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FUSARIUM DNA LEVELS AS COMPARED TO MYCOTOXIN LEVELS IN FINNISH AND ESTONIAN GRAIN SAMPLES

ABSTRACT

In the present work *Fusarium* DNA levels of deoxynivalenol (DON)-producing *F. graminearum* and *F. culmorum* and T-2/HT-2-producing *F. langsethiae* and *F. sporotrichioides* were compared to DON and T-2/HT-2 levels in Finnish (19 oat samples) and Estonian (3 oat, 8 wheat and 9 barley) grain samples of the year 2008. The Finnish oat samples were selected based on high (<870 ppb) DON levels. Some of them also contained high levels of zearalenone (ZEN). The main aim was to find out, which *Fusarium* species is the main DON producer in oats in Finland. In Estonian grain samples all mycotoxin levels were relatively low and below the EU limits. The highest ZEN levels were found in oat in Satakunta in Finland.

This is the first study, in which *F. graminearum* has been found in Estonian grains. The correlation between *F. graminearum* DNA and DON levels in the combined Finnish and Estonian oat samples was higly significant (R^2 = 0.88). In Finnish samples a significant correlation was also found between *F. graminearum* DNA and ZEN levels (R^2 = 0.42). No correlation was found between *F. culmorum* DNA and DON levels in the combined Finnish and Estonian oat samples. There was also a significant correlation between the combined T-2 and HT-2 and combined *F. langsethiae* and *F. sporotrichioides* levels (R^2 = 0.38) in the combined Finnish and Estonian oat samples. According to our results *F. graminearum* is clearly the main DON producer in Finnish oat.

Key words: DON, *Fusarium. culmorum*, *F. graminearum*, TaqMan qPCR, T-2/HT-2.

INTRODUCTION

F. culmorum and *F. graminearum* are the main deoxynivalenol (DON)-producers in Finland (Jestoi *et al.*, 2004, 2008; Yli-Mattila *et al.*, 2008, 2009; Parikka *et al.*, 2010).

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Previously *F. culmorum* has been the main DON producer in northern Europe, but recently *F. graminearum* has been spreading northward in Europe (e.g. Waalwijk *et al.* 2003, Nicholson *et al.* 2003) and has been replacing the closely related *F. culmorum.* According to some investigations based on contamination % values *F. culmorum* is still the main DON-producer in Finland (Parikka *et al*., 2010), while according to qPCR results *F. graminearum* is already the main DON-producer in Finland (Yli-Mattila *et al*., 2008, 2009). The aim of the present work was to use quantitative TaqMan real-time PCR in order to find out which *Fusarium* species is the main DON producer in oats in Finland and if *F. graminearum* can be found in Estonia.

MATERIAL AND METHODS

Grain samples and fungal strains

Nineteen Finnish oat grain samples of the year 2008 containing high levels deoxynivalenol (DON) were received from MTT (Table 1). Estonian grain samples for analyses were harvested in autumn 2008 from Estonian farmers and field trials of ERIA. Estonian amples were subjected to biotest with *Stylonichia mythilus*. 20 grains samples, more or less toxic in this test, were chosen for further analysis of trichothecenes: 8 spring wheat, 9 barley and 3 oat samples (Table 2).

The fungal strains used to generate standard curves for quantitative PCR were single-spore cultures collected from grain samples in Finland and Russia. *F. graminearum* isolate 45773 and *F. culmorum* isolate 45727 are stored NRRL culture collection in Peoria in USA, while *F. sporotrichioides* isolate 11026 is stored in the culture collection of All-Russian Plant Protection Institute in St. Petersburg. The morphological identification and purity of each standard strain for quantitative PCR was confirmed by species-specific primers as described by Yli-Mattila *et al.* (2004b).

