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DISTRIBUTION OF TRICHOTHECENE AND ZEARALENONE PRODUCING *FUSARIUM* SPECIES IN GRAIN OF DIFFERENT CEREAL SPECIES AND CULTIVARS GROWN UNDER ORGANIC FARMING CONDITIONS IN LITHUANIA

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Abstract: *Fusarium* infection level, DNA quantity of the *Fusarium poae*, *F. sporotrichioides*, *F. langsethiae*, *F. culmorum*, *F. graminearum* and *F. equiseti* as well as deoxynivalenol (DON), zearalenone (ZEN) and T-2 toxin (T-2) content were investigated in grain from cultivars of different cereal species grown on organic farming sites during 2005–2006. The *Fusarium* infection level was examined by agar plating of single grains, *Fusarium* spp. DNA content was determined by real-time PCR and the mycotoxins were analyzed by ELISA. Almost all cereal grain samples grown under organic conditions were infected by *Fusarium* spp. The grains of winter cereals were less infected with *Fusarium* compared with those of spring cereals. The presence of *F. culmorum*, *F. equiseti*, *F. sporotrichioides*, *F. poae*, *F. langsethiae* in cereal grain depended on the environmental conditions during the experimental years. Higher *Fusarium* species diversity was found in 2005 when the conditions were more favourable for *Fusarium* infection in cereal grain, whereas *F. poae* and *F. langsethiae* were prevalent in cereal grain in 2006. *F. langsethiae*, identified in Lithuania for the first time, was more frequent in spring cereals than in winter cereals. Almost all grain samples were found to be contaminated with DON, ZEN, T-2 at low concentrations; however, it is known that the action of toxins at low concentrations is slow, the adverse effects are evidenced only after some time and in different forms, which poses a serious risk to human and animal health.

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INTRODUCTION

Fusarium head blight (FHB) is caused by several *Fusarium* species. Many of these are capable of producing mycotoxins, which can cause a wide range of acute and chronic effects in humans and animals through food and feed prepared from contaminated cereal crops [7, 34]. The *Fusarium* species predominantly found in association with FHB in small-grain cereals all over Europe are *F. graminearum*, *F. avenaceum* and *F. culmorum* [3]; however *F. poae*, *F. tricinctum*, *F. sporotrichioides*, *F. equiseti* and *F. langsethiae* are also very common [3, 12]. Trichothecenes and zearalenone (ZEN) constitute the largest group of mycotoxins produced by *Fusarium* in cereal grain. The most common trichothecenes in cereals are deoxynivalenol (DON) produced by certain isolates of *F. graminearum*, *F. pseudograminearum* and *F. culmorum*. ZEN is produsedby the *F. graminearum*, *F. culmorum* and *F. equiseti*. T-2 toxin is produced by *F. sporotrichioides* and

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F. langsethiae, and some isolates of *F. poae* also produce this toxin [3, 36].

Fusarium species involved in FHB differ in pathogenicity and capacity to produce mycotoxins, therefore correct identification and quantification is essential. Classical identification based on morphology requires taxonomic expertise and is time consuming. Molecular methods have several advantages due to their specificity, sensitivity and relatively short test time [9, 13, 20]. Furthermore, quantitative DNA based methods estimate the actual biomass present, whereas classical methods often estimate the percentage of infected seed. Recently, several real-time PCR methods for the detection and quantification of individual *Fusarium* species in infected grain or plant tissues have been developed [13, 24, 27, 39].

Environmental conditions play a vital role in the *Fusarium* infection level in cereals. The prevalence of individual species of *Fusarium* is due to several factors, but climatic conditions, and especially the relationship between humidity and temperature, have influence on the infection level [8, 28]. The same factors affect the content and composition of mycotoxins in cereal grain [25, 26, 28].

