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LEVELS OF FUNGI AND MYCOTOXINS IN SAMPLES OF GRAIN AND GRAIN DUST COLLECTED ON FARMS IN EASTERN POLAND

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Abstract: Ten samples of stored wheat grain and 10 samples of settled grain dust released during machine threshing of wheat grain were collected on 10 farms located in Lublin province (eastern Poland). The samples were examined for the concentration of total microfungi, *Fusarium* species, fusariotoxins (moniliformin, deoxynivalenol, nivalenol), and ochratoxin. Microfungi able to grow on malt agar were present in 30% of grain samples (median for all examined samples = 0, range 0–227.5 × 10^3 cfu/g) and in all samples of grain dust (median = 977.5 × 10^3 cfu/g, range 115.0–16,700.0 × 10^3 cfu/g). *Fusarium* species (*F. avenaceum*) were found only in 10% of grain samples (median = 0, range 0–800.0 × 10^3 cfu/g), but in 90% of grain dust samples (median = 1,150 × 10^3 cfu/g, range 5.5–10,060.0 × 10^3 cfu/g). The species *F. avenaceum, F. culmorum, F. graminearum, F. poae* and *F. sporotrichioides* were isolated respectively from 50%, 10%, 20%, 40% and 20% of examined grain dust samples. The presence of the mycotoxins produced by *Fusarium* (moniliformin, deoxynivalenol, and nivalenol) was found altogether in 70% of wheat grain samples (median = 0.1275 µg/g, range 0–1.480 µg/g) and in 90% of grain dust samples (median = 0.350 µg/g, range 0–1.090 µg/g). Moniliformin (MON), deoxynivalenol (DON), and nivalenol (NIV) were each detected in 40% of grain samples, and respectively in 80%, 40%, and 40% of grain dust samples. Ochratoxin A (OTA) was detected in 60% of grain samples and in 60% of grain dust samples (median in both cases was 0.0005 µg/g). The concentrations of *F. poae* ($p < 0.05$) and of total *Fusarium* species ($p < 0.01$) in grain samples, and the concentrations of *F. culmorum* and *F. graminearum* ($p < 0.05$) in grain dust samples were significantly correlated with the concentration of deoxynivalenol. The concentrations of *F. poae* ($p < 0.05$) and of total *Fusarium* species ($p < 0.01$) in grain dust samples were significantly correlated with the concentration of total fusariotoxins. Moreover, the concentration of total *Fusarium* species in grain dust samples was significantly correlated with the concentration of nivalenol ($p < 0.05$). In conclusion, the majority of samples of wheat grain and grain threshing dust collected on farms in eastern Poland contained notable quantities of fusaria and/or fusariotoxins. This fact poses a potential risk of mycotoxicoses to agricultural workers exposed to grain dust when handling wheat during threshing, unloading, shuffling, and other farm occupations.

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INTRODUCTION

Agricultural workers may be exposed at work to dust-borne fungi and their products which could be released in large quantities into air of breathing zone at harvesting, threshing, and loading of grain, flax threshing, cleaning of herbs and other activities [6, 13, 14, 17]. The exposure may be a cause of allergic and immunotoxic diseases such

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as: bronchial asthma, allergic alveolitis, allergic rhinitis, atopic conjunctivitis, organic dust toxic syndrome, chronic fatigue-like syndrome [6, 10, 14, 17]. Mould fungi of *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium* and *Mucor* genera were identified as the etiologic factors of the above-mentioned diseases. The diseases called mycotoxicoses may be caused by the harmful effects of mycotoxins, which are secondary metabolites of mould fungi [2, 7, 8, 12, 23, 32]. The cytotoxic, neurotoxic, immunosuppressive, teratogenic, mutagenic and carcinogenic effects of mycotoxins following ingestion of contaminated food are well known [16, 36], but little is known about the respiratory effects of these compounds [7].

A serious potential risk for agricultural workers is posed by mycotoxins occurring in grain: aflatoxins, ochratoxin A (OTA), moniliformin (MON) and trichothecenes produced by fungi belonging to genus *Fusarium*: deoxynivalenol (DON, vomitoxin), nivalenol (NIV), zearalenone (ZEA), T-2 toxin and others [1, 2, 3, 4, 5, 9, 16, 18, 21, 26, 33, 34, 35]. Special attention should be paid to toxins produced by *Fusarium* (fusariotoxins), which have been scarcely studied with respect to occupational risk [9, 10, 12]. Most of conducted studies the hitherto on *Fusarium* concern their role as cereals pathogens.

