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Fusarium head blight (FHB) in Flanders: population diversity, inter-species associations and DON contamination in commercial winter wheat varieties

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Abstract The presence of *Fusarium* spp. causing Fusarium head blight (FHB) of wheat was studied in Flanders (Belgium) in 2007 and 2008. Symptoms, deoxynivalenol content (DON), *Fusarium* spp. and trichothecene chemotypes were determined at seven locations on different commercial wheat varieties. Overall, significant differences in disease pressure between locations and varieties were observed within 1 year. In addition, we were able to detect consistent and significant resistance differences among the common varieties both under high disease pressure (2007) and low disease pressure (2008). The accumulation of DON was not related to the presence of *F. graminearum* but showed a clear correlation with rainfall during and after the period of anthesis. During the two-year survey, characterisation of 756 *Fusarium* samples by species-specific PCR designated *F. poae* and *F. graminearum* as the predominant species in Flanders. Furthermore, most of the ears were colonised by multiple FHB pathogens in 2007 whereas the *Fusarium* population was less complex in 2008. Log-linear analysis of these multiple (two- and three-way) species interactions revealed a clear correlation between *F. poae* and several pathogens of the FHB disease complex. Finally, chemotype analysis showed that *F. culmorum* and *F. graminearum* were respectively of the NIV chemotype and DON chemotype. 3-ADON and 15-ADON chemotypes occurred in more or less equal amounts within the *F. graminearum* population both in 2007 and 2008. The congruence of these results with observations throughout Europe are discussed.

Keywords Deoxynivalenol · *Fusarium* · Resistance · Survey

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Introduction

Fusarium head blight (FHB) is a devastating disease of cereals in numerous areas of the world. This disease is caused by a complex of up to 17 species of which *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *Microdochium nivale* (formerly *Fusarium nivale*) are predominant (Brennan et al. 2007; Leonard and Bushnell 2003; Parry et al. 1995). Crop residues are the major reservoir on which pathogens of the FHB complex survive saprophytically. In the subsequent growing season, ascospores, conidia, chlamydospores and hyphal fragments can serve as primary inoculum

(Bai and Shaner 2004). Airborne spores released from crop residue are deposited on or inside wheat florets where they germinate and initiate infection. Anthesis is therefore the most vulnerable growth stage for establishing a successful infection. The abundance of primary inoculum will determine the FHB disease severity. Due to this short vulnerable period, the disease is commonly monocyclic. Under conditions that are particularly conducive for *F. graminearum,* ascospores which are transported by wind can contribute to increased inoculum pressure resulting in two or even more cycles per growing season (Obst et al. 2002). Reduced tillage systems to prevent erosion and increased implementation of corn maize, a suitable host of *F. graminearum*, in crop rotation systems throughout Western Europe favours the outbreak of FHB epidemics in small-grain cereals during the growing seasons by generating increased amounts of primary inoculum (Bai and Shaner 2004).

Until now, no absolute FHB resistance encoded by single dominant resistance genes has been characterised in wheat. Consequently, it is difficult to implement Fusarium resistance into breeding programmes and only few studies address FHB resistance since it is not easy to accurately measure (Brennan et al. 2007; Mesterhazy et al. 1999; Xu et al. 2004). Differences in geographic regions, environmental conditions and periods of anthesis often hamper the interpretation of infection data with regard to varietal resistance (Brennan et al. 2007; Doohan et al. 2003).

The production of mycotoxins is often the major concern in FHB-contaminated fields. FHB pathogens produce a differential spectrum of mycotoxins depending on the species present. Trichothecenes, fumonisins, the oestrogenic zearalenon and moniliformin are the most important mycotoxins produced by FHB species (Bennett and Klich 2003). Trichothecenes, the predominant mycotoxins found in small-grain cereals including nivalenol (NIV), deoxynivalenol (DON) and its acetylated derivatives 3-acetyl deoxynivalenol (3-ADON) and 15-ADON, are inhibitors of eukaryotic protein synthesis and cause food refusal, diarrhoea, vomiting and dermatitits (Fokunang et al. 2006). The set of genes involved in the biosynthesis of these trichothecene metabolites determines the chemotype of each trichothecene lineage of *Fusarium* spp. The production of DON and NIV is mainly restricted to *F. culmorum* and *F. graminearum* although NIV can also be produced by *F. poae* (Quarta et al. 2006).

