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Juliusz Perkowski, Marian Wiwart, Maciej Buśko, Magdalena Laskowska, Franz Berthiller, Wolfgang Kandler, Rudolf Krska

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***Fusarium* toxins and total fungal biomass indicators in naturally contaminated wheat samples from north-eastern Poland in 2003**

Abstract

Concentrations of fungal metabolites were measured in 32 wheat grain samples from north-eastern Poland in 2003. The samples originated from fields cultivated conventionally (but varying in chemical protection level) or cultivated organically. Concentrations of *Fusarium* toxins (HT-2, DON, 3-AcDON, NIV), trichodiene, microbial biomass indicators [fungal ergosterol and general adenosine-5'-triphosphate (ATP)] and seed vigour were assessed. A large variation between samples was observed, depending on their origin. Seed from organic farms contained similar amounts of *Fusarium* toxins but more ergosterol and ATP than conventionally grown and chemically protected seed. The highest levels of toxins and ergosterol were detected in samples from conventional cultivation lacking chemical protection. Intensive agronomic practices (including complete chemical protection) significantly lowered the levels of ergosterol, ATP and trichodiene, as compared with other cultivation systems.

Keywords: wheat, cultivation systems, trichothecenes, ergosterol, trichodiene, ATP

Introduction

In Poland, as in other EU countries, various fungal diseases affect wheat, but special attention is paid to *Fusarium* head blight (FHB). In the literature there is a shortage of convincing data about the influence of cultivation systems on the severity of its disease symptoms (Teich 1989, Champeil et al. 2004, Lemmens et al. 2004, Perkowski et al. 2004). In north-eastern Poland, organic farming is particularly important because of the low level of environmental contamination and the possibility of growing healthy food (Grodzińska et al. 1999, Łukanowski and Sadowski 2002). Literature data reporting contamination by fungal metabolites of organically grown cereals are contradictory (Elmholt and Labouriau 2005, Pussemier et al. 2006). However, some authors point to the significant influence of

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3 environmental conditions (weather in particular) on their concentrations. Malmauret et al.
4 (2002) reported that concentrations of *Fusarium* toxins were higher in wheat and barley seed
5 from organic farms, but their results showed no conclusive evidence whether conventional
6 products were more or less safe than organic ones. Apart from *Fusarium* toxins, also fungal
7 biomass in seed is important. Fungal biomass is usually estimated on the basis of ergosterol or
8 chitin content (Abramson et al. 1998, Ekblad et al. 1998). Also ATP level is measured on the
9 surface of kernels, because it is correlated with levels of some other fungal metabolites
10 (Williams 1989), but it reflects the level of the whole microbial biomass. Seed colonization by
11 fungi often affects its vigour and consequently also sowing value. Infection by pathogens of
12 the genus *Fusarium* often disturbs or even completely inhibits seed germination (Eudes et al.
13 1997, Packa and Koczowska 1987).

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25 This study was aimed to compare concentrations of fungal metabolites in wheat seed from
26 conventional farms (with more or less intensive use of chemical protection) and organic
27 farms. The comparison involved a complex of fungal metabolites: concentrations of selected
28 *Fusarium* toxins (deoxynivalenol, 3-acetyldeoxynivalenol, the sum of 3-acetyldeoxynivalenol
29 and 15-acetyldeoxynivalenol, nivalenol, fusarenon-X, diacetoxyscirpenol, HT-2 toxin, T-2
30 toxin and zearalenone), trichodiene (their precursor), and microbial biomass indicators (fungal
31 ergosterol and general microbial ATP). Differences in the studied characters between four
32 cultivation systems and correlations between concentrations of the studied metabolites were
33 analysed.