An increase in organic farming in the EU has placed more focus on the prevalence of fungal plant pathogens, and in particular mycotoxin producing fungi in organically grown crops. In a study on organically grown winter wheat, *F. avenaceum*, *F. poae*, *F. culmorum* and *F. graminearum* were the most predominant species [2]. The same authors concluded that organic farming systems showed lower rates of infection with ear blight and lower mycotoxin contamination than conventional farming systems. However, others have found no significant differences in mycotoxin contamination levels between conventional and organic systems [6, 42]. In order to prevent *Fusarium* infection and mycotoxin contamination in organically grown cultivars resistance towards FHB is of great importance. A number of experiments in the fields have proved the importance of the genetically determined resistance of some cereal cultivars to FHB [5, 15, 22, 33, 38]; however, there is practically no experimental evidence on FHB resistance of Lithuania-registered cultivars, the data are also scarce on the occurrence of *Fusarium* species on the grain of different cereal species, especially under organic farming conditions.

Since mycotoxin-contaminated food products pose a serious risk for human health, the current study was designed to determine the *Fusarium* fungi infection, with a special focus on the *Fusarium* species producing trichothecenes and ZEN in organically grown spring and winter cereal grain under different environmental conditions using conventional identification based on morphology and species specific real-time PCR assays.

MATERIALS AND METHODS

Field samples. A total of 36 Lithuania-registered cereal cultivars were studied: 9 spring barley (*Hordeum* L.) cultivars – Auksiniai 3, Aidas, Luoke, Ula, Aura, Alsa, Pasadena, Annabell, Barke; 5 oat (*Avena* L.) cultivars – Jaugila, Migla, Cwal, Belinda, Nelson; 3 spring wheat (*Triticum* L.) cultivars – Munk, Hena, Baldus; 1 spring triticale (*xTriticosecale* Wittm.) cultivar – Gabo; 10 winter wheat (*Triticum* L.) cultivars – Sirvinta I, Ada, Seda, Tauras, Alma, Milda, Lina, Bill, Lars, Zentos; 4 winter rye (*Secale* L.) cultivars – Rukai, Duoniai, Joniai, Lietuvos 3; 2 winter barley (*Hordeum* L.) cultivars – Carola, Tilia; 2 winter triticale (*xTriticosecale* Wittm.) cultivars – Tornado, Fidelio. Commercial cultivars (18 of winter and 18 of spring cereals) were grown in a field certified for organic farming at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, during 2005–2006. The cultivars were fully randomized in each block and replicated 3 times. The trial plots were mechanically harvested with a small plot harvester. Grain samples for the mycological and mycotoxins analyses were taken at harvest from each plot.

Plating method. The surface-sterilized seeds (200 per sample) were plated on a Potato Dextrose Agar (PDA) and incubated at 26 ± 2ºC [19] in darkness. The infection level of grain was evaluated in percent (0 – all grains healthy, 100% – all grains infected). Microscopic studies of *Fusarium* fungi were carried out after 7–8 days. The purified single spore cultures of *Fusarium* species were identified on the basis of their cultural and morphological characteristics according to Nelson *et al.* [23] and Leslie *et al.* [14].

Analysis of mycotoxins. The mycotoxins DON, ZEN and T-2 were analysed by ELISA (enzyme-linked immunosorbent assay) [41]. The Veratox test kits (NEOGEN Europe Ltd), approved by the AOAC Research Institute (Certificate N 950702) were used for the analysis. Mycotoxin extraction and tests were performed according to manufacturer's instructions. The optical densities of samples and controls from standard curve were estimated by a multichannel programmable photometer (Multiskan MS, Labsystems, Finland), using a 650 nm filter and calculation mode Point-to-Point. Measured absorbances were automatically converted to the mycotoxin concentration units – μg kg^{-1}. The detection limit for DON, ZEN and T-2 was set at 100.0 μg kg^{-1}, 10.0 μg kg^{-1}, and 7.5 μg kg^{-1}, respectively. While assessing our data with regard to food safety we referred to the EU document No 1126/2007, in which it is specified that the maximum allowable concentration for DON in unprocessed grain (except for hard wheat, oats and maize) is 1,250 μg kg^{-1}, for ZEN – in unprocessed grain (except for maize) – 100 μg kg^{-1}. T-2 assessment was based on research-recommended concentrations in grain and grain products 100 μg kg^{-1} [11].

