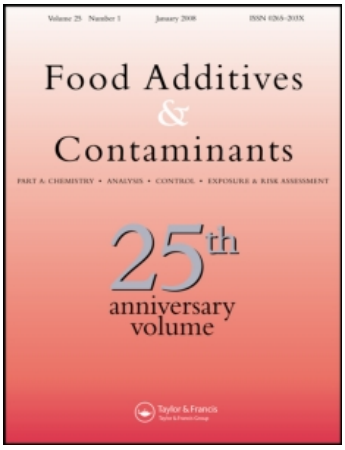

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[a] Department of the Environment and Agro-Biotechnologies, Luxembourg [b] Département de Sciences et Gestion de l'Environnement, Université de Liège, Belgium

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Fusarium head blight and associated mycotoxin occurrence on winter wheat in Luxembourg in 2007/2008

Frédéric Giraud[a]*, Matias Pasquali[a], Moussa El Jarroudi[b], Carine Vrancken[a], Céline Brochot[a], Emmanuelle Cocco[a], Lucien Hoffmann[a], Philippe Delfosse[a] and Torsten Bohn[a]*

[a]*Department of the Environment and Agro-Biotechnologies, CRP-Gabriel Lippmann, 41, rue du Brill, L-4422 Belvaux, Luxembourg;* [b]*Département de Sciences et Gestion de l'Environnement, Université de Liège, B-6700 Arlon, Belgium*

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Fusarium head blight (FHB) is among the major causes of reduced quality in winter wheat and its products. In addition, the causal fungi produce a variety of toxins. A relatively high FHB infection rate in winter wheat was observed in 2007 and 2008 in Luxembourg. A fusariotoxin survey was carried out in 17 different geographical locations. Three groups of *Fusarium* mycotoxins (trichothecenes A and B and zearalenone) were analysed by a multi-detection HPLC–MS/MS method. *Fusarium* strains were also investigated by morphological and molecular methods. In addition, questionnaires relating to cultural practices were sent to the farmers managing the 17 fields investigated. FHB prevalence ranged from 0.3 to 65.8% (mean: 8.5%) in 2007 and from 0 to 24.5% (mean: 8.3%) in 2008. Results of morphological and molecular identification showed that the most common species isolated from diseased wheat spikes was *F. graminearum* (33.1%), followed by *F. avenaceum* (20.3%) and *F. poae* (17.8%). The chemical analysis revealed that 75% of the investigated fields were contaminated by deoxynivalenol (DON, range 0–8111 μg/kg). The preceding crop was highly and significantly correlated to the number of grains infected and had a significant impact on disease prevalence ($p = 0.025$ and 0.017, respectively, Fisher's *F*-test). A trend was found for maize as the preceding crop ($p = 0.084$, Tukey's test) to predict the amount of DON in the fields. This is the first report on the occurrence of DON and ZON in naturally infected wheat grains sampled from Luxembourg.

Keywords: chromatography; LC/MS; mycology; molecular biology; PCR; mycotoxins; trichothecenes; zearalenone; cereals and grain

Introduction

Wheat is, after maize, the most important crop for human consumption worldwide (global production ca. 653 mio t/year in 2006; Economic Research Service, 2007). In Luxembourg, winter wheat is the most important cereal crop (11,947 ha) with an annual production of ca. 75,000 tons in 2006 (Portail des Statistiques Luxembourgeoises, 2006). Even though a number of wheat cultivars exist, the widely planted monoculture makes this cereal prone to several diseases. Among the fungal diseases, FHB or scab is considered the most important worldwide plant disease of the past century, most prominent on small grain cereals, including oat, barley, rye, wheat, triticale, but also on maize, resulting in ear rot (Parry et al., 1995; McMullen et al., 1997). This disease, causing considerable yield and quality losses, has received much attention in recent years as *Fusarium* contaminates the grains with mycotoxins and, therefore, the entire cereal food and feed chain, resulting in grains unusable for further production into, e.g. bakery products, breakfast cereals, pasta, snacks, beer or animal feed (Bullerman and Bianchini, 2007; Dexter et al., 1997; Pirgozliev et al., 2003). FHB caused by *Fusarium* toxigenic species produces several major mycotoxins, namely deoxynivalenol (DON), acetylated-DON (ac-DON), nivalenol (NIV), HT-2 and T-2 toxin, and zearalenone (ZON), which contribute to impaired quality of food products and also cause acute toxic symptoms, such as nausea, or resulting in long-term adverse effects, including interior organ damage, infertility and cancer (Gutleb et al., 2002; Minervini et al., 2004; Rotter et al., 1996; SCF, 1999, 2000a, b, 2001). As a result, there is presently considerable pressure to limit the intake of some of these compounds by setting control levels. Recently, European legislation has established limits for DON and ZON (EU-regulation1881/2006). *F. graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein) Petch] was the most frequently isolated pathogen in warm regions of the world, such as in parts of the USA,

*Corresponding authors. Emails: giraudfrd@mnhn.fr; bohn@lippmann.lu

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Australia and central Europe, whereas *F. culmorum* WG Smith, *F. poae* Peck and *M. nivale* (Fries) Samuel and Hallett are regarded as important causal agents of FHB in the cooler regions of north-west Europe (Nicholson et al., 2004). Even though different species can produce the same mycotoxins, data on *Fusarium* species naturally present in cereal growing areas could aid in predicting the chances for mycotoxin production (Desjardin, 2006). Currently, the differentiation of the various FHB agents is based on physiological and morphological characteristics, such as the shape and size of the macroconidia, the presence or absence of microconidia and chlamydospores, and colony morphology (Nelson et al., 1983). Because classification requires experience and background knowledge, molecular approaches have been developed to facilitate strain identification, including specific diagnostic PCR primers (Nicholson et al., 1998), DNA sequencing (O'Donnell et al., 1998; Yli-Mattila et al., 2004) and genotyping methods, such as randomly amplified length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) (Schmidt et al., 2004).