Fusarium graminearum particularly likes higher temperatures whereas *F. culmorum*, *F. poae*, *F. avenaceum* and *M. nivale* tend to dominate in cooler regions such as Scandinavia and the UK (Doohan et al. 2003; Lukanowski et al. 2008; Parry et al. 1995; Waalwijk et al. 2003; Xu et al. 2005). However, in European climates, temperature is not considered to be a limiting factor for the presence of FHB species. This is illustrated by Hungary, a continental region of Europe with hot summers, hard winters and low precipitation and yet *F. poae* is a predominant component of the FHB disease complex (Xu et al. 2008). Consequently, FHB is often caused by a mixture of the above mentioned species with two- and three pathogen interactions occurring on one plant (Xu et al. 2005). These interactions are not rigid and shifts in the population are reported between (Waalwijk et al. 2003) and within (Xu et al. 2005) growing seasons. Results from The Netherlands obtained by Waalwijk et al. (2003) demonstrate a drift in the FHB population from *F. culmorum* in the early 1990s to *F. graminearum* in 2000 and 2001. An analogous phenomenon was described by Isebaert et al. (2009) for Flanders. These populational drifts not only occur between well-characterised species as sometimes, new species arise and gain importance in FHB disease. In 1999, *F. langsethiae* was firstly characterised on cereals in Europe (Torp and Langseth 1999; Torp and Nirenberg 2004). In recent reports, the presence of *F. langsethiae* has been shown in several countries and is considered a major threat due to the production of T2-toxin (Hudec and Rohacik 2009; Lukanowski et al. 2008).

The driving forces of these population shifts are not unambiguously determined. A combinational effect of geography, weather, fungicide treatment, crop rotation, host resistance and soil tillage will decide on the outcome and structure of the FHB population. Besides the population drifts between growing season, there is also a considerable dynamism in the population within one growing season. Xu et al. (2005) demonstrated an increasing complexity of the population during one growing season with many three- and four-species interactions at the end of the season.

In the present study, we studied the FHB population complexity in two growing seasons (2007 and 2008) and recorded the FHB population on 13 winter wheat varieties in seven wheat-growing areas through-

out Flanders (Belgium). Five varieties were common in both years. This survey allowed us to examine the population structure in each of these locations by analysing the presence of the five predominant FHB pathogens using species-specific PCR reactions. Moreover, using this technique we were able to study multiple-species interactions on the same ear. In addition, rainfall and temperature were monitored in these regions during both growing seasons. Comparing these results with a previous study by Isebaert et al. (2009) gave useful information on the population evolution during the last five years towards the dominance of *F. poae*. The chemotypes of isolated *F. graminearum* and *F. culmorum* were determined with respect to 3-ADON, 5-ADON and NIV production. Since the presence of DON is a major concern in FHB-contaminated fields, DON content in all fields was included in the study and analysed in the light of the population composition at each location and on each variety. To our knowledge this is the first high throughput inventory on the residing FHB population and the attendant DON contamination in Flanders winter wheat fields with emphasis on multiple-species interactions. This study provides new insights into the complexity of the FHB population and DON content in Flanders, a region near the vast wheat-growing areas of northern France and characterised by its typical rotation systems comprising winter wheat combined with silage maize, corn and ryegrass.

Material and methods

Field trials

The FHB symptoms and population were followed during the growing seasons of 2007 and 2008 in seven locations situated in the main agricultural regions of Flanders. Over the two growing seasons a total of 13 commercial varieties were included in the FHB survey. Experimental design was always a completely randomised block design with at least three replications. Elementary plot size varied between 15 and 25 m^2 depending on the location. Normal crop husbandry measures were taken including three nitrogen fertilisations according to the N-index established by Bodemkundige Dienst België and pre- and post-emergence herbicide applications. An overview of husbandry measures is given in Table 1. One fungicide treatment was done at growth stage (GS) 59 (Zadoks et al. 1974). The fungicide treatment was not orientated to *Fusarium* spp. but was included to control other leaf and ear diseases. All plots were harvested mechanically in August according to the ripening stage.