DNA extraction. Genomic DNA was extracted from *Fusarium* pure cultures using DNeasy (QIAGEN) and from 21-field samples using DNeasy for wheat and Nucleo--Spin® Food (Machery-Nagel) for barley and oat according

to the manufacturer's instructions. Ten g of each sample was ground to a fine powder in a Retsch mixer mill (Retsch GmbH, Germany). For extraction, 0.1 g of ground material was used. The quality of all extracted DNA was verified by electrophoresis on 1.0% agarose gel. DNA was visualized by ethidium – bromide and detected under UV lamp. The concentrations of genomic DNA from pure cultures were estimated by visually comparing the band intensities of the DNA to the Lambda DNA/EcoR I + Hind III Markers with known concentrations in a 1% agarose gel.

PCR conditions. Real-time PCR was carried out as described in Nicolaisen *et al.* [24]. PCR reactions were performed in duplicate on all samples. Genomic DNA from grain was diluted 1:20 and PCR was run in a 7900HT Sequence Detection System (Applied Biosystems). Normalization was carried out according to Nicolaisen *et al.* [24] and the values given as the amount of fungal DNA per amount of plant DNA. Ten-fold dilution series of *F. culmorum*, *F. graminearum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. equiseti* DNA were used for standard curves.

RESULTS

Mean values for the percentage of grain infection with *Fusarium* spp. and DON, ZEN and T-2 toxin accumulation ranged from 0.0–67.7, 103.1–913.6, 0.0–21.8 and 0.0–50.2 respectively in fields of spring and winter cereals in 2005 – 2006 (Tab. 1). *Fusarium* infection was numerically higher in spring cereals (on average 44.5% in 2005 and 19.4% in 2006) than in winter cereals (on average 9.5% in 2005 and 8.4% in 2006), and higher in 2005 than in 2006. A multifactor analysis of variance demonstrated a significant effect by cereal type (Tab. 2) and year for *Fusarium* fungi, and no significant effect for DON. Mean values of separate cereal species demonstrated higher *Fusarium* infection level in 2005 than in 2006 of all spring and winter cereals, except winter barley, that was significantly more infected in 2006 (Tab. 3, 4). The highest DON concentration was detected on spring triticale grain in 2005 (913.6 µg kg^{-1}). Spring oat and spring wheat were more contaminated with ZEN than other cereals in 2006. Oat grain had the highest concentrations (on average 32.0 µg kg^{-1}) of T-2 toxin in both years.

Fusarium infection level on cereal grain was highly depended on the cultivar in 2005 (Tab. 5), but in 2006 a significant effect was observed only in spring barley. The grain infection levels for the spring barley cultivars Pasadena and Auksiniai 3 differed over the years (Fig. 1). The local/Lithuanian cultivars Luoke and Ula were among the less *Fusarium* – infected ones in both years.

Fusarium species (*F. graminearum*, *F. culmorum*, *F. poae*, *F. sporotrichioides*, *F. langsethiae*, *F. equiseti*) producing trichothecenes (DON, T-2) and ZEN were detected and quantified in 21 selected grain samples by using species specific primers in real-time PCR. *F. poae* was found to be the most predominant of the trichothecenes producing species in all grain samples (Fig. 2 a, b, c, d). *F. langsethiae* was frequent in spring cereal grain, especially in barley and oat in 2006. *F. sporotrichioides* was found in a nine

Table 1. Minimum, mean (±Se) and maximum values for the percentage of spring and winter cereal grain infection with *Fusarium* spp. and deoxynivalenol, zearalenone and T-2 toxin accumulation, 2005–2006.