DNA extraction and quantification

Grains were ground by an electric coffee mill. DNA was extracted from ground grain samples (100 mg) with the GenElute^{TM} Plant Genomic DNA Kit of Sigma as described by Yli-Mattila *et al.*, (2009). For the isolation of genomic DNA from pure cultures, fungi were grown for four to six days at 24 ^{o}C on PDA. DNA was extracted from pure cultures by the chloroform/octanol method as described by Yli-Mattila *et al.* (1998).

DNA was quantified by two methods. In the first method DNA was run on gel together with known amount of molecular weight marker and the DNA amount was estimated by comparing the intensity of the DNA band to those of molecular weight markers. Similar methods have been used before by Yli-Mattila *et al.* (1996, 2008 and 2009). In the second analysis a fluorescence-based quantitation method by Qubit fluorometer (Invitrogen) was used.

Table 1

Mycotoxins (ppb) in Finnish oat samples as compared to molecularly determined *Fusarium* DNA levels (ng × g^{-1} total DNA) from the same grain samples in 2008.

	Cv	Origin	ZEN	DON	3-AcDON	T-2	HT-2	NIV	Total DNA Ng ×μl^{-1}	Fg12	MGB culm	TMLAN
274-10	Fiia	E-P	n.d.	1100	190	150	550	< 25	9.9	393	0	59394
359-6	Ivory	V-S	< 20	940	89	n.d.	33	72	10.3	680	7.3	14244
359-10	Veli	S	110	3000	430	31	86	87	13.2	829	0	4412
359-11	Belinda	S	360	5600	1500	n.d.	n.d.	< 25	11.3	835	0	387
359-12	Veli	S	170	2600	400	n.d.	n.d.	< 25	15.9	1474	0	177
359-15	Fiia	H	n.d.	940	160	33	64	< 25	12.7	234	0	9436
359-19	Marika	P	n.d.	1300	130	< 25	54	n.d.	18.6	236	1	16198
359-21	Aarre	E-K	n.d.	4100	300	n.d.	n.d.	< 25	13.0	1467	0	376
359-22	Aslak	E-K	n.d.	1300	63	< 25	26	n.d.	10.2	240	0.7	1405
359-26	Aslak	E-S	28	1200	120	41	140	n.d.	12.7	682	0	14265
359-32	Aslak	K-S		1400	160	< 25	60	n.d.	7.9	440	22	10633
359-35	Fiia	K-S	< 20	1700	190	n.d.	n.d.	n.d.	17.2	371	0	1410
359-42	Aslak	E-P	33	2600	290	< 25	< 25	< 25	13.8	1023	0	881
359-45	Fiia	K-P		870	120	n.d.	n.d.	< 26	12.1	150	0	1120
402-1		S		1800	140	< 25	44	n.d.	8.3	290	2.0	8029
402-2		S		1400	280	n.d.	n.d.	n.d.	12.5	461	0	2603
409-2	Fiia	S	65	3100	400	n.d.	n.d.	29	13.3	960	53	2530
409-13	Aarre	P-S	n.d.	1000	130	n.d.	n.d.	n.d.	16.4	159	17	1274
409-14	Fiia	P-S	n.d.	1100	110	n.d.	39	n.d.	15.3	205	1.2	2587

TMFG12 = *F. graminearum* DNA, TMLAN = *F. sporotrichioides*/*F. langsethiae* DNA. MGBculm = *F. culmorum* DNA. n.d. = not detected. white = not analyzed. E-P =Etelä-Pohjanmaa, VS = Varsinais-Suomi, S = Satakunta, H = Häme, P = Pirkanmaa, E-K = Etelä-Karjala, K-S = Keski-Suomi, E-S = Etelä-Savo, P-S = Pohjois-Savo. cv = cultivar