The aim of the present study was to determine the concentration of fungi and selected mycotoxins in wheat grain and grain dust samples collected during threshing, with special attention to *Fusarium* and fusariotoxins, in order to assess potential risk for grain handling farmers.

MATERIALS AND METHODS

Samples of grain and grain dust. Studies were conducted on 10 private farms in the Lublin region (eastern Poland). The samples of wheat grain and grain dust were taken during threshing carried out by the traditional method using on MC-50 thresher. A total number of 20 samples were taken - 10 wheat grain samples and 10 settled dust samples - in which the level of mould fungi was determined, as well as their species composition and level of mycotoxins.

Determination of the concentration and species composition of fungi. The concentration and species composition of microfungi in the samples of wheat grain and grain dust were determined by the dilution plating method [6]. One gram of each sample was suspended in 100 ml of sterile saline (0.85% NaCl) containing 0.1% (v/v) of Tween 80, and after vigorous shaking, serial 10-fold dilutions in saline were made up to 10^{-10}. The 0.1 ml aliquots of each dilution were spread on duplicate sets of plates. Malt agar was used for the determination of the concentration and species composition of microfungi in grain and grain dust, and a mineral medium - SNA (Selective Nutrient Agar which contains the following components: glucose, saccharose, potassium phosphate, sodium nitrate and potassium chloride) [22] as a selective medium for *Fusarium* spp. The malt agar plates were incubated for four days at 30ºC and four days at 22ºC whereas SNA plates - for eight days at 22ºC. After incubation, the number of microfungi in one gram of the sample was calculated and expressed in cfu/g. Then, macro- and microscopic studies of fungi were carried out in order to determine their species composition. Fungi of *Penicillium* spp. were determined according to Ramirez [29] and Pitt [28], while those of *Aspergillus* spp. according to Raper and Fennel [30]. Fungi of *Fusarium* spp. were determined according to Nelson *et al* [22]. The remaining fungal species were determined according to Pidopliczko and Milko [27], and Litvinov [19].

Determination of the concentration of mycotoxins. The thin-layer chromatography method (TLC) and high-performance thin-layer chromatography method (HPTLC) was used [24, 25]. Determination of the concentration of ochratoxin A (OTA), moniliformin (MON), deoxynivalenol (DON) and nivalenol (NIV) was carried out in 10 samples of wheat grain and 10 samples of grain dust released during threshing. Each sample of 10 gm was extracted by the mixture of acetonitryle and water (75:25). The filtrate was divided into three parts: the first part was degreased and cleaned in a column containing Florisil, then the level of moniliformin (MON) was determined by the high-performance liquid chromatography method (HPLC); the second part was cleaned in Ochratest Vicam columns, after which the level of ochratoxin A was determined by HPLC method; the third part of the extract was cleaned in columns containing activated carbon, Celite and Al_2O_3 neutral (1:1:1), and the levels of fusariotoxins of B group: deoxynivalenol (DON) and nivalenol (NIV) were determined with the use of gas chromatograph attached to a mass spectrometer (GC/MS). Chromatographic analysis of mycotoxins was performed in the Department of Chemistry at the Agricultural University in Poznań.

Statistical analysis. The data distribution was checked for normality by the Shapiro-Wilk's test. The significance of the correlations between individual variables was tested by the non-parametric Kendall *tau*-test, and the signicance of differences between matched variables was tested by the Wilcoxon matched pairs test. A p-value of less than 0.05 was considered significant. The analyses were performed with the use of the Statistica for Windows v. 4.5 package (Statsoft©, Inc., Tulsa, Oklahoma, USA).

RESULTS

The results of the study are summarised in Table 1. Total microfungi able to grow on malt agar were present in 30% of wheat grain samples (median for all examined samples = 0, range 0–227.5 × 10^3 cfu/g) and in all samples of grain dust, being much more numerous than in the grain samples (median = 977.5 × 10^3 cfu/g, range

Table 1. Concentrations of total fungi, *Fusarium* spp. and selected mycotoxins determined in samples of grain and grain dust collected on farms in eastern Poland.