Traits	Spring cereal		Winter cereal	
	2005	2006	2005	2006
***Fusarium* spp. infected grain, %**	n=18	n=18	n=18	n=18
minimum	23.7	3.0	0.0	0.0
mean (± Se)	44.5 (2.7)	19.4 (2.3)	9.5 (1.7)	8.7 (3.0)
maximum	67.7	40.0	22.3	44.3
Deoxynivalenol, µg kg^{-1}	n=18	n=18	n=18	n=18
minimum	103.1	106.1	114.7	107.0
mean (± Se)	203.3 (42.8)	192.9 (15.2)	211.0 (20.9)	151.4 (7.5)
maximum	913.6	314.6	454.0	186.3
Zearalenone, µg kg^{-1}	n=5	n=18		n=18
minimum	11.7	0.0		0.0
mean (± Se)	19.5 (1.9)	21.5 (3.3)		9.9 (1.2)
maximum	21.8	20.0		20.0
T-2 toxin, µg kg^{-1}	n=14	n=14	n=8	n=8
minimum	0.0	0.0	0	8.5
mean (± Se)	17.3 (3.6)	18.1 (3.9)	7.9 (1.1)	11.1 (1.8)
maximum	45.9	50.2	9.3	23.6

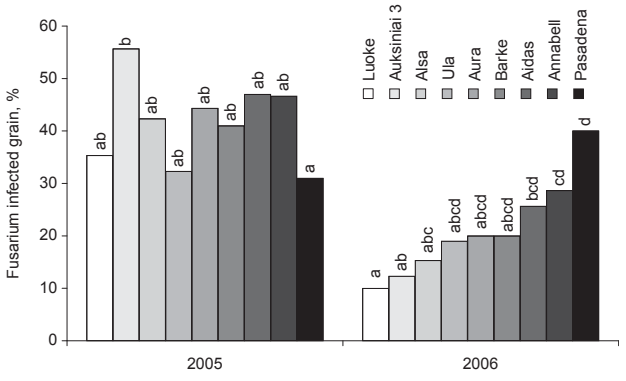

Figure 1. Effect of cultivated spring barley cultivars on *Fusarium* infection level of grain in 2005–2006. Means followed by the same letters are not significantly different for each cultivar ($p \leq 0.05$).

Table 2. Analysis of variance p values from field-testing the *Fusarium* infection level and DON concentration of cereal grain.

Source of variation	ANOVA p values	
	Fusarium infected grain	DON content
Year (A)	0.0017	0.1848
Cereal type (B)	0.0000	0.6869
A x B	0.2275	0.0466

