C-18, 4-mm column and eluted with methanol/acetonitrile (90:10) at a flow rate of 0.6 ml/min. Ergosterol was detected with a Waters 486 Tunable Absorbance Detector (Milford, MA, USA) set at 282 nm. The presence of ERG was confirmed by a comparison of retention times and by co-injection of every tenth sample with an ergosterol standard.

Plate flooding method with decimal dilutions. The count of microscopic fungi per 1 g of grain was assessed using a standard plate method in accordance with the procedure of the standard PN-ISO 21527–2: 2009.

Microbiology of food and feeds

Horizontal method to determine counts of yeasts and moulds. Part 2: Method of counting colonies in products with water activity of max. 0.95. The amount of 10 g comminuted material (in 3 replications) was suspended in 90 ml of 0.1% peptone water. After 30 min samples were shaken for 2.5 min. Next, from the prepared suspension decimal dilutions were prepared in 0.1% peptone water solution. For this purpose 1 ml of suspension was transferred with a sterile pipette from 3 prepared dilutions onto sterile Petri dishes (with 2 for each dilution). In the next stage, the plates were flooded with agar medium containing Rose Bengal and chloramphenicol (15 ml) at a temperature of 45 °C. Plates were incubated under aerobic conditions, placed flat in a heater at a temperature of 25±1 °C for 5–7 days. After incubation, the colonies were counted on selected plates (making it possible to obtain 15–150 colonies per plate), and based on the number of counted colonies, the number of colony-forming units (CFU) of microscopic fungi was calculated in 1 g of tested material (CFU/g). The result was a mean of 2 replications and expressed in log CFU/g.

Analysis of fungi occurrence in wheat. The composition of fungal species occurring in wheat grain samples was analysed. The dilution method used was as follows: 1 g of ground grains was put in 10 ml of sterile distilled water and mixed with a magnetic stirrer for 2 min. Next, 1 ml of the suspension was transferred onto PDA (potato-dextrose agar) medium in Petri dishes and spread with a sterile glass stick on the medium surface. The Petri dishes were incubated at 25 °C for 7 days. Growing mycelia were isolated on the PDA and SNA (synthetic nutrient-poor agar) media to identify fungal species. Identification was carried out on the basis of colony and spore morphology using keys.

Statistical analysis. Results were subjected to statistical analysis using STATISTICA v 8.0 software. In order to compare the contents of individual metabolites in the samples, Tukey's multiple comparison procedure was applied. The values of Pearson's linear correlation coefficients were also determined at the following significance levels: α=0.05, α=0.01 and α=0.001 (*, **, ***) between ERG and trichothecene concentrations and the number of CFU. In order to determine the effect of weather conditions on the level of wheat grain contamination with mycoflora and mycotoxins, multiple regression was applied and Pearson's linear correlation coefficient was calculated at the level of significance α=0.05 between analysed factors.

RESULTS AND DISCUSSION

The level of contamination with microscopic fungi and mycotoxins in wheat is an indicator of its quality. The aim of the analyses conducted within the framework of the presented study in a 4-year cycle was to determine the level of contamination in wheat grain produced in Poland, caused by microscopic fungi and mycotoxins from the group of trichothecenes, through the analysis of 129 wheat grain samples from cereal silos with the application of 2 methods: 1) a classical microbiological method for determination of the number of colony-forming units of microscopic fungi; 2) a chemical method analysing ergosterol concentration. Moreover, genera of microscopic fungi were identified based on observations of colony morphology and propagation organs. Due to the fact that among the 3 most frequently identified genera of microscopic fungi there were fungi from the genus *Fusarium*, mycotoxins from the group of trichothecenes, produced most typically by these fungi, were determined quantitatively and qualitatively.

The contents of microscopic fungi measured in terms of colony-forming units in the case of all wheat grain samples were low, ranging from 1.26 log CFU/g – 1.75 log CFU/g (Tab. 1). Statistical analysis showed no significant differences in the number of CFU between samples tested in individual years. Baliukoniene *et al.* [11] recorded higher results for CFU in tested wheat grain, ranging from 3.44 log CFU/g – 4.2 log CFU/g. Samples came from privately owned farms located in Lithuania. Neagu and Tofan (2008) [12] tested wheat samples from central Romania. They observed low contents of microscopic fungi amounting to 1.2 log CFU/g for analysed samples. Krysińska-Traczyk *et al.* [13] reported that in wheat grain collected in eastern Poland, contamination with microscopic fungi ranged from 0–227.5×10^3 CFU/g. In turn, Stuper *et al.* [14] in 2007, in samples of wheat grain collected in Poland, recorded a mean level of 126 log CFU/g.