Sample	Total fungi (Malt agar) cfu/g × 10^3	*Fusarium* spp. (SNA medium) cfu/g × 10^3						Mycotoxins (µg/g)				
		Fusarium culmorum	*Fusarium graminea*	*Fusarium avenaceum*	*Fusarium poae*	*Fusarium sporotri.*	Total *Fusarium*	*Fusarium* toxins				Ochratoxin
								MON[a]	DON[b]	NIV[c]	Total[d]	
Grain												
1	227.5	0	0	0	1.0	0	1.0	0.180	0.950	0.240	1.370	0.0025
2	60.0	0	0	800.0	0	0	800.0	0.0025	0.280	ND	0.2825	0.0006
3	0	0	0	0	0	0	0	ND	ND	0.015	0.015	ND
4	10.0	0	0	0	0	0	0	ND	ND	ND	ND	0.004
5	0	0	0	0	0	0	0	ND	ND	ND	ND	ND
6	0	0	0	0	0	0	0	0.200	ND	1.280	1.480	0.0005
7	0	0	0	0	0	0	0	ND	0.170	0.110	0.280	0.0005
8	0	0	0	0	0	0	0	ND	ND	ND	ND	0.0005
9	0	0	0	0	0	0	0	ND	0.110	ND	0.110	ND
10	0	0	0	0	0	0	0	0.130	ND	0.015	0.145	ND
Median	0*(O)	0	0	0	0*(D)	0	0**(D)	0	0	0.0075	0.1275	0.0005
Grain dust												
1	115.0	0	5.0	0.5	0	0	5.5	0.260	0.015	ND	0.275	0.0031
2	16700.0	0	0	0	100.0	0	100.0	0.027	ND	ND	0.027	0.0005
3	225.0	0	0	0	0	1500.0	1500.0	ND	0.310	ND	0.310	ND
4	450.0	0	0	50.0	0	0	50.0	ND	ND	ND	ND	0.0009
5	725.0	0	0	40.0	10000.0	20.0	10060.0	0.210	ND	0.880	1.090	0.0016
6	1230.0	0	0	800.0	0	0	800.0	0.360	ND	0.030	0.390	ND
7	1625.0	50.0	0	5000.0	0	0	5050.0	0.180	0.460	0.015	0.655	0.0005
8	2310.0	0	1000.0	0	600.0	0	1600.0	0.025	0.450	0.350	0.825	ND
9	1300.0	0	0	0	2000.0	0	2000.0	0.780	ND	ND	0.780	ND
10	175.0	0	0	0	0	0	0	0.110	ND	ND	0.110	0.0524
Median	977.5	0*(D)	0*(D)	0.25	0*(MDN)	0	1150.0*(N)**(MDN)	0.145	0	0	0.350	0.0005
Total samples												
Median	145.0	0*(D)	0	0	0*(M, MDN)	0	3.25*(M)**(MDN)	0.026	0	0	0.2775	0.0005

Fusarium graminea. = *Fusarium graminearum*, *Fusarium sporotri*. = *Fusarium sporotrichioides*. MON[a] = moniliformin; DON[b] = deoxynivalenol; NIV[c] = nivalenol; Total[d] = total *Fusarium* toxins (MON + DON + NIV). ND = not detected (considered as zero level). *(O): significantly correlated with the concentration of ochratoxin ($p < 0.05$); *(M): significantly correlated with the concentration of MON ($p < 0.05$); *(D): significantly correlated with the concentration of DON ($p < 0.05$); **(D): significantly correlated with the concentration of DON ($p < 0.01$); *(N): significantly correlated with the concentration of NIV ($p < 0.05$); *(MDN): significantly correlated with the concentration of total *Fusarium* toxins ($p < 0.05$); **(MDN): significantly correlated with the concentration of total *Fusarium* toxins ($p < 0.01$).

115.0–16700.0 × 10^3 cfu/g) (Tab. 1). The dominant constituent of fungal flora isolated from grain was *Aspergillus fumigatus* (76.5%) and the other constituents were *Oidiodendron flavum* (16.8%), *Trichothecium laxicephalum* (5.0%) and *Alternaria alternata* (1.7%). Among fungi isolated from grain dust, on average, most common were *Cladosporium* species (*C. atroseptum*, *C. herbarum*) which formed 34.4% of the total, yeast (31.8%), *Fusarium* species (23.0%) while *Alternaria alternata* (7.4%). *Penicillium* spp., *Aspergillus fumigatus*, *Aspergillus repens*, *Mucor mucedo*, *Citromyces* sp., *Oidiodendron rhodogenum* and *Monilia geophila* constituted the remaining 3.4% of the grain dust mycobiota.

The growth of *Fusarium* species on the selective SNA medium was much more abundant compared to malt agar. Among grain samples, *Fusarium* (identified as *F. avenaceum*) was isolated from only one out of 10 samples (10%) (median = 0, range 0–800.0 × 10^3 cfu/g). Similar to

the case of total microfungi, the frequency and concentration of *Fusarium* spp. in grain dust samples was much greater compared to grain samples. Fusaria were isolated from 90% of grain dust samples and their concentrations were large (median = 1150 × 10^3 cfu/g, range 5.5–10060.0 × 10^3 cfu/g) (Tab. 1). The species *F. avenaceum, F. culmorum, F. graminearum, F. poae* and *F. sporotrichioides* were isolated respectively from 50%, 10%, 20%, 40% and 20% of examined grain dust samples. The strains of *Fusarium poae* formed 60.0% of total *Fusarium* isolates recovered from grain dust on SNA medium, followed by *F. avenaceum* (27.8%), *F. sporotrichioides* (7.2%), *F. graminearum* (4.8%), and *F. culmorum* (0.2%).