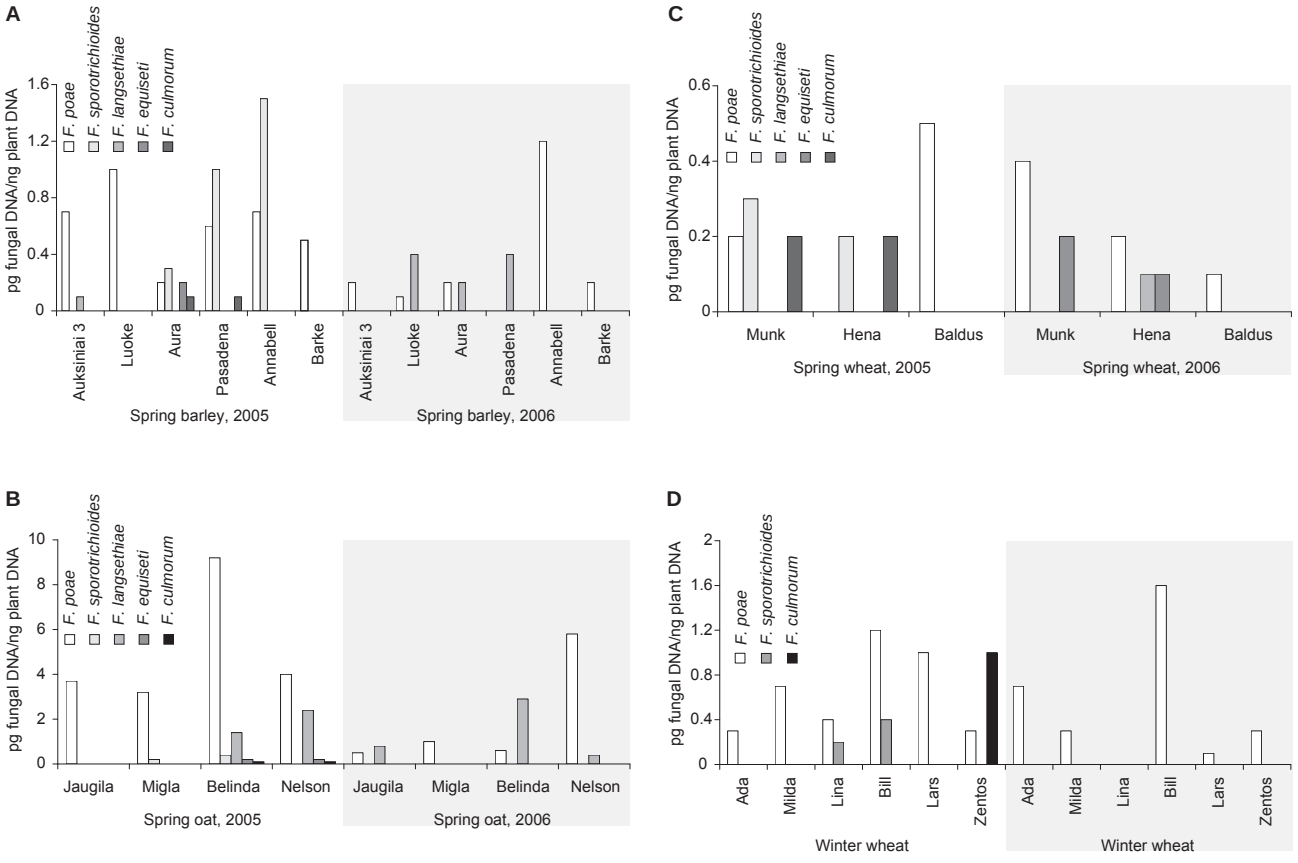

Figure 2. *Fusarium poae*, *F. sporotrichioides*, *F. langsethiae*, *F. equisety* and *F. culmorum* DNA amounts in spring barley (A), spring oat (B), spring wheat (C), winter wheat (D) grain samples assessed by the individual real-time PCR and normalized using the plant EF1α assay in 2005–2006.

Table 3. Mean, standard error (Se), minimum (min) and maximum (max) values for the percentage of every spring cereal species grain infection with *Fusarium* spp. and deoxynivalenol, zearalenone and T-2 toxin accumulation, 2005–2006.

Cereal species, traits	*Fusarium* infected grain,%		DON, µg kg^{-1}		ZEN, µg kg^{-1}		T-2, µg kg^{-1}	
	2005	2006	2005	2006	2005	2006	2005	2006
Barley	n=9	n=9	n=9	n=9	–	n=9	n=6	n=6
mean	41.74	21.22	178.98	149.08		9.51	10.83	11.45
Se	2.63	3.06	9.01	12.72		1.20	2.22	1.93
min	31.00	10.00	111.90	106.10		0.00	7.80	7.90
max	55.67	40.00	200.70	189.90		11.50	21.90	20.90
Oat	n=5	n=5	n=5	n=5	n=5	n=5	n=5	n=5
mean	47.60	26.07	108.54	270.20	19.46	30.08	30.76	33.24
Se	3.26	0.78	1.55	23.87	1.95	5.09	6.26	6.47
min	39.00	24.33	103.10	210.90	11.70	23.80	17.20	21.50
max	56.67	29.00	111.70	314.60	21.80	50.40	45.90	50.20
Wheat	n=3	n=3	n=3	n=3	–	n=3	n=3	n=3
mean	50.78	6.11	197.47	196.40		37.40	7.90	5.93
Se	13.69	2.95	8.08	3.87		1.68	0.06	3.05
min	23.67	3.00	184.80	191.30		34.60	7.80	0.00
max	67.67	12.00	212.50	204.00		40.40	8.00	10.10
Triticale	n=1	n=1	n=1	n=1	–	n=1	–	–
mean	34.3	8.7	913.6	189.5		38.5		

samples in 2005 and just in 2 samples in 2006. *F. culmorum* and *F. equiseti* were found in a few grain samples, while *F. graminearum* was not detected at all.