As no accurate, current data are available in Luxembourg on either FHB or mycotoxin occurrence, the purpose of this study was to (1) provide data on the presence of FHB by monitoring its occurrence, and (2) characterize the related *Fusarium* populations and associated mycotoxin content in winter wheat. For this purpose, observations were carried out at 17 different sites over a 2-year period, 2007 and 2008, covering a wide range of topoclimatological areas in Luxembourg.

Materials and methods

Field survey

FHB was monitored over two seasons (2007 and 2008) in 17 and 16 sites, respectively (Figure 1), in fields selected as representative of different cereal growing areas in Luxemburg and of the three topoclimatological zones (North, East and South).

Determination of the percentage of Fusarium-infected heads

FHB incidence and severity were recorded for each site by carrying out visual evaluations of the disease at the anthesis half-way state (BBCH67, BASF, Bayer, Ciba-Geigy and Hoechst; Landshare et al., 1991) to the late milk state (BBCH77). FHB incidence was calculated as the percentage of wheat plants with recognizable symptoms from an analysis of 150–250 ears per field. FHB severity was calculated as the percentage of kernels with visual symptoms per total kernels present on one ear and computing the average percentage per production field on the basis of 150–250 ears per field.

Grain sample collection

Wheat grains were harvested at BBCH98 (secondary dormancy induced) from the field sites (Figure 1). Spikes that had reached maturity were randomly hand-collected in each plot and manually threshed to recover 500–600 g grains per sample. The samples were packed into paper bags, dried at 60°C for 72 h in an oven (Binder ED 53, Binder Inc., France) to standardize humidity content and then stored at 4 ± 1°C prior to mycoflora and mycotoxin analysis.

Grain mycological analysis

From each sample of ca. 500 g, approximately 50 ml of grains were randomly selected, surface sterilized in 0.37% NaOCl (VWR Prolabo, Briar, France) and 0.1% Tween 20 (Acros, New Jersey, USA) for 10 min and dried on sterile filter paper under a laminar flow hood.

From each of these batches, 100 grains were randomly selected and plated on modified dichloran chloramphenicol peptone agar (DCPA) medium (Andrews and Pitt, 1986; Loos et al., 2004). Grains in DCPA Petri dishes were incubated for 12 days at 22 ± 2°C with a 12-h light period, using a plant incubator (ECPO1E, Snijders Tillburg, The Netherlands).

Fusarium spp. developing on DCPA medium were subsequently transferred to potato dextrose agar (PDA, Merck, Darmstadt, Germany) and incubated at 22 ± 2°C for 6–10 days. Isolates were then identified according to Nelson et al. (1983) based on morphological characters.

Molecular identification of Fusarium strains

To extract DNA, strains were grown in 5 ml potato dextrose broth (PDB, Sigma) with shaking at 150 rpm at 22°C for 6 days (Infors HT Ecotron, Bottmingen, Switzerland). Mycelium was freeze-dried for 20 h and DNA was extracted using a Qiagen Plant DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA was quantified using a Nanodrop (Thermo, Waltham, MA, USA) and dilutions were performed for PCR optimization. Specific primers obtained from Eurogentec (Liège, Belgium) for species confirmation were used (Demeke et al., 2005) to confirm morphological identification.

In particular, Phusion Taq mix (Finzyme, Espoo, Finlande) and 500 μM of each primer in a total volume of 10 μl were used to distinguish *F. poae, F. culmorum, F. graminearum* and *F. avenaceum* (Pasquali et al., 2010). Due to the close genetic relationship to *F. poae*, we also investigated the presence of *F. langsethiae* using specific primers defined by Wilson et al. (2004). The following PCR program was used: annealing