Table 2

Mycotoxins (ppb) in Estonian grain samples as compared to molecularly determined *Fusarium* DNA levels (ng g^{-1} total DNA) from the same grain samples in 2008

	cv	origin	DON	T-2	HT-2	NIV	FX	total DNA ng × μl^{-1}	FG12	MGB-culm	TMLAN
Oat 08T-209	Jaak	V	n.d.	0.4	n.d.	n.d.	n.d.	7.5	1.3	0	4.9
Oat 08T-230	Kerstin	T	n.d.	6.5	5.9	n.d.	n.d.	14.6	13	11	10855
Oat 08T-233	Kerstin	T	n.d.	15.2	23.1	n.d.	n.d.	13.7	2.2	0	26978
Wheat SN 08-028	Vinjet	H	196.1	n.d.	n.d.	n.d.	n.d.	25.7	41	0.3	3.5
Wheat SN 08-046	Vinjet	H	143.0	n.d.	n.d.	n.d.	n.d.	22.5	0	0.4	3.4
Wheat SN 08-102	Trappe	H	n.d.	n.d.	n.d.	n.d.	n.d.	28.1	0	0	0
Wheat 08T-203	Tjalve	J	53.7	n.d.	n.d.	n.d.	n.d.	26.3	164	44	0
Wheat 08T-210	Satu	V	n.d.	n.d.	n.d.	n.d.	n.d.	38.7	200	0	7.0
Wheat 08T-217	Triso	T	n.d.	n.d.	n.d.	n.d.	n.d.	35.3	57	355	1.8
Wheat SN 08T-224	Zebra	V	205.7	n.d.	n.d.	n.d.	n.d.	26.5	3.4	0	3.8
Wheat 08T-229	Ada	T	n.d.	n.d.	n.d.	n.d.	n.d.	23.0	0	0	0
Barley 08T-213	Annabelle	T	n.d.	n.d.	n.d.	n.d.	n.d.	22.1	4.2	0	2120
Barley 08-059	Anni	H	n.d.	n.d.	n.d.	n.d.	n.d.	18.0		0	907
Barley 08-061	Anni	H	n.d.	n.d.	n.d.	n.d.	n.d.	18.5	0.2	0	3342
Barley 08-100	Anni	H	n.d.	n.d.	n.d.	n.d.	n.d.	6.7	0	0	0
Barley 08T-205	Julia	J	n.d.	n.d.	n.d.	n.d.	n.d.	15.9	14	3.5	708
Barley 08T-211	Anni	V	n.d.	n.d.	n.d.	n.d.	n.d.	10.5	0.6	0.6	55
Barley 08T-215	Annabelle	T	n.d.	0.6	n.d.	n.d.	n.d.	21.9	0.4	5.1	2684
Barley 08T-227	Leeni	J	n.d.	n.d.	n.d.	n.d.	n.d.	8.7	2.8	1.7	1448
Barley 08T-232	Gustav	T	n.d.	4.6	2.2	n.d.	n.d.	16.1	1.9	0.3	0
Estimated limit of chromatographic quantitation			50.0	0.4	0.5	120.0	50.0				
Estimated extraction/SPE recovery %%:			74	89	n.d.	n.d.	n.d.				

TMFG12 = *F. graminearum* DNA, TMLAN = *F. sporotrichioides*/*F. langsethiae* DNA. MGBculm = *F. culmorum* DNA. n.d. = not detected. white = not analyzed. cv = cultivar. V = Võrumaa, T = Tartumaa, H = Harjumaa, J = Jõgevamaa

TaqMan primers and, probes and qPCR

The TMFg12 primers and probe have been designed for the *F. graminearum* specific RAPD-PCR product (Doohan *et al.*, 1998, Yli-Mattila *et al.*, 2007a). The TMLAN primers and probe for *F. langsethiae/ F. sporotrichioides* has been designed by another research group (Halstensen *et al.*, 2006), and the culmorumMGB primers and probe for *F. culmorum* by a third research group (Waalwijk *et al.*, 2004). A Bio-Rad IQ^{TM}5 Real-Time PCR Detection System was used for running qPCR samples. Amplification was performed on 96-well iQ Optical PCR plates (Bio -Rad catalog number 223-9441) sealed with microseal "B" adhesive seal (BioRad MSB.1001). The PCR program consisted of 15 min at 95ºC to activate the polymerase, followed by 40 cycles of 15 s at 95ºC and 60 s at 60ºC. Absolute qPCR ROX Mix (ABgene, catalog number AB-139) was used as master mix. Primer and probe concentrations were as described by Yli-Mattila *et al.* (2008, 2009). DNA amount of *Fusarium* species was counted per total DNA, which was estimated by Qubit fluorometer (Invitrogen).