Disease assessment

Fields in all locations were evaluated for the presence of *Fusarium* spp. symptoms between 27 June 2007 and 4 July 2007 and between 24 June 2008 and 2 July

Table 1 Field crop conditions in 2007 and 2008 at the locations in the present study

	Lierde	Zuienkerke	Koksijde	Zwevegem	Verrebroek	Poperinge	Bottelare
Soil type***	Loam	Clay	Clay	Loam	Light loam	Sandy loam	Loamy Sand
Sowing date 2006–2007	27/10/2006	21/10/2006	30/10/2006	27/10/2006	21/10/2006	27/10/2006	7/11/2006
Sowing date 2007–2008	23/10/2007	27/10/2007	5/11/2007	25/10/2007	23/10/2007	25/10/2007	7/11/2007
Precrop survey 2006–2007	Potatoes	Sugar beet	Sugar beet	Potatoes	Sugar beet	Sugar beet	Potatoes
Precrop Survey 2007–2008	Potatoes	Silage maize	Sugar beet	Potatoes	Sugar beet	Seed-potato	Grain maize
Fungicide treatment 2007*	19/05/2007	23/05/2007	24/05/2007	24/05/2007	25/05/2007	24/05/2007	25/05/2007
Fungicide treatment 2008*	3/06/2008	31/05/2008	31/05/2008	1/06/2008	31/05/2008	31/05/2008	27/05/2008
N fertilisation (kg ha^{-1})** 2007	80+50+74	65+80+60	80+82+59	85+58+54	80+40+100	90+65+65	70+33+78
N fertilisation (kg ha^{-1})** 2008	67+46+70	69+48+80	88+60+80	86+50+54	33+44+79	90+65+80	61+51+71

The plant growth regulator chlormequat amended with imazaquin, was applied to reinforce the antilodging effect (Stahli et al. 1995)

*Fungicide treatment was carried out at GS 59 (Zadoks et al. 1974). Both in 2006–2007 and 2007–2008, the fungicide treatment consisted of epoxyconazole (75 g ha^{-1}) and kresoxim-methyl (125 g ha^{-1})

**(kg ha^{-1}): nitrogen fertilisation was applied in three fractions according to the N-index established by Bodemkundige Dienst België

***soil tillage: in all locations a deep plough soil tillage was applied followed by rotor harrow both in 2007 and 2008

2008. In each elementary plot, 100 ears originating from distinct plants were randomly sampled at GS 69 and scored using an ordinal scoring system based on the surface of the ear covered with *Fusarium* spp. symptoms: 1 = healthy; 2 = up to 25%; 3 = 25 to 50%; 4 = 50 to 75%; 5 = 75 to 100% (Isebaert et al. 2009).

Field sampling for species and chemotype determination

In order to obtain a representative image of the FHB population at each location, one symptomatic ear from each variety and each parallel was harvested during the disease assessment period. From each ear, two distinct symptomatic seeds were isolated. As such six to eight seed samples were obtained per location and per variety. This resulted in 54 seed samples per location and per year. These seeds were surface-sterilised for 1 min in 1% NaOCl, washed for 1 min with sterile distilled water, dried for 5 min and subsequently placed on PDA (potato dextrose agar, Oxoid, Belgium) plates. After 5 days incubation at 20°C, outgrowing mycelium was transferred to a new PDA plate. Overall, this procedure resulted in 756 seed samples to be analysed with the species-specific PCR protocol. For species determination, five agar plugs randomly taken from a fully-grown PDA plate were transferred to liquid GPY-broth (10 g glucose; 1 g yeast extract and 1 g peptone, Oxoid, Belgium l^{-1}) and incubated for 5 days at 20°C. After 5 days, mycelium was separated from the culture medium by filtration (Rundfilter, Schleicher & Schuell) and freeze-dried for 6 h at −10°C and 4 h at −50°C (Christ Alpha 1-2 LD Plus, Osterode, Deutschland); samples were stored at −20°C.

DNA extraction and PCR

DNA extraction was a modified CTAB method (Saghaimaroof et al. 1984). One hundred mg of freeze-dried mycelial extracts was placed into 2 ml microcentrifuge tubes. Nine hundred µl of CTAB extraction buffer containing 0.1 M Tris (pH 7.5), 1% CTAB (hexadecyl trimethyl ammonium bromide), 0.7 M NaCl, 10 mM EDTA, 1% β-mercaptoethanol and 0.3 mg ml^{-1} proteinase K was added to the ground samples and homogenised on a vortex for 30 s. The homogenised sample was incubated for 30 min at 60°C after which 900 µl of chloroform:isoamylalcohol (24:1) was added. The mixture was centrifuged for 15 min at 13,200 rpm and 750 µl of the upper water phase was transferred to a new 2 ml centrifuge tube. To precipitate the DNA, 750 µl of ice-cold isopropanol was added, mixed and centrifuged for 10 min at 13,200 rpm. After washing the samples with 250 µl of ice-cold 70% ethanol, the DNA was dried at room temperature under a fume hood for 30 min. PCR for single-species detection was performed in a 25 µl reaction mixture (Demeke et al. 2005).

