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THE EFFECT OF ENVIRONMENTAL CONDITIONS ON ERGOSTEROL AND TRICHOTHECENE CONTENT OF NATURALLY CONTAMINATED OAT GRAIN

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Abstract: Oat plants, similar to other cereals, are susceptible to invasion by fungal pathogens and saprophytes, but the severity of disease symptoms and the extent of fungal growth depend to a considerable degree on environmental conditions. This study aimed to analyse the dependence of ergosterol and trichothecene production in oat grain on environmental conditions. Three oat cultivars were cultivated in 10 localities across Poland under natural conditions of fungal infection. Analysis of the effect of weather conditions during the growing season on ergosterol content and total trichothecene *Fusarium* toxin content in grain showed that they are negatively correlated with the sum of precipitation in the dry month of June, i.e. at the flowering stage of oats. Significant rainfall in July (256% multiannual average) resulted in a considerable growth of saprophytic fungi and, as a consequence, in high ERG levels in grain (mean 14.0 mg/kg). Although the total trichothecene content was relatively low (< 150 μg/kg), a significant correlation was observed between this trait and ergosterol content of grain (r = 0.7313). Higher values of correlation coefficients were recorded for the dependence of trichotecene A, as well as trichothecene A and NIV, and ERG levels, amounting to r = 0.8703 and r = 0.7748, respectively. This was probably caused by specific weather conditions manifested by slight precipitation during panicle flowering, which promoted the growth of pathogens (*F. poae*, *F. sporotrichioides*) producing trichothecenes A (T-2 toxin, HT-2 toxin and NIV). In addition, a significant influence of locality on values of both traits was recorded. Variation between cultivars was not significant.

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INTRODUCTION

The occurrence of mycotoxins in small-grain cereals is of worldwide concern. They are frequently present in processed feeds and foods and have been associated with chronic or acute mycotoxicoses in livestock and in humans [33].

The differentiation of host resistance is greatly influenced by an array of non-genetic factors (macro-environmental, microclimate, host growth stage, host organ) that show significant interactions with the host genotype. Highly resistant lines accumulate little toxin, even under ideal disease conditions [13].

Mycotoxin accumulation can be high both in artificially and naturally infected plant stands. The correlations between plant resistance and mycotoxin content are moderate and depend on the environment [13, 14].

However, the influence of several factors (climatic and agronomic conditions), favourable to the infection may lead to a change in the *Fusarium* profile from region to region, as well as from year to year [1]; moreover, the *Fusarium* population structure in grain depends mostly on microclimatic conditions [31]. Perkowski *et al.* [25] found significant correlations between the formation of various *Fusarium* toxins (i.e. trichothecenes) in grain and

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the dominant *Fusarium* species found in a given region. Mesterhazy [12] showed clearly that variation in deoxynivalenol (DON) production was partly due to a highly significant year-climate interaction. Additional symptoms or yield reduction, as well as the development of mycelium in grain (as ergosterol content – the predominant sterol component of mycelium, which can be used as an indicator of the extent of fungal infection of grain) of infected heads and toxin production may be influenced by environmental conditions, such as temperature, humidity and mineral fertilization [15].

Precipitation level at anthesis, and in the periods immediately before and after it, is the most important factor determining the infestation of panicles by pathogenic fungi from genus *Fusarium*, and thus their colonization of grain [23]. This affects the formation of mycotoxin profiles. Slight precipitation at anthesis, or even a complete lack of rainfall, followed by heavy rainfall during grain ripening cause an intensive development of saprotrophic fungi. Vozenilkova *et al.* [36] reported that, irrespective of the infestation with pathogenic *Fusarium* species, considerable amounts of fungi from genus *Alternaria* spp. were always isolated from grain of naked oat grass, with the grain discoloration rate depending closely on the system of soil and climatic factors and the cultivar. According to Moudry *et al.* [17], high humidity during grain ripening and its irregular development have a significant effect on infestation by saprotrophic and weakly pathogenic fungi, which is evident especially in the case of small kernels which developed the latest [3, 23]. Studies on the content of mycobiota indicate that increased fungal infestation of grain is connected with increased ergosterol content in grain [24, 29]. It may be expected that the presence of mycoflora definitely results in an increased ergosterol content in grain.

There is little published information on representative experimental results concerning relationships between the above traits. Hence, this study was designed to analyse correlations between the trichothecene content of grain, mycobiota in grain (assessed on the basis of ergosterol content) and environmental conditions, with the use of 3 naturally infected oat cultivars grown in 10 localities.