Table 1. Range and mean concentrations of total toxins, ERG and number of CFU in samples of wheat grain in 2006–2009

Year	No. of samples	Mean ERG concentration (mg/kg)		Total of mean concentrations of trichothecenes (mg/kg)		Log CFU/g	
		Range	Mean	Range	Mean	Range	Mean
2006	35	1.14–7.51	4.41^a	0.011–0.062	0.031^a	1.23–2.67	1.75^a
2007	31	0.38–4.61	1.90^b	0.004–0.026	0.018^b	1.29–2.43	1.26^a
2008	35	0.47–5.21	2.01^b	0.012–0.033	0.025^b	1.13–2.10	1.30^a
2009	28	0.94–3.91	2.27^b	0.009–0.048	0.020^b	1.34–2.55	1.43^a
Σ	129	0.38–7.51	2.64	0.004–0.062	0.023	1.13–2.67	1.43

Identical letters in columns – lack of differences at significance level α=0.05

Chemical analysis of ERG concentration as a specific marker of fungal biomass, similarly for CFU levels, showed low contamination with microscopic fungi in wheat grain. Mean concentration of this metabolite was 2.64 mg/kg (Tab. 1). The performed Tukey's test showed a significant difference in the contents of the analysed metabolite in samples collected in 2006 in comparison to the other years of the study.

Thus, the recorded results need to be compared with the current trends in this field since, to date, no legal regulations have been prepared in this respect. A concentration of ERG amounting to 3 mg/kg was adopted as the safe content of mycoflora in healthy grain, while Schnürer and Jonsson [10] proposed a range of concentration for this metabolite of 1–9 mg/kg as the boundary value for grain for human consumption. Mean ERG concentrations for all analysed samples did not exceed the boundary value specified in the literature. Schnürer and Jonsson [10] determined in

wheat grain similar ERG concentrations, ranging from 0.32–4.40 mg/kg. Perkowski *et al.* [10] determined average ERG concentration in samples of wheat grain at 1.26 mg/kg. Stuper *et al.* [15] also observed a mean ERG concentration of 2.87 mg/kg.

Apart from the content of mycoflora in cereal samples, their concentrations of mycotoxins were also observed. The level of type A and B trichothecenes in the 4 years of the study remained low and the concentration of DON did not exceed in any of the tested samples the value of 1250 μg/kg, established as safe for grain to be used for human consumption. Analogously, as in the case of ERG concentration, the content of these toxic metabolites was significantly higher in 2006 than in the other years of the study. The sum of mean concentrations of trichothecenes was 0.023 mg/kg (Tab. 2). Among type A trichothecenes STO, T-2 tetraol, T-2 triol, DAS and HT-2 were identified in the analysed wheat grain samples. Among the above, STO was the most frequently identified toxin, detected on average in 14% of all samples. The highest concentration of this toxin was recorded in 2009–0.042 mg/kg. Apart from type A trichothecenes, type B trichothecenes were also determined (Tab. 3). The following toxins from this group were identified: DON, Fus-X, 3-AcDON, 15-AcDON and NIV. The most frequently detected toxins included DON (found in 99% of all samples) and NIV (on average, in 54% of all samples). Maximum DON concentration was determined in 2008 and was 0.051 mg/kg. The highest recorded concentration of NIV was 0.036 mg/kg in 2007.

Table 2. Range of concentrations, mean concentrations of type A trichothecenes and percentages of positive samples

	Year	Concentrations of type A trichothecenes (mg/kg)				
		STO	T-2 Tetraol	T-2 Triol	DAS	HT-2
Mean Range Possitive samples (%)	2006	0.001 <LOD – 0.012 23%	0.005 <LOD – 0.019 9%	0.001 <LOD – 0.005 2%	0.001 <LOD – 0.007 2%	0.001 <LOD – 0.005 2%
	2007	0.004 <LOD – 0.017 15%	<LOD - 0%	0.001 <LOD – 0.004 6%	<LOD - 0%	0.001 <LOD – 0.03 1%
	2008	0.006 <LOD – 0.022 17%	0.003 <LOD – 0.007 10%	<LOD - 0%	0.001 <LOD – 0.010 8%	0.007 <LOD – 0.024 11%
	2009	0.011 <LOD – 0.042 10%	0.002 <LOD – 0.009 6%	0.001 <LOD – 0.003 2%	<LOD - 0%	<LOD - 0%
	Σ	0.005 <LOD – 0.042 14%	0.002 <LOD – 0.019 3%	0.001 <LOD – 0.005 1%	0.001 <LOD – 0.010 1%	0.002 <LOD – 0.024 3%