Analysis of mycotoxins

Finnish grain samples

DON, 3AcDON, 15AcDON, HT-2 and T-2 were extracted and determined as described by Hietaniemi *et al.* (2004). ZEN was extracted as trichothecenes, but small amount of acetic acid was added in extraction solvent in oats sample (Romer Labs, 2003, mod.). The ZEN extract was cleaned up with $SiOH_2$ –SPE column (Bakerbond, J.T.Baker) (AOAC, 1984, mod). ZEN was determinated by HPLC and FLD with the exitation wavelenght 236 nm and the emission wavelenght 465 nm.

Estonian grain samples

A domestic coffee mill was used for grinding. Deionised water was produced with MilliQ, from Millipore Corp., Billerica, MA, USA. HPLC grade methanol was got from Rathburn Chemicals Ltd, Walkenburg, Scotland. Standards of nivalenol and fusarenon were purchased from Riedel-de-Haen, (Seelze, Germany) as OEKANAL analytical standard solutions in acetonitrile, deoxynivalenol from Sigma-Aldrich, USA, as solid substance cat. no. D 0156, T-2 and HT-2 toxins from Serva, Germany, as solid substance cat. No. 36737 and 36739 respectively. Prepurification cartridges MycoSep 225 Trich and MultiSep 216 Trichothecene were got from Romer Labs, Tulln, Austria. Circulate shaker type WU-4 (Premed, Poland) was used for extraction. Rotary vacuum evaporator (NE -1, Weaton, USA) was used for removal of extraction solvent and type Sigma 113 micro centrifuge (B. Braun Biotech International GmbH, Mel-

sungen, Germany) was used for precipitation of solid debris before chromatography. HPLC instrument Series 200 from Perkin Elmer, Norwalk, CT, USA was used for chromatography, including low pressure gradient pump system, Rheodyne manual injector, column GL Nucleosil ODS 250 x 2 mm, 5 mm from SGE, Ringwood, Australia, guard column AJO-42864 x 3 mm from Phenomenex, Torrance, California, USA. The single quadrupole mass spectrometer type API 150 EX with Turbo Ionspray (ESI) ion source from PE Sciex (Toronto, Canada) served as detector for identification and quantitation of trichothecenes. The chromatograph was controlled by the soft TotalChrom Workstation v. 6.3.1 and MS by Analyst v. 1.1.

Grain samples were 2.5–3 kg. After thorough mixing with disinfected spoon, 100 g of cereals were taken for grinding. Ground grain subsample 5.0–5.1 g was extracted with 25 ml of mixture 1 of acetonitrile:water 84:16 (v/v) on the circulate shaker 60 min and sufficient amount of the extract was filtered through the 225 Trich cartridge as suggested by the producer. Cartridge 216 was preliminary washed subsequently with 5 ml of mixture 2 of acetonitrile:water 90:10 (v/v) and 5 ml of extraction solvent, then 6.5 ml of filtrate from 225 was loaded to the cartridge and allowed to drop through without any pressure or vacuum, followed by 9 mL of the mixture 2 for elution of trichothecenes. First 2.5 ml of extraction liquid dropping out from the 216 was discarded, all the rest of the eluate was collected into the evaporation bulb and evaporated to dryness at 40°C in the flow of nitrogen. Dry residue was dissolved in chromatography eluent A, centrifuged at 10000 rpm for precipitation of undissolved matter and used for further analysis as specified above