MATERIALS AND METHODS

Samples. Oat grain samples (n = 90, 1,000 g each) of 3 cultivars (Kwant, POB 3295, Skrzat) were collected randomly at harvest (late August) in 1997 at 10 Experimental Stations for Variety Testing of the Research Centre for Cultivar Testing (COBORU) (Bezek, Białogard, Bukówka, Kościelec, Łopuszna, Marianowo, Naroczyce, Przecław, Uhnin, Wrocikowo) in different regions of Poland. Conventional cultivation systems with complete chemical plant protection were applied at all stations. All agricultural measures were performed in an identical manner following the COBORU recommendations. Artificial plant inoculation was not applied at any location. Each of the 3 cultivars in each location was randomly assigned to experimental plots in each of the 3 replications.

Table 1. Monthly precipitation and average temperature from April–August in 1997 in the 10 localities where samples were grown.

Localities	April	May	June	July	August
	mm/°C	mm/°C	mm/°C	mm/°C	mm/°C
Bezek	38/4.5	81/14.4	37/16.9	170/17.5	57/17.8
Białogard	34/4.9	98/11.3	91/16.1	80/17.5	20/19.4
Bukówka	51/3.2	79/11.6	94/14.6	388/15.2	40/16.4
Kościelec	23/5.4	95/13.2	42/16.5	188/18.0	26/19.3
Łopuszna	87/1.9	110/11.5	77/14.4/	318/15.1	79/14.8
Marianowo	24/4.5	40/12.4	29/16.4	153/18.0	22/19.0
Naroczyce	29/5.9	50/13.5	46/16.9/	236/18.0	83/19.5
Przecław	68/5.2	91/14.9	63/17.1	188/17.7	63/17.7
Uhnin	46/4.6	122/13.9	45/17.0	208/18.0	47/17.7
Wrocikowo	30/4.0	78/11.6	50/15.7	150/17.5	21/18.4
Mean*	43/4.4	84/12.8	57/16.2	208/17.3	46/18.0
	113%/56%	143%/98%	81%/101%	256%/100%	66%/100%

* percent of mean for analogous locations in Poland, 1985–2005

The mean monthly values for temperature and rainfall in the experimental season (April–August 1997) in comparison to the mean for 10 locations presented above in the period of 1985–2005 are given in Table 1.

Ergosterol analysis. Samples containing 100 mg of ground grains were placed into 17 ml culture tubes, suspended in 2 ml of methanol (MeOH), treated with 0.5 ml of 2 M aqueous sodium hydroxide and tightly sealed. The culture tubes were then placed within 250 ml plastic bottles, tightly sealed and placed inside a microwave oven (Whirlpool, model AVM 401/1WH) operating at 2450 MHz and 900 W maximum output. Samples were irradiated (370 W) for 20 s and after ca. 5 min for an additional 20 s. After 15 min, the contents of the 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 nitrogen stream. Prior to analysis, samples were dissolved in 4 ml of MeOH, filtered through 13 mm syringe filters with a 0.5 μm pore diameter (Fluoropore Membrane Filters), evaporated to dryness in an N_2 stream and dissolved in 1 ml of MeOH. Such prepared samples were analysed by high performance liquid chromatography (HPLC). Separation was achieved on a 150 × 3.9 mm, 4 μm particle size) Nova Pak C-18 column and eluted with methanol-acetonitrile (90:10 v/v) at a flow rate of 0.6 ml min^{-1}. Ergosterol (ERG) was detected with a Waters 486 Tunable Absorbance Detector set at 282 nm. ERG contents were estimated by comparison of peak areas with those of the original external standards of ERG (Sigma-Aldrich, USA). Confirmation of ERG was achieved by comparison of retention times or by co-injection with a ERG standard. The detection limit was 0.01 mg kg^{-1} [24].

Analysis of trichothecenes. Subsamples of 20 g were used for analysis of each toxin. All the subsamples were prepared in an identical way. They were ground in a WŻ-1 laboratory mill (Research Institute of Baking Industry Ltd. Bydgoszcz, Poland), designed specially for grinding cereal samples.

Sample were extracted overnight with 100 ml of solvent acetonitrile–water (82:18, v/v), and filtered (Whatman No. 5 filter paper), then the extracts were purified on a column (5 ml) of mixed alumina (neutral activated, 70–230 mesh), Darco G 60-charcoal–(100 mesh), Celite 545 4:3:4 [w/w/w]. The extracts were evaporated to dryness using a rotary evaporator. The residue was dissolved using 2 aliquots of 2 ml ethyl acetate and 2 ml of chloroform-acetonitrile (4:1, v/v) and divided into 2 portions.