DISCUSSION

Our experimental evidence indicated that in 2005–2006 *Fusarium* fungi affected from 0–67.7% of the tested grain. In 2005, the *Fusarium* – affected grain accounted on average for 26.8%, in 2006 – 14.2%. In 2005, *Fusarium* infection level on spring wheat, barley, oat, winter wheat, rye, and triticale grain was higher than in 2006, and that on winter barley grain was higher in 2006. Data analysis showed that these differences mostly depended on the weather conditions during (or pre-) cereal anthesis stage. The beginning and duration of anthesis varied in relation to the meteorological conditions in separate years; however, winter barley was the first to start flowering (end of May in 2005; beginning of June in 2006), followed by rye, triticale and wheat, the latter finished flowering most often in the first 10-day period of July; spring barley, spring wheat and oats started flowering the latest. Many foreign authors have noted that *Fusarium* infection level in cereal grain is mostly dependent on the amount of precipitation and air temperature during the cereal flowering period [2, 8, 28], since it is the most favourable time for the infection to penetrate grains, less favourable periods are pre- and post-anthesis [32]. Warm and rainy weather (with relative air humidity of ≥ 80%) during cereal anthesis stage is the most conducive to the occurrence of *Fusarium* fungi infection in cereal grain [29]. During flowering of most of the winter cereals there was more rainfall and days with relative air humidity of ≥ 80% in 2005 than in 2006 (Fig. 3). However, before and during winter barley anthesis there were more days with favourable relative air humidity and rainfall in 2006 than in 2005.

Table 5. ANOVA – table of the *Fusarium* infection level in cereal grain as affected by cultivated cultivar.

Effect of cultivars	n	*Fusarium* infection, 2005		*Fusarium* infection, 2006	
		F-value	Probability	F-value	Probability
Spring barley	9	3.0	0.0292	7.3	0.0004
Spring oat	5	4.62	0.0316	0.3	0.8670
Spring wheat	3	50.62	0.0014	3.35	0.1396
Winter wheat	10	10.19	0.00002	0.27	0.9752
Winter rye	4	8.4	0.0144	1.70	0.2655

There was markedly less *Fusarium* – affected grain in winter cereal than in spring. The differences in the *Fusarium* infection level between winter and spring cereals in Lithuania were noted in previous research too [31]. The obtained results also agree with those obtained by B. Kosiak *et al.* [12]. However, these results are controversial compared with those reported by Tekauz *et al.* [35], which shows that oats are less infected than wheat and barley. Edwards SG [10] has reported that the differences between crop species appear to differ between countries, and this is probably due to differences in the genetic pool used by the breeding programme in each country, and the different environmental and agronomic conditions in which crops

Table 4. Mean, standard error (Se), minimum (min) and maximum (max) values for the percentage of every winter cereal species grain infection with *Fusarium* spp. and deoxynivalenol, zearalenone and T-2 toxin accumulation, 2005–2006.

Cereal species, traits	*Fusarium* infected grain,%		DON, µg kg^{-1}		ZEN, µg kg^{-1}		T-2, µg kg^{-1}	
	2005	2006	2005	2006	2005	2006	2005	2006
Wheat	n=10	n=10	n=10	n=10	–	n=10	n=6	n=6
mean	12.17	6.20	224.47	136.16		7.60	8.95	9.22
Se	2.32	0.84	37.99	9.92		1.67	0.11	0.22
min	0.00	3.33	114.70	107.00		0.00	8.50	8.50
max	22.33	11.00	454.30	175.10		11.50	9.20	9.80
Rye	n=4	n=4	n=4	n=4	–	n=4	–	–
mean	8.08	1.42	190.88	159.08		13.53		
Se	3.32	0.84	2.08	12.84		2.16		
min	1.00	0.00	185.60	120.60		11.20		
max	15.67	3.33	195.70	173.00		20.00		
Barley	n=2	n=2	n=2	n=2	–	n=2	n=2	n=2
mean	2.67	42.83	197.65	185.05		11.95	4.65	16.90
Se	0.67	1.50	2.65	1.25		0.05	4.65	6.70
min	2.00	41.33	195.00	183.80		11.90	0.00	10.20
max	3.33	44.33	200.30	186.30		12.00	9.30	23.60
Triticale	n=2	n=2	n=2	n=2	–	n=2	–	–
mean	6.17	1.17	197.00	178.85		11.85		