34 35 36 37 38 39 40 41 42 **Material and methods**

43 44 45 *Samples*

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47 The analysis involved 32 grain samples (of 1000 g each) of winter and spring wheat from
48 conventional farms (20 samples) and organic farms (12 samples) located in 7 districts in
49 north-western Poland (Figure 1). Conventional farms were divided into 3 groups: CON I,
50 CON II and CON III. In CON I (9 samples from 6 districts), complete antifungal protection
51 and pesticides were applied. CON II (6 samples from 6 districts) included large fields (over
52 30 ha) subject to a lower level of chemical protection (fungicidal protection against main
53 foliar diseases, first of all mildew and leaf septoriosiis), while CON III (5 samples from one
54 district Ostróda) was an experimental farm Bałcyny, where neither fungicides nor pesticides
55 were used. Production in organic farms conformed to the rules applied in this cultivation
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3 system (the seed had the Polish ECO certificate). Production in conventional farms followed
4 the rules of the currently recommended intensive methods of cereal cultivation.
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8 *Determination of major A- and B-trichothecenes and zearalenone by LC-MS*

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10 Deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), the sum of 3-
11 acetyldeoxynivalenol and 15-acetyldeoxynivalenol (AcDONs), nivalenol (NIV), fusarenon-X
12 (FUS-X), diacetoxyscirpenol (DAS), HT-2 toxin, T-2 toxin and zearalenone (ZON) were
13 determined by liquid chromatography – triple quadrupole mass spectrometry as described
14 previously by (Berthiller et al. 2005). 10 g of the ground samples were extracted with 40 mL
15 acetonitrile/water (84/16, v/v) and purified using MycoSep 227 columns (RomerLabs, Tulln,
16 Austria). 4 mL of the purified extracts were evaporated to dryness and dissolved in mobile
17 phase (methanol/water 20/80, v/v containing 5 mM ammonium acetate). An Agilent
18 (Waldbronn, Germany) 1100 HPLC system was used in conjunction to a QTrap MS/MS from
19 Applied Biosystems (Foster City, USA). Atmospheric pressure chemical ionisation was used
20 in negative mode for B-trichothecenes and in positive mode for A-trichothecenes. 25 µl were
21 injected onto a Thermo Aquasil RP-C18 column (4.6 x 100mm, 3µm), thermostated to 30°C.
22 The multiple reaction monitoring mode was used for quantification of the samples. This
23 method was carefully optimized for the quantification of selected Fusarium mycotoxins in
24 cereal. Its limits of quantification (LOQs) are 3 µg/kg DON, 13 µg/kg 3-AcDON, 4 µg/kg
25 AcDONs, 18 µg/kg NIV, 6 µg/kg FUS-X, 1 µg/kg DAS, 4 µg/kg HT-2, 1 µg/kg T-2 and 3
26 µg/kg ZON. The limits of detection (LODs) were about 3 times lower than the LOQs.
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43 *Determination of ergosterol*

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45 Samples containing 100 mg of ground grains were placed into 17 mL culture tubes,
46 suspended in 2 mL of methanol, treated with 0.5 ml of 2 M aqueous sodium hydroxide and
47 tightly sealed. Then the culture tubes were placed within 250 mL plastic bottles, tightly sealed
48 and placed inside the microwave oven (Model AVM 401/1WH, Whirlpool, Sweden)
49 operating at 2450 MHz and 900 W maximum output. Samples were irradiated (370 W) for 20
50 s. and after ca. 5 min. an additional 20 s. After 15 min contents of culture tubes were
51 neutralized with 1 M aqueous hydrochloric acid, 2 mL MeOH were added and extraction with
52 pentane (3 x 4 mL) were carried out within the culture tubes. The combined pentane extracts
53 were evaporated to dryness in a nitrogen stream. Prior to analysis samples were dissolved in 4
54 mL of MeOH, filtered through 13 mm syringe filters with 0.5 µm pore diameter (Fluoropore
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3 Membrane Filters, Millipore, Ireland) and evaporated to dryness into N₂ stream. The sample
4 extract was dissolved in 1 mL of MeOH and 50 µL was analysed by HPLC technique.
5 Separation was achieved on 150 x 3.9 mm Nova Pak C-18, 4 µm column and eluted with
6 methanol/acetonitrile (90:10) at a flow rate of 0.6 mL /min. Ergosterol was detected with
7 Waters 486 Tunable Absorbance Detector set at 282 nm. Quantification of ERG was made by
8 a comparison of peak areas with those of external standards of ERG. Confirmation of ERG
9 was achieved by a comparison of retention times with the external standard and by co-
10 injection every tenth sample with an ergosterol standard.
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19 *Determination of trichodiene*