The trichothecenes group A (H-2 toxin, T-2 toxin. T-2 tetraol, diacetoxyscirpenol (DAS)) were analysed as trifluoroacetic (TFA) derivatives. To the dried sample, 100 µl of 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 or 2 µl were injected onto a gas chromatograph-mass spectrometer (Hewlett Packard GC 6890, MS 5972 A, Waldbronn, Germany).

The trichothecenes group B (DON, NIV (nivalenol), 3-AcDON (3-acetyldeoxynivalenol), 15-AcDON (15-acetyldeoxynivalenol)) were analysed as TMS (trimethylsilyl ethers) derivatives. The amount of 100 µl TMSI/TMCS (trimethylsilyl imidazole/trimethylchlorosilane 100:1, v/v) mixture was added to the dried extract. After 10 min, 500 µl of isooctane were added and the reaction quenched with 1 ml of water. The isooctane layer was used for analysis and 1 or 2 µl of sample were injected on a GC/MS system.

The column used was an HP-5MS (30 m). The injection port temperature was 280°C, the transfer line temperature was 280°C and the analysis performed with the programmed temperature (from 80°C [1 min] to 280°C at 25°C/min), the final temperature being kept for 10 min. The helium flow rate was constant at 0.7 ml min^{-1}. Trichothecenes were quantified in the secondary ion-monitoring (SIM) mode in comparison with mycotoxin standards supplied by Sigma (St. Louis, USA). The following ions were used to detect trichothecenes: deoxynivalenol – m/z 235, 422; 3-acetyldeoxynivalenol-117, 482; 15-acetyldeoxynivalenol-193, 482; nivalenol-585, 289; HT-2 toxin (HT-2) toxin-180, 455, 532, 327; T-2 toxin-180, 205, 401; T-2 tetraol, 455, 568; DAS-402, 374, 329. The first ion in each set was used for quantification. The detection limit was 0.001 mg kg^{-1}. To confirm the identities of the toxins, full scan analysis in the range of 100–600 amu was performed. Average recoveries of the toxins were as follows: trichothecenes group A – 86% ± 3.8 for T-2, 88% ± 4.0 for T-2 tetraol, 91% ± 3.2 for HT-2, 84% ± 4.6 for DAS; trichothecenes group B - 84% ± 3.8 for DON, 81% ± 4.4 for NIV, 74% ± 2.2 for 15 Ac-DON, 78% ± 4.8 for 3-AcDON.

Statistical analysis. Basic statistics were calculated for the recorded results and the analysis of variance and Pearson's simple correlation analysis were performed.

RESULTS AND DISCUSSION

In 1997, when this study was conducted, the mean precipitation in the compared localities from across Poland was higher than the long-term mean (Tab. 1). In April, the mean for the studied localities amounted to 43 mm (113% of the long-term mean). In May, the mean precipitation for all localities in the test reached 84 mm, i.e. 143% of the long-term mean. In June, when oats were flowering, precipitation in the studied localities varied from 29 mm–94 mm, with the mean of only 57 mm (81% of the long-term mean). In July, precipitation was extremely high, with an average of 208 mm (265% of the long-term mean), and varied from 80 mm in Białogard to 388 mm in Bukówka. In August, precipitation was low again, with an average of only 46 mm (66% of the long-term mean).

Low temperatures in early April delayed seedling emergence, but weather conditions between the emergence stage (BBCH 09) and the beginning of the flowering stage (BBCH 51) [11] were favourable for plant growth. Excessive precipitation in July caused lodging of oat plants, probably increasing colonization and infection of seed plants with fungal pathogens and saprophytes [3, 23].

Weather conditions in 1997 did not favour the infection of panicles and grain with *Fusarium* fungi or the accumulation of trichothecenes in grain, as severe symptoms are usually observed under conditions of high relative humidity and frequent rainfall at the flowering stage of cereals [23].

To date, weather conditions similar to those presented in this study have not been analyzed, but rather analyses were limited to correlations between precipitation at the flowering stage and parameters describing fusariosis [12, 13], mainly after artificial inoculation, when the proportion of infected spikes exceeded 5% and the total *Fusarium* toxin content exceeded 1 mg/kg [12, 13, 16, 26, 27, 34].

Investigations presented in this paper concern a low level of infection, characteristic for years with no precipitation during anthesis and with rainfall occurring at a later time. Such environmental conditions resulted in the infection level being at least 10 times lower than after artificial inoculation and the total trichothecene content below 150 µg/kg.