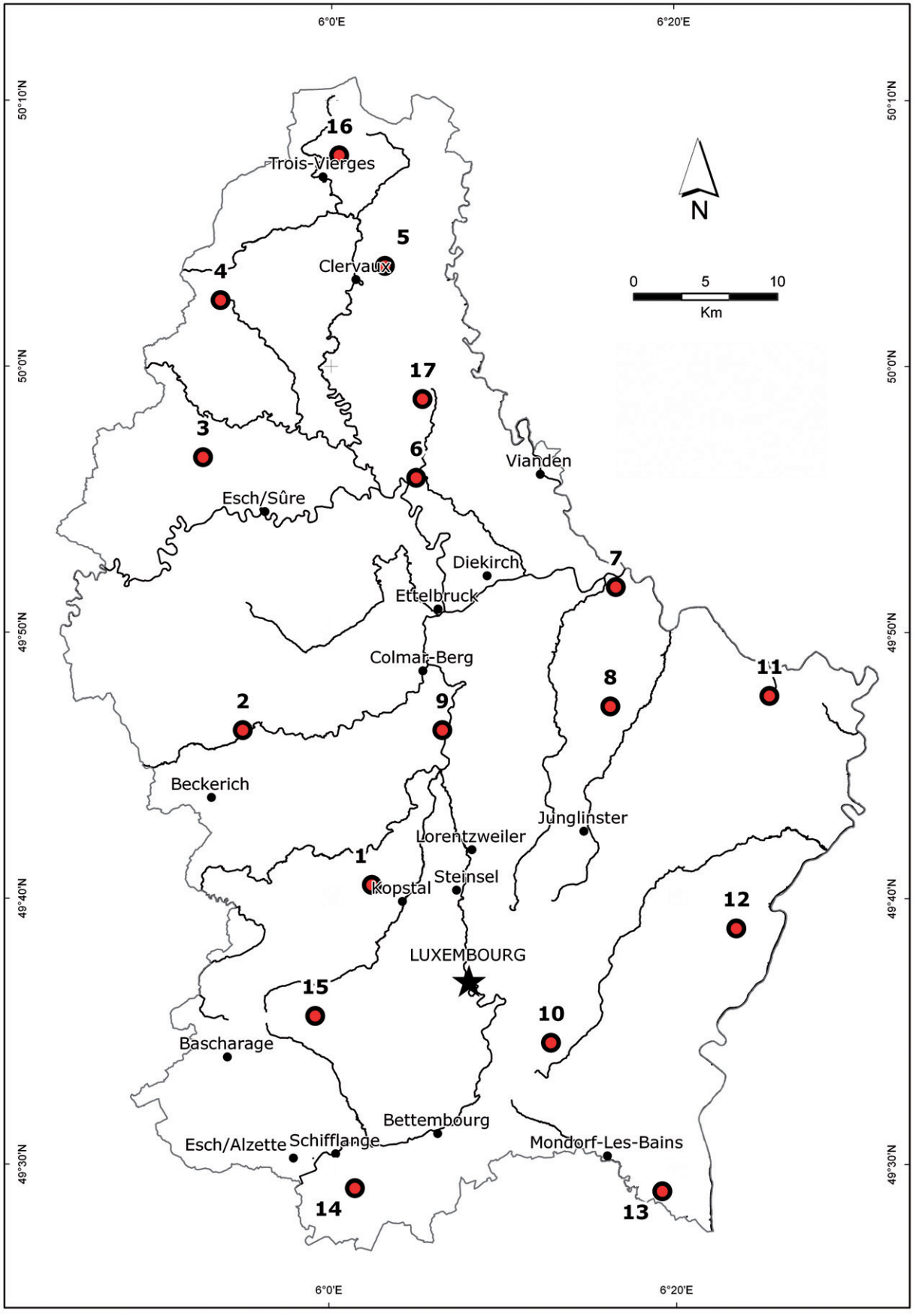

Figure 1. Map of Luxembourg showing the location of the fields where wheat samples was collected in 2007 and 2008.

temperature was 66°C for the first five cycles and 64°C for the next five cycles, followed by 25 cycles at 62°C. The temperature cycle used consisted of denaturation (98°C) for 20 s, annealing (as described above) for 20 s and extension (72°C) for 45 s with maximal ramping rates between temperatures. A final extension step of 5 min was incorporated followed by cooling to 4°C until recovery of samples. All amplicons were visualized on agarose gel 3%, separating PCR fragments with a 100-V tension (Biorad Power Pac 300) for 40 min.

Detection of the primary inoculum for the 2008 season

During the month of May, to verify whether soil and residues could serve as a source for primary inoculum and impacting the development of the FHB, the presence of the four *Fusarium* species detected on the wheat spikes was also evaluated in the first 10 cm of soil and on the residues, as described by Maiorano et al. (2008).

Soil samples, residues (five plastic-boxes with 80 g for each field) and weeds (between 10 and 20 plants per field) were randomly hand-collected in fields localized at three sites (Burmerange, Christnach and Reuler), representing the three major agro-climatological areas (South, East and North) of Luxembourg. Weeds were also sampled from the field borders, sent to the laboratory and then dried for 24 h at 60°C in a ventilated oven. Weeds and residues were cut into 3-cm long sections and sieved to remove gravel. The samples were then analysed as described previously. Weed identification was based on Mamarot (2002).

Fusarium mycotoxins content

Mycotoxin analysis was based on the procedure described by Spanjer et al. (2008), but modified as follows. For mycotoxin assays, 500 g of wheat grains per field were dried for 48 h at 30°C for humidity standardization and aliquots (200 g) were obtained by milling with a Cyclotec^{TM} 1093 (Foss, Belgium). For further extraction of the mycotoxins, 5 g of the obtained flour was homogenized together with 15 ml of acetonitrile/water (80 : 20, v/v), and sonicated for 15 min (Elma Transonic TS540). After centrifugation (720 *g*, 20°C; Sigma 3K12 Bioblock Scientific), a supernatant aliquot was filtered through a 0.20-μm GHP membrane filter (PAL, MI, USA) and diluted 10 times in water.