Elution solvent was mixed as the following: eluent A – 10% (v/v) methanol and 0.26g/L ammonium acetate in water, eluent B – 10% (v/v) water and the same salt in methanol, linear gradient from 10%B to 30%B in 10 min, then 75%B in 4 min. followed B up to 99% in 14 min., total flow constantly 0.2 ml/min., column temperature 35°C. MS was tuned to the max. count of ions 353 (fusarenon X M-1) in the negative mode of scanning for detection of NIV, DON and FX, followed by positive mode of scanning tuned to max count of ions 489 (T-2.Na^{+}, M+23). Total ion chromatograms were registered in the range of 250-750 Da in both cases of polarity of scanning and selected ions chromatograms were extracted from this database for integration and quantitative calculation as the following (Table 3):

Table 3

Total ion chromatograms registered in the range of 250-750 Da in both cases of polarity of scanning and selected ions chromatograms, extracted from the database for integration and quantitative calculation

Target toxin	Ion interpretation	Counting range	Ion interpretation	Counting range
NIV	M-H	310 – 311.5	M-H.CH_3COOH	370 – 371.5
DON	M-H	294 – 297.5	M-H.CH_3COOH	354 - 357
FX	M-H	352 - 354	M-H.CH_3COOH	412 - 414
HT-2	M+NH_4	440.8 – 443.8	M+Na	445.7 – 448.7
T-2	M+NH_4	482.5 - 487	M+Na	487.5 - 492

Owing to some thermal fragmentation (ionisation at 400°C), weak signals of fragment ions 281, 265 and 263 were available for confirmation of identifications of NIV, DON and FX respectively, but quantitative calculations were made on the basis of the sum of two main ions in all cases.

Statistical analyses

R^2 (= coefficient of determination), regression slope and P (= significance of regression slope) were calculated using the program SigmaPlot 2001 version 7.1 (SPSS Inc.). The original DNA and toxin concentrations were transformed to logarithmic values in order to obtain a more normal distribution for the values of toxin and DNA concentrations

RESULTS AND DISCUSSION

Both methods for estimating the total amount of DNA in grain samples gave similar results (not shown). We think that >99 % of the total DNA measured by Qubit fluorometer was plant DNA from grains. The fungal DNA amount was counted per total DNA in order to compensate the differences in sample sizes and DNA extraction. Total DNA levels were 7.5-18.6 ng μl^{-1} in oats, 6.7-21.9 ng × μl^{-1} in barley and 22.5-38.7 ng × μl^{-1} in spring wheat.

TMLAN-DNA (combined DNA amount of T-2/HT-2-producing *F. langsethiae* and *F. sporotrichioides*) levels were in most cases clearly higher (highest level 59 µg g^{-1} total DNA) than *F. graminearum* and *F. culmorum* DNA levels, especially in oat (and barley, except in Estonian wheat samples, in which *F. graminearum* DNA levels were higher than those of TMLAN (Tables 1,2). The highest *F. graminearum* DNA level (1.5 µg × g^{-1} total DNA) was found in one Finnish oat sample, while the highest *F. culmorum* DNA level was found in one Estonian wheat (0.35 µg × g^{-1} total DNA) and in one Finnish oat

sample (0.05 μg × g^{-1} total DNA). In Finnish oat samples *F. graminearum* DNA levels were usually between 0.2-1 μg × g^{-1} total DNA (Table 1).

In Estonian grain samples only three wheat samples contained DON. Low levels of T-2 and/or HT-2 toxins were found in two oat and barley samples. Alternariol was found in three Estonian wheat and in one Estonian barley sample (results not shown). In addition moniliformin was found in one Estonian wheat sample. The Finnish oat samples contained 870-5600 ppb of DON and 63-1500 ppb of 3AcDON. No 15AcDON was found. ZEN was found in eight samples and T-2 and/or was found in 14 samples (Table 1).