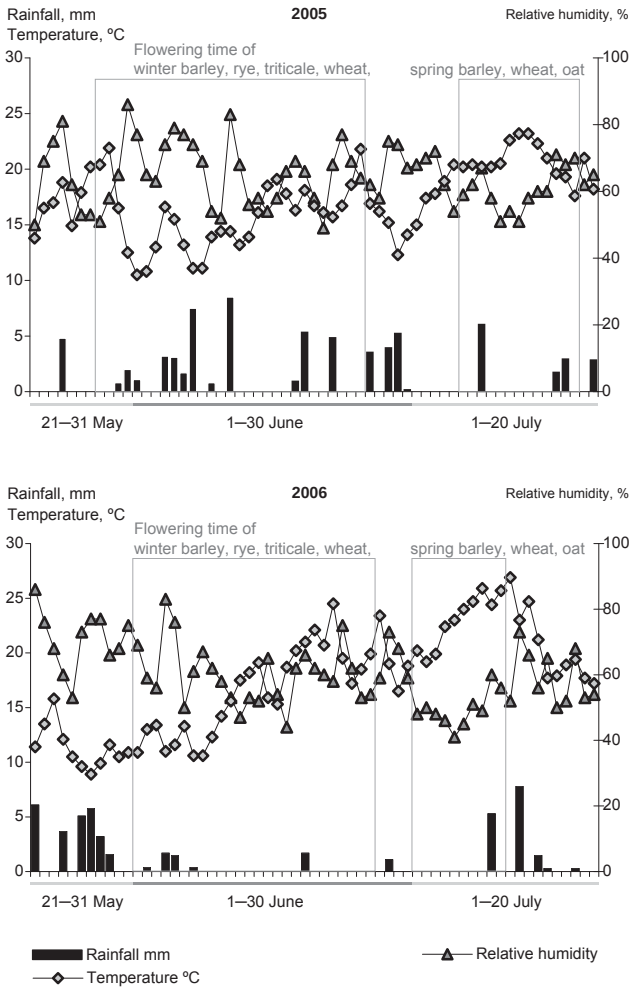

Figure 3. Meteorological conditions during cereal flowering stage in Dotnuva, Kedainiai district, Lituania 2005–2006.

are cultivated. As we indicated, *Fusarium* infection was affected by some environmental factors: meteorological conditions, time and length of flowering period and source of infection. During our experiments the amount of precipitation was similar during the spring and winter cereal flowering stages (Fig. 3). However, the average air temperature was 5.5ºC higher during spring cereal flowering (on average 20.8ºC during 6–18 July in 2005, and 22.6ºC during 1–9 July in 2006) compared with that during winter cereal flowering (on average 15.7ºC during 28 May–26 June in 2005, and 16.8ºC during 2–27 June in 2006) in both years. As the data of previous research suggest, a 4-degree rise in temperature (from 16–20ºC) had a marked effect on FHB [4]. The meteorological conditions during winter cereal anthesis controlled *Fusarium* infection level on winter cereal and the source of inoculum for spring cereal. More favourable meteorological conditions for increasing of the source of inoculum in June and sufficiently long flowering period of spring cereal resulted in a higher *Fusarium* infection level on spring cereals, and bigger differences between spring and winter cereals infection in 2005 than in 2006. As a result, it is very important to take notice of the above-described factors, especially meteorological conditions, during winter cereal flowering while forecasting *Fusarium* infection in spring cereals.