Detailed information on the content of trichothecenes in cereal grain in Europe is supplied in numerous literature reports. Keblys *et al.* [16] analysed DON content in samples of naturally infested wheat grain and it was lower than the detection level of 0.001 mg/kg. Similar mean results for DON concentration were recorded by Garaleviciene *et al.* [17]. Šliková *et al.* [18] in 2004–2006 observed much higher DON concentration and found significant differences between DON concentrations in the tested wheat samples. Concentration of this metabolite in individual years was

Table 3. Ranges of concentrations, mean concentrations of type B trichothecenes and percentages of positive samples

	Year	Concentrations of type B trichothecenes (mg/kg)				
		DON	Fus-X	3-AcDON	15-AcDON	NIV
Mean Range Positive samples (%)	2006	0.017 0.001 – 0.035 100%	<LOD - 0%	0.001 <LOD – 0.010 21%	0.002 <LOD – 0.009 5%	0.009 <LOD – 0.018 20%
	2007	0.013 0.004 – 0.040 100%	<LOD - 0%	<LOD - 0%	0.004 <LOD – 0.023 6%	0.012 <LOD – 0.036 75%
	2008	0.025 0.001 – 0.051 100%	<LOD - 0%	0.003 <LOD – 0.017 14%	0.001 <LOD – 0.012 10%	0.009 <LOD – 0.020 48%
	2009	0.019 <LOD – 0.033 94%	0.004 <LOD – 0.015 11%	<LOD - 0%	0.004 <LOD – 0.009 17%	0.010 <LOD – 0.029 38%
	Σ	0.018 <LOD – 0.051 99%	0.001 <LOD – 0.015 3%	0.001 <LOD – 0.017 9%	0.003 <LOD – 0.023 7%	0.010 <LOD – 0.036 54%

0.56 mg/kg (2004), 0.23 mg/kg (2005) and 0.73 mg/kg (2006). In 2001, under the SCOOP project [19], studies were conducted on the occurrence of *Fusarium* toxins in food and their daily intake in EU member countries. The results obtained concerned the contents of DON, NIV, HT-2 and T-2. Analyses were conducted in the following countries: Austria, Belgium, Denmark, Finland, France, Germany, Italy, Norway, Portugal, Sweden, England and Holland. Among 11,022 tested samples, 57% contained DON, while 16% of 4,166 samples analysed for NIV content contained this toxin. Out of 3,490 samples, 20% were contaminated with the T-2 toxin, while among 3,032 samples 14% contained the HT-2 toxin. Among the analysed cereal samples wheat grain was characterised by the highest number of positive samples containing DON, of which 7% contained more than 750 μg/kg.

The performed qualitative analysis of the composition of fungal microflora, based on colony morphology and observations of specimens *in vivo,* showed that the most frequently found genera of microscopic fungi in samples of wheat grain tested in the years 2006–2009 included *Penicillium*, *Aspergillus* and *Fusarium* (Tab. 4). When comparing the percentage shares of individual identified genera of microscopic fungi in the total microflora, it was found that 20% of them comprised fungi from the genera *Penicillium* and *Aspergillus*, with slightly lower proportions of fungi from the genera *Fusarium* and *Mucor*, 13% and 11%, respectively (Fig. 1). Champeil *et al.* [20] observed in the tested cereal samples that *Penicillium* and *Fusariumwe* were the most commonly found genera of microscopic fungi in Europe. In turn, Nicholson *et al.* [21] stated that the most frequently identified genera of fungi detected in samples of wheat grain included *Alternaria*, *Penicillium*, *Aspergillus* and *Fusarium*. Čoncová *et al.* [22], on the basis of studies conducted in 2008 on the composition of mycoflora in wheat grain collected in Central Europe, found that among the identified species of microscopic fungi the highest proportions in the total mycoflora were recorded for fungi from the genera *Penicillium* (24%), *Aspergillus* (17%), *Alternaria* (15%) and *Fusarium* (11%).