The following mycotoxins were investigated: trichothecenes of group A (T2, HT2), group B (deoxynivalenol (DON), nivalenol (NIV) and the sum of 3- and 15-acetylated deoxynivalenol (acDONs) and zearalenone (ZON). Separation and detection were achieved by HPLC coupled to tandem mass spectrometry (LC–MS/MS, Dionex Ultimate 3000; Applied Biosystems API 3200) in the multiple reaction monitoring (MRM) positive mode for group A trichothecenes and the negative mode for group B and ZON. An Altima HP RP-C_{18} column was used (150 × 2.1 mm; 3 μm; Grace Davison, Deerfield, IL, USA) with a mobile phase consisting of methanol/water (80 : 20, v/v) containing 2.5 mM ammonium acetate. The detection and quantification limits obtained by extracting 5 g of sample were 35 and 70 ng/g for the trichothecenes and 20 and 40 ng/g wheat for ZON, following the US-EPA approach (Zorn et al., 1999). Quantification was based on external standards and by measuring the following fragment ions of the parent molecules: 371 → 311 for NIV, 355 → 295 for DON, 397 → 337 for acDONs, 442 → 323 for HT2, 484 → 215 for T2 and 317 → 131 for ZON.

Statistical analysis

Statistical analyses were carried out using SPSS 16.0 (Chicago, IL, USA). Normality of data was tested with Q–Q plots and Kolmogorov–Smirnoff tests, equality of variance by Box plots and Levene's test. For further data analyses, data were transformed by the root mean square operation. Comparison of prevalence and severity between different areas was carried out in a linear mixed model using prevalence, severity, DON concentration, yield of wheat and percentage of infected grains as dependent variables, and location, field site, year and previous crop as independent variables. A post-hoc test (Tukey's) was carried out given significant results by the Fisher *F*-test. A *p*-value below 0.05 (two-sided) was considered as significantly different. For correlations between previous crops and FHB, Spearman correlation coefficients were obtained. Unless otherwise stated, all values represent mean ± SD (standard deviation).

Results

Prevalence, severity and percentage of grains infected by Fusarium

The results are summarized in Table 1. No significant difference was found for the prevalence between the two years. However, we observed a trend for a lower severity in 2008 compared to 2007 ($p = 0.053$). A significant difference ($p < 0.01$) for infected grains was found between 2007 (37.7 ± 27.5%) and 2008 (10.5 ± 11.6%). The location had a significant impact on both prevalence and severity ($p = 0.012$ and $p = 0.020$, respectively, Fisher's *F*-test). The eastern part of Luxembourg showed the highest prevalence and severity in both years, confirmed by a high level of *Fusarium* contamination, especially in

Table 1. Prevalence (% infected wheat spikes), severity (% infected grains/spike), occurrence of grains infected by *Fusarium* spp. and preceding crop studied in various geographical areas of Luxembourg ($n = 17$) over 2 years.

Year	Site	Location	Preceding crop	Prevalence	Severity		% of grains infected by FHB agents
				%	Mean (%)	SD	
2007	1	Dondelange	maize	6.00	8.8	6.3	44
	2	Everlange	pea	0.3	nd	nd	8
	3	Nothum	nd	1.0	10.0	0	38
	4	Hamiville	colza	2.8	6.3	2.3	25
	5	Reuler	maize	5.8	21.6	16.7	5
	6	Lipperscheid	maize	2.6	11.5	5.6	39
	7	Reisdorf	nd	16.1	14.7	15.4	92
	8	Christnach	maize	10.2	16.1	20.2	40
	9	Essingen	maize	12.3	19.6	11.1	81
	10	Contern	wheat	1.0	6.2	0	42
	11	Echternach	maize	65.8	46.4	20.5	88
	12	Oberdonven	colza	9.6	39.5	28.1	26
	13	Burmerange	colza	0.5	61.9	38.6	43
	14	Kayl	maize	3.9	40.8	26.1	24
	15	Dippach	triticale	1.1	31.2	0	18
	16	Troisvierges	nd	1.4	5.5	0	4
	17	Hoscheid	colza	3.6	5.6	0.5	19
Average 2007				8.5	21.6	17.8	37.7
2008	1	Kehlen	maize	0.6	5.6	0	10
	2	Everlange	fallow	23.3	36.2	39.8	13
	3	Nothum	oats	0	0	0	3
	4	Hamiville	maize	12.2	7.8	3.5	18
	5	Reuler	colza	10.3	7.8	8.9	9
	6	Lipperscheid	nd	nd	nd	nd	nd
	7	Reisdorf	barley	9.9	52.4	37.1	17
	8	Christnach	maize	24.5	16.6	11.3	4
	9	Essingen	nd	1.6	24.1	19.5	6
	10	Contern	nd	0	0	0	4
	11	Echternach	maize	31.5	34.8	25.9	49
	12	Oberdonven	wheat	0	0	0	1
	13	Burmerange	colza	2.4	22.9	28.1	1
	14	Kayl	wheat	0	0	0	7
	15	Dippach	lucerne	0	0	0	2
	16	Troisvierges	barley	16	8.1	3.1	10
	17	Hoscheid	colza	0	0	0	14
Average 2008				8.3	13.5	16.2	10.5

Note: No data are available for site 6 (Lipperscheid) in 2008 because the cereal planted was spelt wheat and not winter wheat. nd: no data.

Echternach: prevalence and severity from the East were significantly higher compared to the North ($p = 0.049$ and $p = 0.012$, respectively, Tukey's) and to the South ($p = 0.003$ and $p = 0.020$, respectively, Tukey's). A significant correlation was found for the prevalence and the number of grains infected by *Fusarium* strains and maize as the preceding crop ($p = 0.017$ and $p = 0.025$, respectively) as opposed to other crops, but no relation was found for severity.