Species-specific primers are listed in Table 2. DNA amplification was performed in an Applied Biosystems GeneAmp PCR System 9700. Amplicons were separated on 1.5% (wt/vol) agarose gels stained with 0.1 µg ml^{-1} ethidium bromide. PCR was validated by including reference strains obtained from the MUCL/BCCM collection in each PCR run: *F. graminearum* MUCL 42841; *F. culmorum* MUCL 555; *F. poae* MUCL 6114; *M. nivale* MUCL15949; *F. avenaceum* MUCL 6130. PCR identification of *F. graminearum* chemotypes was based on sequences of *Tri13* and *Tri3* genes (Ji et al. 2007).

DON analysis

At harvest (mid-August 2007/2008), all elementary plots were harvested and a representative sample was ground using a Retsch GmbH grinding station (Mill: SM100, DR 100/75, divider: PK1000 Haan, Germany). This sample was used for DON determination. Ground samples were analysed in duplicate for DON presence in all individual plots by ELISA using the Veratox DON 5/5 kit (Biognost, Neogen, Leest, Belgium). As such, six to eight DON measurements were obtained per variety and location. Ten g of ground sample was amended with 100 ml of water and shaken for 30 min at room temperature. The suspension was filtrated (Rundfilter, Schleicher and Schuell) and the through flow analysed for DON presence according to the manufacturer's instructions. The lowest limit of detection was 0.1 ppm. A standard curve was established using 0, 0.25, 0.4, 1 and 2 ppm DON. The ELISA kit provides 100% specificity for DON but also shows minor cross-reactivity with 3-ADON.

Statistical analysis

Disease levels were scored with an ordinal scoring system. Differences in DON levels and disease severity between winter wheat varieties were detected using a

Table 2 Primers used for the species and chemotype determinations

	Sequence (5′–3′)	Size (bp)	Reference
Species			
F. avenaeceum	GCTAATTCTTAACTTACTAGGGGCC CTGTAATAGGTTATTTACATGGGCG	220	(Turner et al. 1998)
F. culmorum	ATGGTGAACTCGTCGTGGC CCCTTCTTACGCCAATCTCG	570	(Nicholson et al. 1998)
F. graminearum	CTCCGGATATGTTGCGTCAA GGTAGGTATCCGACATGGCAA	450	(Nicholson et al. 1998)
F. poae	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	220	(Parry and Nicholson 1996)
M. nivale	GGTGCTGTCTCTCGGGAC TCCTCCGCTTATTGATATGC	628	(Glynn et al. 2007)
Chemotype			
NIV vs DON	TACGTGAAACATTGTTGGC GGTGTCCCAGGATCTGCG	415/234	(Waalwijk et al. 2003)
3-ADON	GATGGCCGCAAGTGGA GCCGGACTGCCCTATTG	583	(Jennings et al. 2004a)
15-ADON	CTCGCTGAATTGGACGTAA GTCTATGCTCTCAACGGACAAC	863	(Jennings et al. 2004a)

NIV nivalenol; *DON* deoxynivalenol; 3-*ADON* 3-acetyldeoxynivalenol; 15-*ADON* 15-acetyldeoxynivalenol

non-parametric Kruskall-Wallis and Mann-Whitney test with a sequential Bonferroni correction for multiple comparisons. Differences between DON levels and disease severity were considered at $P=0.05/(n-1)$ with n the number of varieties in the study. Relationships between the weather data, DON data and disease severity were investigated using the Pearson product moment correlation analysis at a significance level of $P=0.05$. Two-way interactions and higher between *Fusarium* spp. and *Microdochium* were analysed using a simple straightforward log-linear model as described by Xu et al. (2005) assuming a Poisson sampling in each location. K-way and higher-order effects were determined to significance levels of $P=0.05$ and $P=0.001$. All data were analysed using SPSS-software (Originally: Statistical Package for Social Sciences) version 15.0 for WindowsXP.