Table 4. Genera of microscopic fungi identified in wheat samples collected in Poland in 2006–2009

Genus of fungi	Percentage contents of identified genera of fungi in a given year (%)				
	2006	2007	2008	2009	2006–2009
Penicillium spp.	92	85	97	94	92
Aspergillus spp.	100	90	76	97	90
Fusarium spp.	54	32	61	83	57
Mucor spp.	74	47	24	64	52
Rodothorulla spp.	45	26	82	38	47
Alternaria spp.	60	72	26	21	44
Stemphylium spp.	21	44	37	10	28
Cladosporium spp.	29	30	9	37	26
Rhisopus spp.	12	31	16	12	17

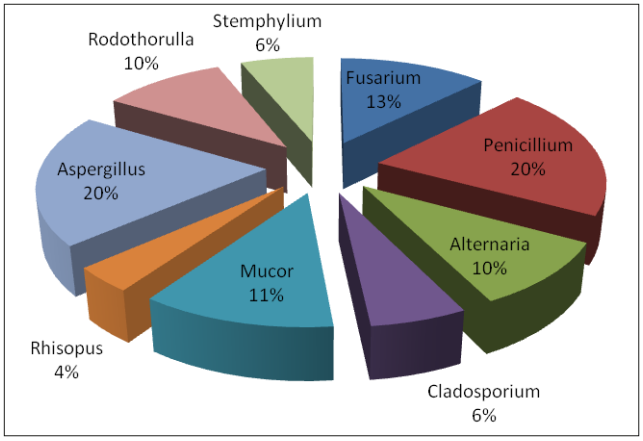

Figure 1. Percentage proportions of individual genera of microscopic fungi in total mycoflora in samples of wheat grain collected in Poland in 2006–2009

In recent years, much attention has been devoted in the literature on the subject to the determination of specific weather conditions required for the occurrence of ear blight and increased toxin accumulation in grain. It is reported that the most significant effects are connected with precipitation during flowering and elevated humidity during plant vegetation. These factors are also significantly influenced by temperature [23]. When analysing weather conditions in the 4 years of the study on the basis of data obtained from the Institute of Meteorology and Water Management, it was found that in the analysed years 2006–2009 the highest mean temperature in the period from May – August was observed in 2006, while the lowest in 2007 (Tab. 5). On average, precipitation levels in 2006 and 2009 were similar, amounting to approx. 80 mm (Tab. 6). When analysing recorded results concerning both ERG concentration and the content of trichothecenes in tested grain (Tab. 1), it was

Table 5. Means for temperature in the seasons of 2006–2009 in Poland and their percentage relationships to the means for the last two decades.

Month	Temperature (°C)			
	2006	2007	2008	2009
May	18.0 (83%)	18.0 (83%)	22.0 (102%)	22.3 (103%)
June	25.0 (116%)	15.5 (81%)	19.5 (97%)	21.0 (107%)
July	30.0 (138%)	15.0 (70%)	28.0 (132%)	25.0 (120%)
August	28.0 (122%)	25.0 (116%)	25.0 (116%)	20.0 (101%)
mean	25.2	18.3	23.6	22.0

Table 6. Means for rainfall in the seasons of 2006-2009 in Poland and their percentage relationships to means for the last two decades

Month	Rainfall (mm)			
	2006	2007	2008	2009
May	110.0 (143%)	90.0 (87%)	100.0 (90%)	60.0 (71%)
June	120.0 (140%)	130.0 (151%)	110.0 (134%)	60.0 (70%)
July	90.0 (78%)	131.0 (142%)	70.0 (62%)	100.0 (92%)
August	90.0 (81%)	20.0 (32%)	50.0 (46%)	80.0 (88%)
Mean	80.0	92.7	82.5	75.0

found that their mean contents differed significantly for 2006 in relation to the other years; this is reflected in the presented meteorological data (Tab. 5, 6). In June 2006, when ear inoculation occurred, the highest temperature of 25 °C (116%) was observed and this tendency was manifested also in July and August. At the same time, high humidity was recorded in June (precipitation was 140% of the multiannual mean), which influenced ear infestation, particularly when also in May precipitation considerably exceeded the multiannual mean (143%), which resulted in plant wetting. In the other years, such trends were not observed. The importance of interactions of temperature and humidity under field conditions (see 2006) is shown by conditions observed in 2007, with heavier precipitation, in June amounting to 130.0 and in July to 131.0 mm, while the temperature did not promote the development of fusariosis, whereas precipitation in August was slight, amounting to only 32% of the multiannual mean. Despite such heavy precipitation occurring in 2007, it did not contribute to a significant increase in the risk expressed both by the concentration of mycotoxins and the number of fungi in grain. Thus, the observed trends for the occurrence of a dry and cool spring may result in a low level of contamination with microscopic fungi in wheat grain. Similar dependencies were observed in the case of oat samples analysed by Perkowski *et al.* [10]. Data recorded within this study supplement and confirm investigations conducted by Mesterhazy and Miedaner [10], showing the effect of weather conditions on the development of fusariosis.