20 Volatile metabolites were extracted from grain using solid phase microextraction method
21 (SPME). Grain samples (8 g) were placed in 20 mL vials and extracted by means of
22 headspace SPME during 30 min at 40°C with 100 µm polydimethylsiloxane (PDMS) fibre.
23 The analyses were run on a gas chromatograph mass spectrometer (Hewlett Packard GC
24 6890, MS 5972 A), using an HP – 5MS (0.25 mm x 30 m) capillary column. The injection
25 port temperature was 250°C, the transfer line temperature was 280°C and the analyses were
26 performed with programmed temperature: initial temperature 40°C hold 3 min, from 40°C -
27 200°C at 10°C/min, 200°C - 280°C at 20°C/min, the final temperature being maintained for 3
28 min. The helium flow rate was held constant at 0.7 mL/min. Quantitative analysis of
29 trichodiene was performed in selected ion monitoring (SIM) mode, using following ions for
30 trichodiene detection: m/z 67, 93, 108, 109. The amount of trichodiene was estimated
31 comparing the area of its total ion current (TIC) peak with internal standard (tridecane, 25 ng
32 in pentane) and expressed as their ratio (RU). Trichodiene was identified by comparing its
33 mass spectrum with literature data (Jeleń et al. 1995).
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48 *Determination of ATP*

49 Adenosine-5'- triphosphate (ATP) measurement was carried out using luciferase enzyme.
50 ATP was extracted from 1.00 g grain samples using 8 mL boiling Tris buffer (0.1 molar
51 solution, pH – 7.75). 100 µL of this extract were mixed with 100 µL reagent containing
52 luciferin and luciferase (LuminATE (QM), Celsis, Netherlands). The emitted light was
53 quantified by Lumac Biocounter M 1500 luminometer and readout as Relative Light Units
54 (RLU). The emitted light correlates with the amount of ATP in the sample.
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Seed vigour test

The test was performed in two replicates, according to the guidelines of the International Seed Testing Association (Hampton and Tekrony 1995). Subsamples of 50 surface-sterilized kernels each were placed on strips of filter paper (50 cm × 25 cm). Next the strips were rolled up and deposited in beakers containing a small amount of sterile distilled water and kept in a phytotron in darkness at 22°C. After 7 days, proportions of non-germinated and abnormally germinated grains and of grains with *Fusarium* mycelium were assessed.

Statistical analyses

The significance of differences in mean values was evaluated with the use of Student's *t*-test, and linear correlation analysis was made for all studied parameters.

Results and discussion

The wheat grain samples classified into 4 different groups (CON I, CON II, CON III and ECO) markedly differed in the analysed parameters: HT-2 toxin, DON, 3-AcDON, NIV, sum of the most important group B trichothecenes in grain (DON+NIV together), ERG, TRICH, ATP and seed vigour parameters (non-germinated and abnormally germinated grains as well as grains with *Fusarium* mycelium). The results are presented in Tables 1 and 2 (except of ZEA concentrations because their were below the limit of detection).

The grains were characterized by a low proportion of infestation by fungi of the genus *Fusarium*, ranging from 0 to 4.0%, but differences between the groups were not significant. The proportion of grains with *Fusarium* mycelium was positively correlated with the proportion of abnormally germinated grains, although value of Pearson correlation coefficient was not significant, because during vegetation *Fusarium* is not the only factor influencing abnormal grain germination, but also harvest conditions, kernel damage as well as preinfestation changes. Additionally, since these were productive farms, an accurate determination of the above mentioned factors was difficult. A significant correlation was found between the proportion of non-germinated grains and the proportion of abnormally germinated grains. Mean values of both these parameters were the lowest in CON I, and the highest in ECO.