The correlation between *F. graminearum* DNA and DON levels was highly significant in combined Finnish and Estonian oat samples, while no clear correlation was found between *F. culmorum* DNA and DON levels (Fig. 1a). There was also a clear correlation between *F. langsethiae/F. sporotrichioides* DNA and T-2/HT-2 and *F. graminearum* DNA and ZEN levels in the combined Finnish and Estonian oat samples (Figs 1b,c). No correlation was found between *F. culmorum* DNA and ZEN levels (Fig.1b).

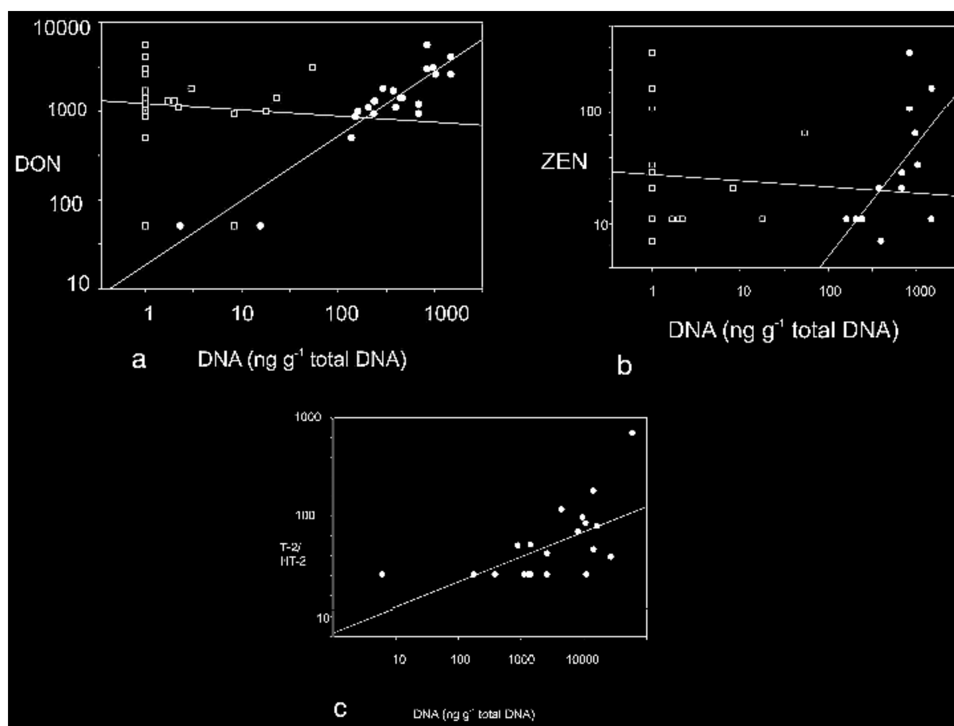

Fig. 1a,b,c. Correlation between log *Fusarium* DNA and mycotoxins in combined Finnish and Estonian oat grains in 2008. Black circles *F. graminearum* DNA and white squares *F. culmorum* DNA in Figs. 1a,b. In Figure 1c black circles *F. langsethiae+ F. sporotrichioides* DNA. Regression slopes are shown.

This is the first study, in which *F. graminearum* has been found in Estonian grains. The coefficient of determination (R^2) was 0.88^{***} between *F. graminearum* DNA and DON levels in the combined Finnish and Estonian oat samples, while between *F. graminearum* DNA and ZEN levels R^2 was 0.42^{**} in Finnish oat samples. No correlation was found between *F. culmorum* DNA and DON levels. There was also a significant correlation between the combined T-2 and HT-2 and combined *F. langsethiae* and *F. sporotrichioides* levels (0.38^{**}) in the combined Finnish and Estonian oat samples.

The high TMLAN levels in oat and barley were probably due to high *F. langsethiae/F. sporotrichioides* contamination in the outer grain layer, which is removed during the de-hulling process before they are used for food (Hietaniemi *et al.*, 2009). According to our results *F. graminearum* is clearly the main DON producer in Finnish oat. The results are in agreement with the previous results of Yli-Mattila *et al.* (2008, 2009).

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