The results presented above, collected within the 4-year cycle of investigations concerning the content of mycoflora and trichothecenes in wheat grain, clearly indicate a low level of contamination with microscopic fungi and toxins in cereals for human consumption in Poland, while the differences determined statistically between the contents of analysed metabolites between the years of the study confirm observations on the significance of the effect of weather conditions on the development of mycoflora and production of mycotoxins, and they concern objects characterised by low infestation.

Correlations between the amount of mycoflora and analysed metabolites were calculated for the results obtained in 2006–2009 and presented in this study. These correlations were significant in over 90% of investigated cases. In turn, when considering all samples tested in the 4-year cycle and 3 possible combinations for the computation of correlations (Tab. 7), the calculated correlation coefficient at the confidence level P=0.001 for ERG/Σ_{Ctrich} was 0.7096, for ERG/log CFU/g – 0.6086, while for the concentration Σ_{Ctrich}/log CFU/g – 0.4016. Similar or even higher results for correlation coefficients concerning the presented characteristics were obtained by other authors [4]. The analysed wheat samples indicated a highly significant correlation coefficient between ERG and

Table 7. Results of internal correlation analyses

Year	Correlation coefficient				
	ERG/Σc_{trich}	ERG/DON	ERG/NIV	ERG/log CFU/g	Σc_{trich}/log CFU/g
2006	0.7886***	0.3213	0.5278**	0.6056***	0.3219
2007	0.7032***	0.4867**	0.4957**	0.7215***	0.4284*
2008	0.6419***	0.3183	0.3631*	0.5016**	0.5172**
2009	0.8012***	0.2998	0.4667*	0.6459**	0.4788*
2006–2009	0.7096***	0.3665*	0.4631**	0.6086***	0.4016**

Σc_{trich} – total concentration of trichothecenes
*, **, *** – Pearson's linear correlation coefficients taking into consideration the following significance levels α=0.05, α=0.01, α=0.001

DON of 0.83. In turn, Wiśniewska and Buśko [24] reported in their study a correlation coefficient for ERG/DON of 0.91 at the significance level P=0.01. Saxena *et al.* (2001) [25], Gawrysiak-Witulska *et al.* (2008) [8] also reported significant correlations between ERG concentration and CFU/g within a range of 0.70–0.90. Similar to these results, Stuper *et al.* (2010) [14] indicated a higher correlation coefficient for ERG/Σ_{Ctrich} (0.8032 at the confidence level P=0.001) than correlation coefficients for ERG/log CFU/g (0.7115) and Σ_{Ctrich}/log CFU/g (0.4284) in the case of wheat grain.

Highly significant correlations between the content of trichothecenes and the concentration of ERG, which were stronger than the correlation for the total toxin concentration/log CFU/g, indicated that the level of this metabolite is inseparably connected with mycotoxin content in grain.

CONCLUSIONS

The presented study conducted in 2006–2009 on the contents of ERG, type A and B trichothecenes, as well as the number of CFU in samples of wheat grain collected in Poland, indicated their slight contamination with microscopic fungi and their metabolites. The EU admissible DON concentration in unprocessed cereals was not exceeded in any of the 129 analysed samples. The most frequently found mycotoxins included DON and 3-AcDON, whose presence was detected in 80% of samples. Moreover, the concentration of type A trichothecenes was also determined, but they were present in a lower percentage of samples. In comparison with other cereals tested in the same period (rye, barley, oat), it was found that wheat grain contained significantly lower amounts of mycotoxins and microscopic fungi. A significant effect of weather conditions on increased contents of mycoflora and the formation of *Fusarium* toxins in grain was observed particularly in 2006, which was manifested in a higher ERG concentration and mean total trichothecene concentrations.

Among the methods analysing the content of mycoflora in grain, a higher correlation with mycotoxin concentration was observed for the relationship of ERG/Σ_{Ctrich} than for Σ_{Ctrich}/log CFU/g, which indicates a cheaper and simpler method for determining the level of contamination with microscopic fungi in cereal grain using a specific fungal marker, such as ERG.

The conducted studies definitely indicate the necessity to conduct comprehensive annual grain monitoring in terms of its contamination with microscopic fungi and *Fusarium* toxins, and indicate that long-term efforts aimed at improving the cultivation conditions in Poland have been successful.

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