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3 The mean concentration of trichothecene toxins in CON I and CON II can be regarded as low,
4 and it is comparable with results reported by (Pettersson 1996, Perkowski et al. 1997). In
5 those groups, NIV+DON concentration reached 43.0 $\mu\text{g kg}^{-1}$ (CON I) and 30.0 $\mu\text{g kg}^{-1}$ (CON
6 II). In organically grown wheat, the NIV+DON level was similar, but the concentration of
7 DON was higher than the concentration of NIV. In comparison with CON I, CON II and
8 ECO, significant differences were found in CON III (conventional but lacking chemical
9 protection), where NIV+DON content was over 14 times higher than the mean for the other
10 groups (635.2 $\mu\text{g kg}^{-1}$ versus 44.3 $\mu\text{g kg}^{-1}$), and the major contributor was DON.
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19 Concentrations of trichodiene (TRICH), which is a precursor of trichothecenes (Jeleń et al.
20 1995), were the highest in samples containing the highest levels of trichothecenes (CON III),
21 while the lowest in organically grown wheat seed. A positive correlation ($r=0.788$, $P=0.05$)
22 was observed between TRICH level and concentration of NIV+DON in seed.
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29 In this study, with the use of so wide-ranging wheat materials, concentrations of ERG
30 (ergosterol, indicator of fungal biomass) and ATP (indicator of microbial biomass in the outer
31 parts of the grain) were measured. To be exact, Reid et al. (1999) examined disease severity,
32 ergosterol content, fungal DNA content and mycotoxin (DON and fumonisin B₁)
33 concentration in silk maize ears channel-inoculated with *F. graminearum* and *F. moniliforme*.
34 The authors reported that although biomass may be the best predictor of disease ratings, the
35 DNA analysis demonstrated that this biomass was not always produced by the expected
36 *Fusarium* sp. However, the above experiments were carried out for maize. Similar
37 conclusions were indicated by Marfleet et al. (1991) as well.
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47 The highest ERG level was 2.39 mg/kg (CON III), but it reached a similar value in ECO.
48 These values were significantly higher than in CON I and CON II, but still were below the
49 threshold of 3 mg/kg reported by Schnürer (Schnürer and Jonsson 1992) for seed showing no
50 symptoms of fungal colonization. In CON I, the lowest ERG level was recorded, and ATP
51 content was also low (2153 RLU). It is interesting that such a low ATP concentration was
52 recorded in all analysed samples of this group. This value was about 8-fold lower than in
53 organically grown wheat seed (16128 RLU). It is probably a result of the lack of chemical
54 protection in organic farming, which is favourable for microbial growth in the outer parts of
55 the grain. The lack of chemical protection and high mineral fertilization combined with all
56 other agronomic practices used in conventional cultivation in CON III could favour fungal
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3 growth at the expense of other microorganisms. This was reflected in a high ERG and
4 mycotoxins concentration and relatively low ATP content. Because of the varied cultivation
5 systems, only a low positive correlation between ERG and ATP content was found when all
6 four groups were analysed jointly. When the groups were analysed separately, the highest
7 correlation between these characters ($r=0.726$, significant at $P<0.01$) was recorded in CON I,
8 which seems to be the most common in that part of Poland.
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16 In conclusion, results of this study show that wheat seed in north-eastern Poland in 2003 was
17 characterized by a low level of colonization by fungi of the genus *Fusarium*, but considerable
18 differences were observed depending on cultivation system. Seed samples from organic farms
19 contained similar amounts of *Fusarium* toxins but higher levels of ERG and ATP than
20 conventionally grown and chemically protected seed samples did. A slightly higher DON
21 content in ECO samples in comparison to CON II samples, and especially CON I samples,
22 and a very low NIV content suggest that while observing the principles of organic farming,
23 especially appropriate crop rotation, the absence of chemical protection does not necessarily
24 have to led to increased concentrations of these metabolites in grain, which was also
25 emphasized by Champeil et al. (2004). At the conventional cultivation system cereals,
26 especially wheat and maize, account for a very large share of crops in crop rotation.
27 Necrotrophic pathogens from genus *Fusarium* develop dynamically on post-harvest residue of
28 these crops, which promotes the accumulation of FHB symptoms in the successive years,
29 especially in wheat following maize. Dill-Macky and Jones (2000) reported that in case of
30 wheat grown after soy FHB symptoms occur in a much lesser degree. The application of
31 ploughing additionally limits the incidence of this disease, as post-harvest residue is
32 transferred to deeper soil layers. Thus it seems that the application of an appropriate crop
33 rotation scheme in organic farming (with papilionaceous plants), together with comprehensive
34 mechanical soil cultivation reduces the risk of pathogenic species from genus *Fusarium* to
35 such an extent that a lack of chemical protection does not have a significant effect on an
36 increase of *Fusarium* toxin concentrations in wheat kernels. It is possible that a significantly
37 higher dry matter content (including that of nutrients and minerals) in tissues of plants grown
38 in the organic farming system (Kouba 2003) is also accompanied by a higher concentration of
39 phytochemical substances exhibiting fungistatic action connected with resistance to fungal
40 pathogens. It is of interest that conventional farming with no fungicides applied (CON III)
41 resulted in a drastic increase of *Fusarium* toxin concentrations in kernels. This result is
42 especially important in view of the increasingly promoted and implemented sustainable
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3 cultivation system, in which chemical crop protection is reduced, at the simultaneous
4 maintenance of major elements of the conventional farming system (e.g. the application of
5 mineral fertilizers) and at the same time placing more emphasis on crop rotation and
6 mechanical tillage. The most intensive agronomic practices resulted in significantly lower
7 concentrations of ERG, ATP and TRICH, as compared to the other systems of cultivation.
8 The highest levels of ergosterol were detected in samples from conventional cultivation
9 lacking chemical protection. These results form the basis for further research on this subject.
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20 cooperation no. 7/2004.
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Table 1. Concentration of trichothecenes ($\mu\text{g kg}^{-1}$) and ergosterol (ERG, mg kg^{-1}) and amount of trichodiene (TRICH), adenosine triphosphate (ATP) and vigour test parameters for 32 examined wheat grain samples obtained from different cultivation systems.