Results

Evaluation of FHB symptoms on different winter wheat varieties

In order to evaluate disease pressure on different wheat varieties, a total of 13 varieties were scored for the presence of *Fusarium* spp. symptoms during the growing season of 2006–2007 and 2007–2008. Premature head bleaching in the different wheat varieties revealed major, consistent and significant differences between wheat varieties in all locations. Figure 1 clearly shows Tulsa, Patrel, Kaspart and Limes to be the most resistant varieties to *Fusarium* spp. infection in 2007 compared to varieties Lexus, Tuscan, Toisondor and Rosario which were more susceptible to FHB. In 2008, Waldorf and Sahara were the most resistant varieties to *Fusarium* spp. infection compared to varieties Toisondor and Lexus which were more susceptible to FHB. When focusing on the five common wheat varieties in both sampling years, it is clear that Toisondor and Lexus were the most susceptible while Limes was the best performing variety over the two-year period. Although in the present study no artificial infections were conducted, susceptibility of the commercial wheat varieties was consistent among locations and sampling year although differences were much smaller in 2008 compared to 2007 due to lower infection levels. Besides differences in resistance between wheat varieties, infection intensity varied between different locations both in 2007 and 2008. Finally, in 2007 under high infection pressure, class 3 and 4 symptoms

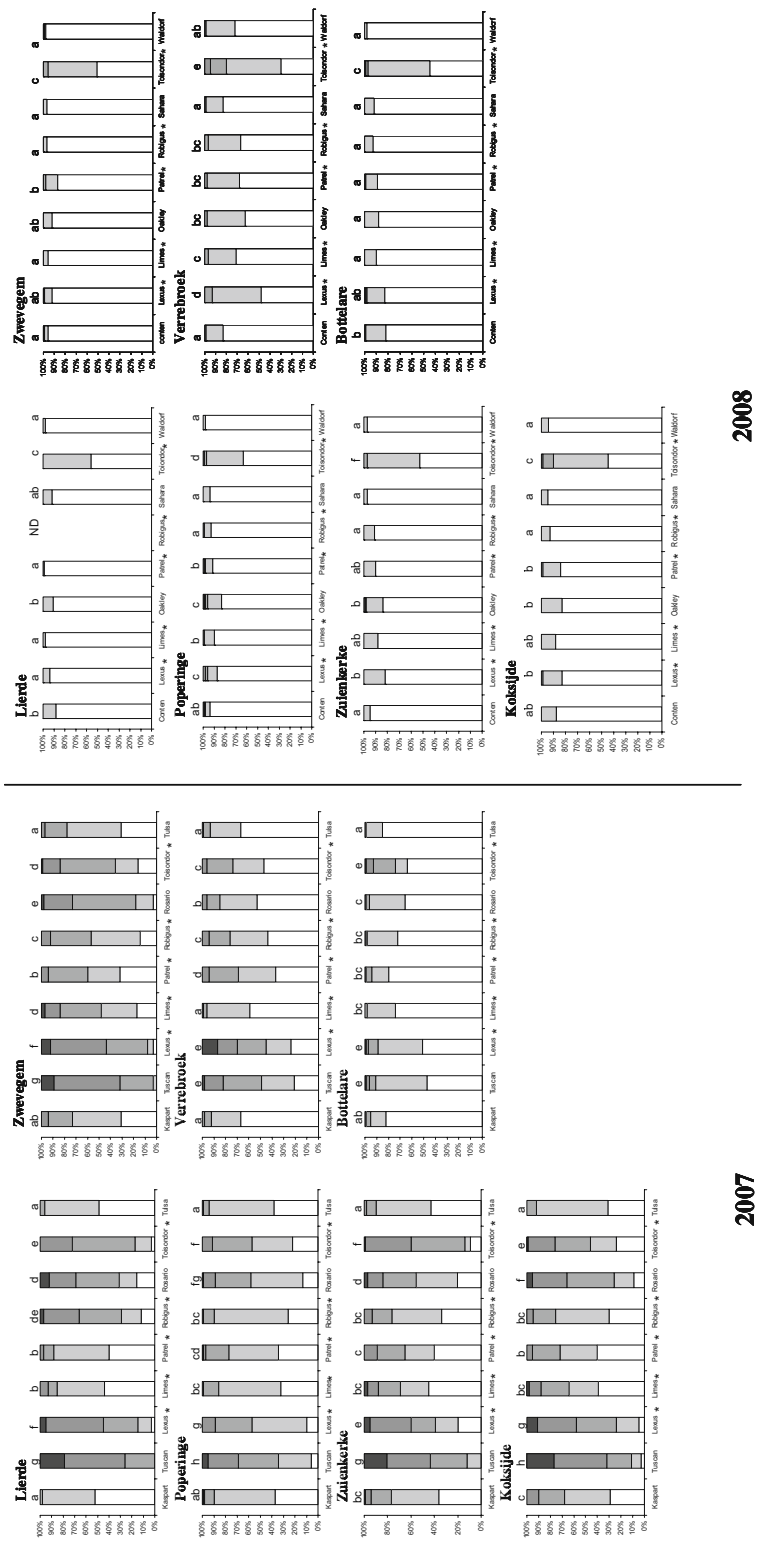

Fig. 1 Evaluation of FHB symptoms at seven locations and on nine commercial wheat varieties in 2007 and 2008. Infection was scored using an ordinal scoring system with 5 classes: 1: no disease; 2: < 25%; 3: 25–50%; 4: 50–75%; 5: >75% of the ear covered with FHB symptoms. Data were statistically analysed using a non-parametric Kruskall-Wallis and Mann-Whitney test with a Bonferroni correction for multiple comparisons (P=0.05/11) for main effects and shifts between classes

Table 3 Correlative interactions between DON content, disease severity, rainfall in June (mm m^{-2}) and May, number of days with rainfall in June or May after analysis with a Pearson product moment correlation analysis (P=0.05)

		DON	Disease	Rain in May	Number of days in May	Rain in June	Number of days in June
2007							
	DON	1	0.129	0.430	−0.631[a]	0.706[a]	0.501
	Disease		1	−0.188	−0.088	0.056	−0.227
2008							