Identification and distribution of the Fusarium species isolated in Luxembourg

During the biannual survey, more than 800 strains (Table 2) were identified by morphological characteristics and bio-molecular tools using specific primers. The most common species isolated from FHB diseased wheat spikes were *F. graminearum* (33.1%) followed by *F. avenaceum* (20.3%) and *F. poae* (17.8%). The distribution of the *Fusarium* species varied strongly from year to year and also from location to location (Figure 2).

In 2007, 640 strains were identified and the five most predominant fungal pathogens are shown in Figure 2. *F. graminearum*, *F. culmorum*, and *F. poae* are known to be potential trichothecenes producers, while *F. avenaceum* can produce another mycotoxin, moniliformine. *M. nivale* is described as unable to synthesize *Fusarium* mycotoxins. The distribution of the *Fusarium* species varied strongly between agroclimatological zones (North, South and East; Figure 2A). In 2008, fewer *Fusarium* strains were identified (167)

Table 2. Occurrence of grains infected by *Fusarium* spp. studied in 17 geographical areas of Luxembourg.

			F. poae		*F. avenaceum*		*F. graminearum*		*F. culmorum*		*Fusarium spp.*		*M. nivale*	
Location	No	Year	*X*/*n*	%	*X*/*n*	%	*X*/*n*	%	*X*/*n*	%	*X*/*n*	%	*X*/*n*	%
Dondelange	01	2007	17/48	35.4	8/48	16.6	5/48	10.4	0/48	0	1/48	2.1	17/48	35.4
		2008	2/10	20.0	3/10	30.0	1/10	10.0	4/10	40.0	0/10	0	0/10	0
Everlange	02	2007	2/8	25.0	3/8	37.5	1/8	12.5	2/8	25.0	0/8	0	0/8	0
		2008	2/13	15.4	5/13	38.5	1/13	7.7	3/13	23.0	2/13	15.4	0/13	0
Nothum	03	2007	14/38	36.8	3/38	7.9	0/38	0	5/38	13.1	0/38	0	16/38	42.1
		2008	2/3	66.6	1/3	33.4	0/3	0	0/3	0	0/3	0	0/3	0
Hamiville	04	2007	1/25	4.0	4/25	16.0	0/25	0	0/25	0	0/25	0	20/25	80.0
		2008	4/21	19.0	4/21	19.0	9/21	42.8	2/21	9.5	2/21	9.5	0/21	0
Reuler	05	2007	1/5	20.0	3/5	60.0	0/5	0	0/5	0	1/5	20.0	0/5	0
		2008	4/9	44.4	1/9	11.2	4/9	44.4	0/9	0	0/9	0	0/9	0
Lipperscheid	06	2007	22/39	56.4	11/39	28.2	0/39	0	0/39	0	4/39	10.2	2/39	5.1
		2008	–	–	–	–	–	–	–	–	–	–	–	–
Reisdorf	07	2007	0/92	0	35/92	38.0	47/92	51.0	8/92	8.7	2/92	2.1	0/92	0
		2008	1/18	5.5	1/18	5.5	13/18	72.2	0/18	0	3/18	16.6	0/18	0
Christnach	08	2007	3/40	7.5	8/41	20.0	10/40	25.0	18/40	45.0	1/40	2.5	0/40	0
		2008	0/4	0	0/4	0	4/4	100.0	0/4	0	0/4	0	0/4	0
Essingen	09	2007	9/81	11.1	4/81	4.9	44/81	54.3	3/81	3.7	0/81	0	21/81	25.9
		2008	1/7	14.3	3/7	42.8	1/7	14.3	2/7	28.6	0/7	0	0/7	0
Contern	10	2007	3/42	7.1	13/42	30.9	7/42	16.6	7/42	16.6	1/42	2.3	11/42	26.2
		2008	2/4	50.0	1/4	25.0	1/4	25.0	0/4	0	0/4	0	0/4	0
Echternach	11	2007	0/88	0	12/88	13.7	64/88	72.7	9/88	10.2	2/88	2.2	1/88	1.1
		2008	0/57	0	2/57	3.5	50/57	87.7	0/57	0	5/57	8.8	0/57	0
Oberdonven	12	2007	12/26	46.1	7/26	27.0	1/26	3.8	0/26	0	0/26	0	6/26	23.1
		2008	0/1	0	1/1	100	0/1	0	0/1	0	0/1	0	0/1	0
Burmerange	13	2007	0/43	0	14/43	32.5	2/43	4.6	27/43	62.8	0/43	0	0/43	0
		2008	0/1	0	0/1	0	0/1	0	1/1	100.0	0/1	0	0/1	0
Kayl	14	2007	11/24	45.8	0/24	0	4/24	16.6	1/24	4.1	6/24	25.0	2/24	8.3
		2008	2/7	28.6	0/7	0	0/7	0	0/7	0	5/7	71.4	0/7	0
Dippach	15	2007	8/18	44.4	5/18	27.7	0/18	0	0/18	0	0/18	0	5/18	27.7
		2008	2/2	100	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0
Troisvierges	16	2007	0/4	0	1/4	25	0/4	0	0/4	0	0/4	0	3/4	75.0
		2008	6/10	60.0	0/10	0	3/10	30.0	0/10	0	1/10	10.0	0/10	0
Hoscheid	17	2007	5/19	26.3	8/19	42.1	0/19	0	0/19	0	2/19	10.5	4/19	21.1
		2008	5/14	35.7	6/14	42.8	0/14	0	1/14	7.1	2/14	14.3	0/14	0