Sample	HT-2 ($\mu\text{g kg}^{-1}$)	DON ($\mu\text{g kg}^{-1}$)	3-AcDON ($\mu\text{g kg}^{-1}$)	NIV ($\mu\text{g kg}^{-1}$)	NIV+DON ($\mu\text{g kg}^{-1}$)	ERG (mg kg^{-1})	TRICH (RU) $\times 10^{-3}$	ATP (RLU)	Non-germinated (%)	Abnormal (%)	<i>Fusarium</i> (%)
CON I											
1	13	31	-	-	31	1.08	2.085	3096	0.0	0.0	1.5
2	-	-	-	26	26	0.88	1.579	2844	0.0	0.0	0.0
3	12	-	-	75	75	0.86	5.026	1754	0.0	6.0	2.0
4	53	36	-	39	75	0.79	3.061	2313	1.0	4.0	2.0
5	-	-	-	80	80	0.37	6.630	1874	0.5	2.0	0.0
6	-	-	-	33	33	0.33	0.394	1834	0.5	1.0	0.5
7	-	-	-	-	0	0.64	0.520	1329	0.0	6.0	1.5
8	-	67	-	-	67	0.62	0.467	1110	1.5	2.5	2.5
9	-	-	-	-	0	1.85	0.955	3228	1.5	2.5	4.0
CON II											
10	-	14	-	-	14	2.11	7.768	15821	2.5	4.5	1.0
11	-	11	-	22	33	1.47	7.711	18166	1.5	3.5	0.0
12	-	11	-	(12)	23	0.66	3.116	13582	3.0	3.0	1.5