Analysis of weather conditions in relation to ergosterol and total trichothecene levels in grain of the studied oat cultivars revealed strong negative correlations between the total trichothecene content and monthly precipitation in April, May and June (respective values of Pearson correlation coefficients of -0.72, -0.71 and -0.65, significant at $p < 0.01$). In the case of ergosterol, a significant negative correlation was recorded between ergosterol and monthly precipitation only in June, while in the other months the

correlations between its content and monthly precipitation and temperature were not significant.

However, our data concern oat plants, which have panicles instead of spikes, and no artificial inoculation was applied in our study. It is also noteworthy that oat plants often start to flower while still in the leaf sheath, before the panicle emerges. This implies a different type of head blight pathogenesis than in the case of wheat spikes, where infection takes place at the flowering stage when spikes are already exposed [5, 35]. Also, oat seeds are hulled and the kernels can be colonizes with many fungi, especially when the plant lodges.

Under conditions of natural infection of inflorescences, the pathogenicity and toxinogenicity of isolates present in their environment are of considerable importance [12, 13]. Moreover, Rohacik and Hudec [31] showed that the structure of species in grain depends mostly on microclimatic conditions. The species spectrum increases with an increasing rainfall rate and altitude. Similar conclusions can be drawn from earlier results on natural infection of grain in various barley cultivars, as significant correlations between toxins attest to the formation of *Fusarium* toxins in grain within the dominant *Fusarium* species found in that region, depending on its toxin profile [25].

Confirmation of this thesis required the collection of representative samples of naturally infected grain. This seemed particularly justifiable after a previous study of *Fusarium* toxin levels in barley grain, which revealed significant correlations between the formation of various *Fusarium* toxins in grain by the dominant *Fusarium* species found in a given region [25].

Despite some controversy related to this, we measured the total concentration of all identified trichothecenes of type A (DAS, HT-2 toxin, T-2 toxin), type B (DON, NIV, 3-AcDON), type A and NIV, respectively (Tab. 2). DAS was detected in 7% of samples, HT-2 toxin in 37%, T-2 toxin in 13%, NIV in 13%, while DON in 23%. However, 15-Ac DON and T-2 tetraol were not detected, whereas 3-Ac DON was found in 17%, but only at a level below the limit of quantitation. Results presented above concerning the incidence of trichothecenes in oat grain are lower in relation to studies conducted in 1994 and 1995, where a higher frequency was observed for DON and NIV [28].

High contents of trichothecenes A and NIV definitely indicate relatively strong colonization of analyzed grain samples by *F. poae* and *F. sporotrichioides*, forming primarily T-2 toxin, HT-2 toxin and NIV. These fungi are considered to be weakly pathogenic and accompanying strong pathogens, such as *F. culmorum* and *F. graminearum*, forming mainly DON. Conditions recorded in the year of the study generally did not promote infestation by *F. culmorum* and *F. graminearum*, which could have resulted in an increased share of less pathogenic species, such as *F. poae*, which was isolated in Poland in considerable amounts from infested oat panicles also in 1999–2001 [6].

The mean total *Fusarium* toxin content varied from 0 (i.e. below the detection limit) for samples from Białogard and Przecław, to 146 µg/kg for samples from Naroczyce (Tab. 2).

Table 2. Mean trichothecene content and ergosterol level in 3 cultivars of naturally contaminated oat grain grown of 3 cultivars in 10 localities in Poland in 1997.

Localities	Trichothecenes µg/kg					ERG mg/kg
	Total	Group A (T-2, HT-2)	Group A + NIV	Group B (DON, NIV)	DON	
Bezek	85	74	85	11	0	28.8
Białogard	0	0	0	0	0	6.7
Bukówka	52	52	52	0	0	17.6
Kościelec	100	84	92	16	8	18.9
Łopuszna	8	0	0	8	8	5.6
Marianowo	101	10	91	91	8	12.7
Naroczyce	146	76	136	60	10	21.3
Przecław	0	0	0	0	0	9.0
Uhnin	29	21	21	8	8	13.0
Wrocikowo	112	80	89	32	32	17.8
Mean	63	40	57	23	6	14.0

The values recorded in this study were very low in comparison with other published data on oat grain contamination with trichothecenes [2, 4, 7, 21, 30]. In turn, when comparing these results in terms of their contents of trichothecenes type A (T-2 toxin, HT-2 toxin) it may be stated that they are comparable.