Notes: X = number of samples infested with the respective specific *Fusarium* species.
n = total number of analysed samples infested with any of the six head blight causing species.
No data available for site 6 (Lipperscheid) in 2008 because the cereal was spelt and not winter wheat.

compared to 2007. However, the same predominant fungal pathogens as in 2007 were identified, with the exception of *M. nivale*, which could not be detected at all (Table 2). The proportion of strains isolated and identified as *F. poae*, *F. avenaceum* and *F. culmorum* contributed to 20, 17 and 6% of the total population, respectively. The distribution of these three strains did not appear to be homogeneous in Luxembourg (Figure 2). Using specific primers for *F. langsethiae*, this species could not be detected.

Primary inoculum: plant debris, soil and weeds

In crop residues and soils from Burmerange and Reuler, none of the four *Fusarium* species was detected; contrarily, *F. culmorum* was identified in the soil and in the residues (maize) from East Luxembourg (Christnach). An additional strain (*F. avenaceum*) was also detected in the soil from this site.

Most weeds recovered at three different sites (Burmerange, Reuler and Christnach) were from the plant family of *Poaceae* (*Bromus, Dactylis, Lolium, Festuca, Alopecurus myosuroides* Hudson, *Poa*) and *Asteraceae* (*Taraxacum officinale* Weber, *Achillea millefolium* L., *Centaurea cyanus* L., *Matricaria* spp.). In Reuler *F. graminearum* was isolated from blackgrass (*Alopecurus myosuroides* Hudson), a common weed in cereal rotations in Europe.

Mycotoxins

Only three toxins were identified in winter wheat: DON, NIV and ZON with DON being the predominant one (range 70–8000 µg/kg). Table 3 summarizes

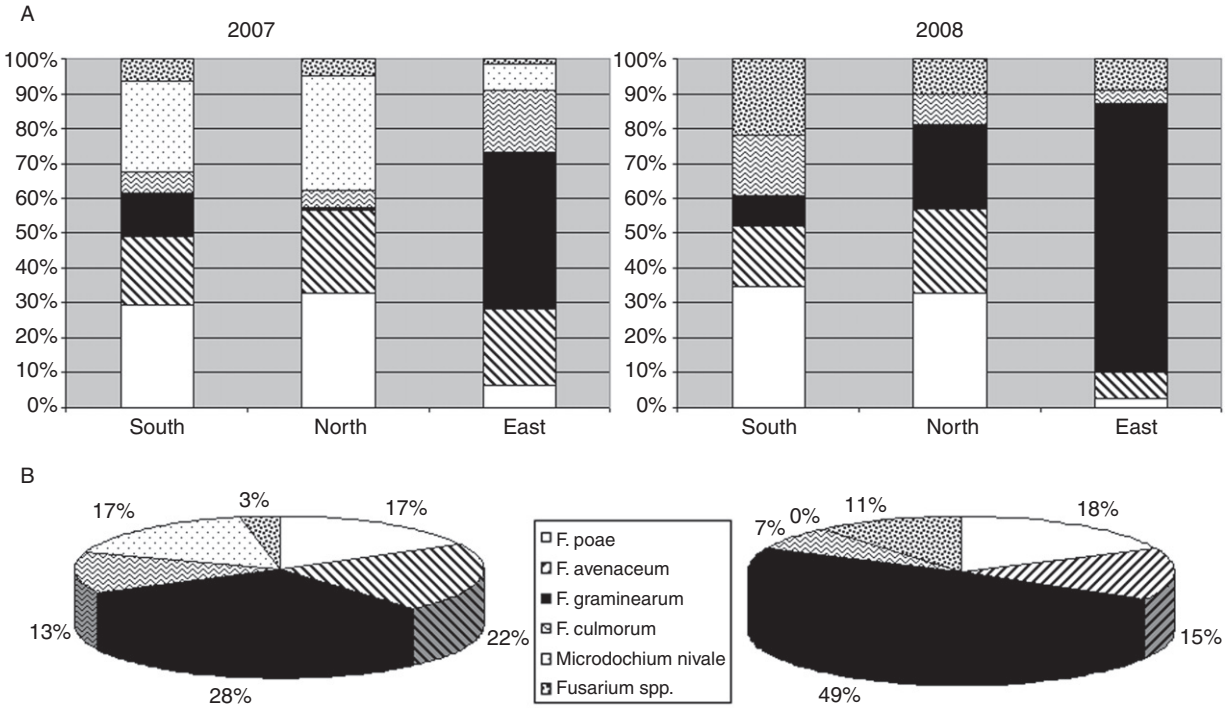

Figure 2. (A) Occurrence (% of contaminated samples) of the different *Fusarium* spp. in Luxembourg in 2007 and 2008 based on an investigation of 17 and 16 sites, respectively, at the time of harvest, and distribution of the *Fusarium* species according to the three agro-meteorological regions in 2007. (B) Circle diagrams represent the overall average of *Fusarium* occurrence for each individual field for both years.