13	4	26	-	-	26	0.79	3.649	16199	4.5	7.0	1.5
14	6	53	-	(13)	66	0.55	1.319	12706	3.5	4.5	0.5
15	-	17	-	-	17	0.46	2.385	17262	6.0	8.0	3.5
CON III											
16	-	784	18	27	811	1.83	6.293	16642	2.0	4.5	0.0
17	-	480	-	43	523	2.9	3.961	14893	3.0	2.5	2.0
18	66	607	19	(7)	614	2.01	7.866	10944	1.5	3.0	0.5
19	-	190	-	-	190	3.34	8.581	5663	4.0	1.0	1.5
20	-	997	37	41	1038	1.87	3.285	10009	1.5	1.5	0.0
ECO											
21	-	-	-	28	28	3.34	1.528	10010	2.0	6.5	5.0
22	8	-	-	13	16	3.26	1.262	10477	3.5	3.5	2.5
23	-	-	-	-	0	2.01	1.404	12740	4.5	9.5	2.0
24	-	380	-	-	380	2.95	4.843	9772	2.5	10.5	1.5
25	-	12	-	-	12	1.65	0.644	15843	4.5	9.5	2.0
26	-	-	-	-	0	2.44	0.799	14828	3.5	7.5	1.5
27	-	7	-	-	7	0.92	2.009	14287	2.5	7.0	3.5
28	-	60	-	-	60	2.88	0.472	22062	10.5	7.5	1.0
29	-	-	181	-	0	2.84	1.410	31273	3.5	2.0	0.5

30	-	-	-	-	0	2.66	0.501	18701	3.5	2.5	0.0
31	-	-	-	-	0	1.67	0.943	23831	3.5	4.0	3.0
32	-	16	-	-	16	1.39	0.753	9714	0.5	3.0	2.5

T-2, DAS were not detected in the samples (LOQ: 1 µg/kg)

FUS-X was detected only in sample 15 at 12 µg/kg (LOQ: 6 µg/kg)

“-“ means that no peak was found

Results in brackets are below the limit of quantitation

Table 2. Mean values of analysed characters in wheat seed samples from four cultivation systems

Cultivation System	Mycotoxins ($\mu\text{g kg}^{-1}$)					Proportion of grains (%)					
	HT-2	DON	3-Ac DON	NIV	NIV+	TRICH (RU) $\times 10^{-3}$	ERG (mg kg $^{-1}$)	ATP (RLU)	Non-germ.	Abnorm. germ.	<i>Fusarium</i> mycelium
CON I	8.7	14.9 ^a	0.0 ^a	28.1 ^b	43.0 ^b	2.302 ^a	0.82 ^a	2153 ^a	0.6 ^a	2.7 ^a	1.6
CON II	1.7	22.0 ^b	0.0 ^a	7.8 ^a	29.8 ^a	4.325 ^{ab}	1.01 ^a	15623 ^b	3.5 ^b	5.1 ^b	1.3
CON III	13.2	611.6 ^c	14.8 ^b	23.6 ^b	635.2 ^c	5.997 ^b	2.39 ^b	11630 ^b	2.4 ^b	2.5 ^a	0.8
ECO	0.7	39.8 ^b	15.3 ^b	3.4 ^a	43.3 ^b	1.381 ^a	2.3 ^b	16128 ^b	3.7 ^b	6.1 ^b	2.1

^{a, b, c} - Values marked with the same letters within columns do not differ significantly ($P > 0.05$).

Figure 1. Location of sites of seed samples collection on a map of Poland.