Thus it seemed difficult to detect correlations between such low values and ergosterol content (the latter reflecting the mycobiota in grain). In the studied oat grain samples, ergosterol content proved to be high. The highest values were detected in samples from Bezek, Naroczyce, Kościelec and Wrocikowo: 28.8, 21.3, 18.9 and 17.8 mg/kg, respectively. Schnürer and Jansson [32] postulated that the maximum admissible ergosterol content of cereal grain used for human consumption should be below 3 mg/kg. This value is very low in comparison with data found in literature. Maupetit *et al.* [10] detected on average 4.2 mg/kg of this metabolite in wheat flours and 5.9 mg/kg in grain. Müller and Schwadorf [18] and Müller *et al.* [20] reported that mean ergosterol content of wheat grain and flour samples was 4.7 and 14.5 mg/kg in 1985, 9.8 and 19.6 mg/kg in 1987, 3.8 and 11.0 mg/kg in 1988, and 4.8 and 11.7 mg/kg in 1989, respectively. The same research team [19] showed that ergosterol content of wheat grain was 2.64 ± 0.97 mg/kg (mean ± standard deviation), while that of oats was 3.51 ± 2.16 mg/kg. Similar values were recorded by Young and Loughnen [37]. In contrast, Majunder [9] found that ergosterol content of freshly collected, healthy grain was only 0.2 mg/kg.

It seems that the only reason for the high mycobiota recorded in our study was the excessive precipitation in July, which caused lodging of oat plants and enabled severe infection of oat plants by fungal pathogens and saprophytes.

Table 3. Correlation coefficients between trichothecenes and ergosterol.

	Trichothecenes				
	Total	Group A (T-2, HT-2)	Group A + NIV	Group B (DON, NIV)	DON
Group A (T-2, HT-2)	0.8041**	–			
Group A + NIV	0.9924**	0.8066**	–		
Group B (DON, NIV)	0.7007*	0.1375	0.6791	–	
DON	0.4980	0.4087	0.3904	0.3559	–
ERG	0.7313**	0.8703**	0.7748**	0.1785	0.1029

* Significant at $p < 0.05$; ** Significant at $p < 0.01$.

Champeil *et al.* [3] pointed to the fact that agronomic factors causing lodging may result in a stronger colonization of grain not only by pathogens from genus *Fusarium*, but also by other mould fungi. In turn, Newton *et al.* [22] stated that oat irrigation performed from anthesis until full ripeness results in a stronger discoloration of oat groats as a result of increased colonization of grain by different fungal species from genera *Fusarium* and *Alternaria* as well as *Microdochium nivale*. This was indirectly confirmed by the results of this study, since in the locations where grain samples were collected, anthesis of oat plants occurred at the end of the last decade of June and the first decade of July, and extremely high rainfall was recorded immediately after flowering.

The analysis of variance revealed a significant variation in mean values of ergosterol and total *Fusarium* toxin content between localities (F – statistic values of 29.49 and 3.74, respectively; $p \leq 0.01$). Our results attest to a very strong relationship between sample origin and both ergosterol content and mean total trichothecene toxin content of grain.

The mean ergosterol content of grain from the ten samples ranged from 5.6 mg/kg (Łopuszna) to 28.8 mg/kg (Bezek). No significant variation between cultivars was observed, both in terms of ergosterol content (on average 13.1 mg/kg in Skrzat, 15.9 mg/kg in POB 3295 and 13.0 mg/kg in Kwant) and mean total trichothecenes content (on average 215 µg/kg in Skrzat, 233 µg/kg in POB 3295 and 185 µg/kg in Kwant). The ranges of variation in ergosterol content were wide: 4.4–28.4 mg/kg in Skrzat, 4.6–27.6 mg/kg in POB 3295 and 4.5–30.3 mg/kg in Kwant. This confirms an earlier suggestion [25] that at low concentrations (< 1 mg/kg), characteristic for natural contamination, the mechanism of toxin formation by each of the studied *Fusarium* species is similar in various cultivars. The simultaneous production of various toxins by several *Fusarium* species competing with one another was certainly due to favourable climatic conditions. The importance of climatic conditions for *Fusarium* toxin formation in oat grain in Norway was emphasized by Langseth and Rundberget [8], who showed that the concentrations and profiles of those metabolites depended on the region where grain samples were collected.

This strong linear correlation between ergosterol and total trichothecene content of grain ($r = 0.7313$; $p \leq 0.01$) is of particular importance. Wet conditions after flowering favoured the growth of non-DON forming *Fusarium* Link and saprophytic fungi, which is confirmed by the determined correlation coefficients presented in Table 3.

The patterns of plant growth and development of mycobiota, as well as mycotoxin concentrations in grain, depend mainly on weather conditions, among which the most significant are precipitation and temperature. Results of this study indicate that the influence of precipitation during the growth period of oats is greater than that of temperature.

The results of this study should inspire further research on the influence of environmental conditions on the mycobiota and mycotoxin production in naturally infected cereal grain.

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