the results of the mycotoxin analyses for the 2 years of survey. NIV was only found in three locations (in 2007, 293 µg/kg for site 8 and 236 µg/kg for site 11; in 2008, 241 µg/kg for site 01; Figure 1), mostly in the East and with concentrations below the EU guidelines. For ZON, we observed two contaminated sites (in 2007, 200 µg/kg for site 7 and 113 µg/kg for site 11) with concentrations higher than that specified by the European Union for unprocessed wheat (100 µg/kg; EU directive 856/2005). The major contamination in Luxembourg came from DON with 75% (25 of 33 sites) of the sites contaminated by this toxin. Its distribution differed strongly and significantly between the three regions (North, South and East, $p = 0.008$) but no significant difference was found between the 2 years studied. Twenty one percent (7 of 33 sites) of the samples contained more than 750 µg/kg DON, 9% (3 of 33 sites) showed a value higher than 1250 µg/kg, the maximum level for unprocessed wheat recommended by the EU (EU directive 856/2005). When maize was the preceding crop, there was a trend ($p = 0.084$, Tukey's) for high DON contamination.

Very high DON levels were observed in the eastern parts of Luxemburg (average of 1915 µg/kg), which were significantly different compared to the North (average of 340 µg/kg, $p = 0.007$, Tukey's) and to the South (average of 396 µg/kg, $p = 0.007$, Tukey's). The highest average DON levels detected for the 2 years came from the same location in Echternach (Table 3).

Discussion

This study is the first report on fusariotoxin contamination in Luxembourg and is an integrated approach to mycotoxin and FHB (prevalence and severity) occurrence, identification of the responsible fungi strains, together with climatological data and farming practices.

As no accurate data are available in Luxembourg on FHB occurrence, this study has provided information on the presence of this disease by mapping the prevalence and severity, and characterizing the *Fusarium* populations involved in the disease.

One of the major environmental factors influencing FHB occurrence is climate. Frequent rainfall, high humidity and warm temperatures, coinciding with flowering and early kernel filling, favor infection and development of the disease (Doohan et al., 2003). For the 2 years of surveys, the average winter temperature was comparatively warm (4.6 and 3.2°C for 2007 and 2008, respectively) compared to the average temperature recorded in previous years (2.0°C for 2000–2007 period), presumably increasing the survival of the FHB causal agents. In addition, it is likely that the "relatively warm" spring temperature, combined with rain and high humidity, promoted the development of FHB. It also can be speculated that the difference observed for FHB severity between 2007 and 2008 ($21.0 \pm 17.8\%$ versus $13.5 \pm 16.2\%$) may, at least in part, be explained by the warmer temperature observed

Table 3. DON content determined in wheat samples collected from different locations and growing seasons.

Location			Year 2007		Year 2008	
			DON (μg/kg)		DON (μg/kg)	
Name	No	Situation	Average	Range	Average	Range
Dondelange	1	South	278	[255–301]	220	[201–240]
Contern	10		657	[627–687]	<LOQ	–
Kayl	14		365	[358–372]	<LOQ	–
Dippach	15		<LOQ	–	463	[443–483]
Everlange	2	North	249	[238–260]	301	[286–316]
Nothum	3		139	[129–148]	<LOQ	–
Hamiville	4		88	[86–91]	928	[871–984]
Reuler	5		89	[84–94]	291	[291–291]
Lipperscheid	6		<LOQ	–	n.d.	–
Troisvierges	16		<LOQ	–	487	[461–512]
Hoscheid	17		147	[136–157]	681	[673–688]
Reisdorf	7	East	1213	[1202–1224]	1014	[1004–1025]
Christnach	8		501	[492–510]	204	[199–210]
Essingen	9		1933	[1593–2273]	<LOQ	–
Echternach	11		4506	[4366–4645]	8111	[7604–8945]
Oberdonven	12		382	[350–414]	101	[94–107]
Burmerange	13		1189	[1178–1200]	<LOQ	–

Notes: Data based on means of duplicate analyses.
Level of quantification or LOQ was 76 μg/kg (for DON) in Luxembourg (nd: no data).

in 2007 (11.9°C) than in 2008 (9.4°C). as stated by Doohan et al. (2003).

In addition to favorable climatic conditions, soil conditions are also likely to impact on FHB development. Several reservoirs (seeds, residues of previous crops, etc.) of the FHB agents are known and have been described (Champeuil et al., 2004; Doohan et al., 2003; McMullen et al., 1997; Parry et al., 1995). However, the role of weeds and grasses has recently been investigated as an alternative potential reservoir (Inch and Gilbert, 2003), with contradictory results (Xu, 2003). The results obtained in the present study are especially interesting in the case of one specific location (Reuler), where identical *Fusarium* species (*F. graminearum*) were found in grains and in blackgrass, suggesting that blackgrass could be a host and a source of *F. graminearum* pathogens in wheat. Nevertheless, these preliminary results require further investigations on a larger set of samples.

In addition to weed, debris from the previous crop and soil are considered the principal inoculum sources for *F. graminearum* and *F. culmorum (*Champeuil et al., 2004; Osborne et al., 2007*)*. *F. culmorum* was detected in both soil and maize residues in one location (Christnach), and data from the cropping systems (questionnaires, data not shown) of the 17 different studied sites made clear that FHB prevalence was higher following maize as the previous crop. This finding confirms the importance of the inoculum for the spread of FHB disease on wheat, in agreement with previous reports (Champeuil et al., 2004; Osborne et al., 2007).