The concentrations of both total fungi and *Fusarium* species were significantly greater in the samples of grain dust compared to grain samples ($p < 0.05$).

The presence of the mycotoxins produced by *Fusarium* (moniliformin, deoxynivalenol, and nivalenol) was found altogether in 70% of wheat grain samples (median for all examined samples = 0.1275 µg/g, range 0–1.480 µg/g) and in 90% of grain dust samples (median = 0.350 µg/g, range 0–1.090 µg/g) (Tab. 1). Separately, moniliformin (MON), deoxynivalenol (DON), and nivalenol (NIV) were each detected in 40% of grain samples, and respectively in 80%, 40%, and 40% of grain dust samples. The average concentrations of MON, DON, NIV, and the total (summarised) *Fusarium* toxins (fusariotoxins) in positive grain samples were respectively 0.1281 µg/g, 0.3775 µg/g, 0.3875 µg/g, and 0.5261 µg/g, whereas in positive samples of grain dust - respectively 0.2440 µg/g, 0.3087 µg/g, 0.3187 µg/g, and 0.4958 µg/g. No significant differences were found between the concentrations of fusariotoxins in the samples of grain and grain dust ($p > 0.05$).

The occurrence of ochratoxin was very similar in the samples of grain and grain dust. This mycotoxin was detected in 60% of both kinds of samples and also the median was the same in both cases, equal to 0.0005 µg/g (range for grain samples was 0–0.004 µg/g, and for grain dust samples 0–0.0524 µg/g) (Tab. 1). The average concentrations of ochratoxin in positive samples of grain and grain dust were 0.0014 µg/g, and 0.0098 µg/g, respectively.

Significant relationships were found between the concentrations of particular *Fusarium* species and the concentrations of fusariotoxins. As regards grain samples, the concentrations of *F. poae* and of total *Fusarium* species were significantly correlated with the concentration of deoxynivalenol ($p < 0.05$ and $p < 0.01$, respectively). As regards the samples of grain dust, the concentrations of *F.culmorum* and *F. graminearum* were significantly correlated with the concentration of deoxynivalenol ($p < 0.05$), the concentration of *F. poae* was significantly correlated with the concentration of total (summarised) fusariotoxins (MON+DON+NIV) ($p < 0.05$), and the concentration of total *Fusarium* species was significantly correlated with the concentration of nivalenol ($p < 0.05$) and the concentration of total fusariotoxins ($p < 0.01$). As regards total examined samples (grain + grain dust), the concentration of *F. culmorum* was significantly correlated with the concentration of deoxynivalenol ($p < 0.05$), the concentration of *F. poae* was significantly correlated with the concentrations of moniliformin and total fusariotoxins ($p < 0.05$), and the concentration of total *Fusarium* species was significantly correlated with the concentration of moniliformin ($p < 0.05$) and the concentration of total fusariotoxins ($p < 0.01$) (Tab. 1).

The concentration of total fungi (grown on malt agar) in grain samples was significantly correlated with the concentration of ochratoxin ($p < 0.05$) (Tab. 1).

DISCUSSION

Mycotoxins detected in the present study are described as etiologic factors of mycotoxicoses creating a health risk for humans [6, 8, 14, 16, 36].

Moniliformin (MON) produced by *Fusarium avenaceum*, causes acute, degenerative lesions in the myocardium, the symptoms being similar to those for arsenic poisoning [31].

Deoxynivalenol (DON) and nivalenol (NIV) produced by *F. culmorum* and *F. graminearum* are trichothecene mycotoxins causing vomiting, diarrhoea and dermal reaction. An epidemic of alimentary mycotoxicosis in the Kashmir Valley was described by Smith *et al* [31]. The symptoms of poisoning were: abdominal pain, feeling of fullness of the stomach within 15–60 minutes after eating, irritation of the throat, diarrhoea, vomiting, blood in the stools and allergic dermal reactions. These symptoms, called 'red mould disease' were caused by the consumption of mouldy wheat and rice contaminated with DON and other fusariotoxins.