Significant differences in infection level were detected between 9 spring barley cultivars in both 2005 and 2006; however the results were inconsistent – some of the cultivars that were less infected in 2005 had higher infection in 2006. The results indicated that these cultivars are insufficiently resistant to FHB because resistant cultivars maintain FHB resistance in the changing environment conditions, whereas more susceptible cultivars are highly dependent on the environmental changes [21]. All other analysed cultivars significantly differed in *Fusarium* infection only in 2005; therefore, it was difficult to estimate the resistance of organically produced cereal cultivars to *Fusarium* fungi.

The presence of *F. culmorum*, *F. equiseti*, *F. sporotrichioides*, *F. poae*, *F. langsethiae* in cereal grain depended on the environmental conditions during the experimental years. Higher *Fusarium* species diversity was found in 2005 when the conditions were more favourable for *Fusarium* infection in cereal grain, whereas *F. poae* and *F. langsethiae* were prevalent in cereal grain in 2006. *F. poae* was found to be the most predominant species in all tested grain samples. A number of studies performed in Scandinavia, Hungary and other mid-European countries identified *F. avenaceum* and *F. poae* as the dominating species in association with FHB in small-grain cereals [2, 3, 12, 16]. Due to the morphological similarity between *F. poae* and *F. langsethiae* the latter has not been recognized in Lithuania before. Our investigation showed that *F. langsethiae* was mostly present in spring cereal grain, especially in barley and oat in 2006. Early investigations indicated that grain infection level with *F. langsethiae*, which was temporally designated "powdery *Fusarium poae*" [37], was detected in 80, 73 and 70% of oats, barley and wheat grain samples, respectively, in 1994 [12]. In 2004, *F. langsethiae* strains were found in northern and central Europe (Norway, Austria, Germany, Czech Republic, Denmark and England) by Torp and Adler [37].

F. graminearum was not detected, which is in accordance with previous studies on Lithuanian cereals [17, 18].

Grain samples were tested for DON, ZEN and T-2 toxin contamination. The concentrations of all mycotoxins tested in cereal grain samples did not exceed the maximum allowable limits specified in the EU directives. All samples were found to be contaminated with DON at levels from 106.1–454.3 μg kg^{-1}. DON is the most frequently encountered *Fusarium* mycotoxin in cereals throughout Europe [3] and also in Lithuania [1, 17]. The main producers of DON *F. graminearum* were absent in the investigated samples and *F. culmorum* was only found in a few grain samples in 2005 where the highest levels of *F. culmorum* DNA and DON were found in winter wheat Zentos.

ZEN was detected in all oat grain samples (from 11.7 μg kg^{-1}–21.8 μg kg^{-1}) in 2005; therefore, in 2006 we analysed

all grain samples. The analysis revealed that ZEN content in spring oat, spring wheat and spring triticale was higher than that in spring barley and all analysed winter cereal grain. Similar results were obtained for conventionally grown spring and winter cereals [17, 18].

The detected T-2 concentration in grain samples was from 0–47.8 μg kg^{-1}. This does not exceed the proposed limit for T-2 in grain which is 100 μg kg^{-1} [11] , but as was shown by our previous study in 2004, oat grain can be contaminated at critically high levels (121.5 μg kg^{-1}) [17]. Although we did not find a good correspondence between this toxin and the DNA amount of the main producers (*F. langsethiae* and *F. sporotrichioides*), there was good correspondence between total DNA amounts of *F. langsethiae*, *F. sporotrichioides*, *F. poae* and T-2 contents. Thrane *et al.* [36] have reported that some strains of *F. poae* can produce T-2 toxin, thus it is likely that *F. poae* strains producing T-2 toxin are more frequent in Lithuania.

Almost all grain samples were found to be contaminated with DON, ZEN, T-2 at low concentrations; however, it is known that the action of toxins at low concentrations is slow, the adverse effects are evidenced only after some time and in different forms, which poses a serious risk to human and animal health.

F. langsethiae was identified in Lithuania for the first time; however, its potential capacity to produce T-2 did not materialize; therefore, the results of this study are inconclusive and the research needs to be continued.

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