Field surveys conducted elsewhere have indicated that the prevalence and severity of FHB varied strongly from year to year and also from location to location (Isebaert et al., 2009; Tomczak et al., 2002). Nevertheless, few data are available in Europe and, often, comparability to our situation is limited due to difference in methodology used to describe the impact of the disease. For Luxembourg, different climatological conditions around flowering stage and changes in *Fusarium* population composition may account for the variability in prevalence and severity during the observation period. No effect on yield was recorded; therefore, it is difficult to forecast the consequences of mycotoxin production without possessing further data over an extended time period.

FHB is caused by a complex of species (up to 17 species have been associated with this disease), but the main causal agents are *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum* and *Microdochium nivale* (Champeuil et al., 2004; Doohan et al., 2003; McMullen et al., 1997; Parry et al., 1995; Xu, 2003). However, the distribution of these five major species was not homogenous and the *Fusarium* population patterns observed in Europe showed large variations between countries (Adler et al., 2002; Snijders and Perkowski 1990; Tomczak et al., 2002; Waalwijk et al., 2003). In the present study, a high prevalence of *F. graminearum* (45% in 2007 to 77% in 2008) and decrease in *F. culmorum* (17% in 2007 to 3.4% in 2008) was found in the eastern part of Luxembourg where *F. graminearum* dominated. As mentioned by Isebaert

et al. (2009), temperature plays a role in the composition of *Fusarium* spp. population and proximity to the Moselle river valley may partly explain the dominating presence of *F. graminearum*.

F. avenaceum distribution in Luxembourg was homogeneous among the three regions (19–21% for 2007) and was apparently stable over the years (below 20% deviation). This species appeared to be a common contaminant of winter wheat with a level of occurrence less important than previously described in northern Europe (Loos et al., 2004; Stepien et al., 2008; Waalwijk et al., 2003). High frequencies of *M. nivale* were detected in 2007 in the north of Luxembourg (32%) but this strain was not observed in 2008. The epidemiology of this fungus is quite different from the *Fusarium* species and its incidence is mainly influenced by climatic conditions (Loos et al., 2004). In 2008, the conjunction of weakly favorable conditions, interaction with other *Fusarium* species and application of chemical treatment (e.g. triazoles are known to be effective against *Fusarium* sp. but not against *Microdochium* sp.) may explain the large decrease of its incidence.

Despite the high occurrence of FHB and the presence of diverse *Fusarium* sp. producing different mycotoxin profiles in Luxembourg, winter wheat was mainly contaminated with DON, partly above levels recommended in the European directive 856/2005, but generally (for 91% of the sites) 15AcDON, 3AcDON, T2 and HT-2 were not detected in the present samples.

The frequency of ZON (two positive sites) was found to be low but the concentrations measured were above the European guideline recommendation (100 μg/kg). According to Desjardin et al. (2006), previous data indicated that ZON levels in Europe in cereal grains rarely exceed this limit, a level associated with visible effects in swine. Based on its estrogenic properties and its negative impact on human and animal health, a dedicated survey in the eastern part of Luxembourg, looking for high contamination levels, is recommended.

Maize as a preceding crop, the wheat cultivars used and seasonal and local weather conditions explained most of the variations observed in DON concentration. However, no clear explanation could be found for the high level of contamination detected in Echternach. Even though the range of DON level contamination detected in Luxembourg was comparable to other European situations (Belgium: Chandelier et al., 2003, Poland: Tomczak et al., 2002), Luxembourg showed sites with extremely high contamination rates, higher than other reported contaminated European sites and closer to results found in South America or Africa (Desjardin et al., 2006).

Many efforts were made to relate measurements from the fields (disease prevalence or severity) to mycotoxin concentrations; however, results were very variable, from weak to strong positive correlations as well as negative ones (Isebaert et al., 2009; Paul et al., 2005). In 2008, the average DON contents were similar to the previous season despite the fact that field severities were lower. High disease pressure of *Fusarium* is not automatically related to a higher DON content (Everlange in 2008, severity: 36% and DON: 301 μg/kg). Edwards et al. (2001) suggested that some pathogens do not produce mycotoxins, which means that mycotoxin contamination is not related to visual symptoms of disease severity. Opinions also vary strongly on the existence of a relationship between DON and the amount of FHB pathogen (Isebaert et al., 2009). For Luxembourg, no clear relation could be detected between DON level and the species profile of the agents causing FHB. This result is probably due to the large variability in toxin production between and within *Fusarium* spp. and to micro-environmental factors (climate, use of several chemical treatments inducing high toxin levels).

Conclusion

This large-scale sampling provided an overall picture of FHB occurrence in Luxembourg. *Fusarium* populations causing FHB in naturally infected winter wheat at the different sites differed considerably, indicating that the profile of the *Fusarium* population is influenced by several regionally linked parameters, and comparisons with other countries should be carried out cautiously. Further epidemiological surveys are required to adapt specific preventive practices, such as chemical treatment application, to reduce the infection level. Mycotoxin levels identified here are unlikely to present a health risk to animal/human consumers, but deserve further investigation. Future studies are warranted to follow *Fusarium* distribution and to improve climatological models capable of predicting *Fusarium* occurrence.

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