Ochratoxin (OTA) produced by *Aspergillus ochraceus* and some *Penicillium* species (*P. viridicatum, P. cyclopium, P. chrysogenum*) caused Balkan endemic nephropathy (BEN) observed among the population of the Balkan countries [31]. The consumption of food contaminated with ochratoxin resulted in pathologic symptoms such as: great reduction in kidney size, tubular degeneration, interstitial fibrosis and hyalinization of glomeruli.

Until now, no internationally accepted threshold limit values (TLV) for the concentration of mycotoxins in wheat and other food products have been established. The proposals for such values for DON range between 0.5–2.0 µg/g [24]. In this study, the lower limit of this range was exceeded in only one sample.

The common infestation of wheat with fusaria and fusariotoxins, in particular with deoxynivalenol (DON) and nivalenol (NIV), has been reported from many countries of Europe, Asia and America [5, 9, 11, 18, 24, 25, 26, 33, 34, 35]. In the examined wheat, DON was detected in 60–100% of samples [3, 5, 9, 11, 18, 33, 34,

35] and NIV in 4–96% of samples [9, 11, 18, 26, 33, 34, 35]. The concentration of DON in the positive samples ranged between 0.031–1.257 µg/g [11, 18, 33, 34, 35] and that of NIV - between 0.023–0.566 µg/g [11, 18, 33, 34, 35].

The frequency of the occurrence of DON in the samples of wheat grain examined in the present study (40%) was distinctly lower compared to values reported by other authors, while that of NIV (50%) was in the middle of the range of hitherto reported data. The average concentrations of DON and NIV in positive grain samples (respectively 0.3775 µg/g and 0.3875 µg/g) stated by us were greater than most of the values hitherto reported from other countries, except for DON concentration found in Canada [34] and NIV concentrations found in Korea [18] and Scotland [33].

Abramson *et al.* [1] determined the production of mycotoxins by different *Fusarium* cultures and concluded that DON was produced mostly by the strains of *F. culmorum* and *F. graminearum*, while moniliformin by the strains of *F. avenaceum*. The results of these authors concerning DON conform to those found in the present study, as a significant correlation between the concentrations of *F. culmorum* and *F. graminearum* in grain dust samples and the concentration of DON in these samples was found. A significant correlation between the concentration of *F. graminearum* and DON has been also found by Dalcero *et al.* [5] who examined wheat samples in Argentina. In contrast, Moreno Contreras *et al.* [21] failed to find any significant correlation between the concentration of *Fusarium* spp. and DON in grain samples collected in Venezuela. In our study, the concentration of moniliformin was not significantly correlated with the concentration of *F. avenaceum*, but with that of *F. poae*. The concentrations of *F. poae* were also significantly correlated with the DON concentration (in grain samples) and with the concentration of total fusariotoxins (in grain dust samples and total samples of grain and grain dust). These results suggest that most probably *Fusarium poae*, the species distinctly prevailing among *Fusarium* strains isolated from grain and grain dust in eastern Poland, poses in this area the main hazard as the source of adverse mycotoxins.

Ochratoxin was detected in 1.0–57.6% of wheat samples collected in various countries [3] and its concentration in positive samples varied widely between 0.005–27.5 µg/g [3]. In the present study, the frequency of occurrence of positive samples in grain and grain dust (60%) was higher compared to hitherto reported results, but the average concentration of ochratoxin in these samples was much smaller (0.0005 µg/g).

So far, little is known about the concentrations of fusariotoxins and other mycotoxins in grain dust with respect to a potential health hazard. Low DON concentrations (0.003–0.020 µg/m^3) were found in the air during milling of grain in Finland [9]. May *et al.* [20] detected low concentration of DON (0.1–0.2 µg/g) in the sample of silage associated with a febrile illness in farmers. Palmgren *et al.* [23] examined for mycotoxins 15 samples of settled grain dust collected in elevators in the New Orleans area and found in 10 samples zearalenone at levels from 0.025 to 0.1 µg/g, but no ochratoxin A or aflatoxin. Ehrlich and Lee [7] found in 80% of the examined samples of grain dust the presence of ochratoxin, deoxynivalenol, secalonic acid, zearalenone, and aflatoxin in the concentrations of 0.0005–0.02 µg/g, 0.0005–0.02 µg/g, 0.0005–0.02 µg/g, 0.02 µg/g, and 0.0005 µg/g, respectively. It is noteworthy that the average concentration of deoxynivalenol in the grain dust samples found in the present study is 19–755 fold greater compared to the data obtained by these authors.

In conclusion, the majority of samples of wheat grain and grain threshing dust collected on farms in eastern Poland contained notable quantities of fusaria and/or fusariotoxins. This fact poses a potential risk of mycotoxicoses to agricultural workers exposed to grain dust when handling wheat during threshing, unloading, shuffling, and other farm occupations.

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