	DON	1	0.138	0.031	0.119	0.324	0.198
	Disease		1	0.013	0.268	0.258	0.117

[a] correlation coefficient after a Pearson product moment correlation analysis

differed between resistant and susceptible varieties. In resistant varieties, class 3 and 4 scores mainly consisted of several scattered individual grains throughout the ear with FHB symptoms possibly reflecting different infections sites under conditions of high disease pressure. On the other hand, in susceptible varieties, class 3 and 4 scores were mainly composed of whole areas of the ear covered with FHB symptoms.

There was no correlation between disease severity and rainfall in May and June or between disease severity and the number of days with rainfall in May or June (Table 3).

DON content in different winter wheat varieties

For all replications and wheat varieties, DON content was measured in the different locations (Table 4). Although the infection pressure was very high in 2007, the DON thresholds as stated by the European Commission were rarely exceeded except for the location Poperinge in 2007 where almost all varieties

Table 4 DON levels (mg kg^{-1}) in nine winter wheat varieties at seven different locations

DON (mg kg^{-1})	Lierde	Zuienkerke	Koksijde	Zwevegem	Verrebroek	Poperinge	Bottelare
2007							
Kaspart	**1.273**[b]	0.533[ab]	0.596[a]	0.183[a]	0.213[a]	1.215[ab]	0.120[a]
Lexus*	0.795[a]	0.188[a]	0.379[a]	0.115[a]	0.468[ab]	**2.325**[bc]	0.289[b]
Limes*	0.789[a]	0.184[a]	0.547[a]	0.127[a]	0.284[a]	**1.540**[abc]	0.142[a]
Patrel*	0.736[a]	0.180[a]	0.618[a]	0.142[a]	0.266[a]	0.980[a]	0.127[a]
Robigus*	0.508[a]	0.310[a]	0.373[a]	0.271[a]	0.319[a]	**1.678**[b]	0.182[ab]
Rosario	0.616[a]	0.637[ab]	1.110[a]	0.432[ab]	0.611[b]	**3.685**[c]	0.172[ab]
Toisondor*	0.542[a]	0.166[a]	0.466[a]	0.106[a]	0.410[ab]	**1.523**[b]	0.110[a]
Tulsa	**1.701**[b]	0.177[a]	0.438[a]	0.118[a]	0.423[ab]	**2.406**[bc]	0.141[ab]
Tuscan	1.108[a]	0.707[b]	0.848[a]	0.570[b]	ND	0.781[a]	ND
2008							
Conten	0.411[b]	0.461	0.135[a]	0.313[b]	0.205[a]	0.434[b]	0.129[ab]
Lexus*	0.315[b]	0.430[a]	0.299[b]	0.169[a]	0.684[bc]	0.467[b]	0.401[c]
Limes*	0.218[a]	0.478[a]	0.131[a]	0.511[b]	0.628[b]	0.331[ab]	0.331[c]
Patrel*	0.356[b]	0.489[a]	0.510[c]	0.786[c]	0.655[b]	0.567[bc]	0.144[b]
Robigus*	0.512[b]	ND	ND	0.453[b]	0.292[a]	0.504[b]	0.192[b]
Sahara	0.289[b]	0.599[a]	0.145[a]	0.193[a]	0.787[c]	0.563[bc]	0.117[ab]
Toisondor*	0.499[b]	**1.464**[b]	0.109[a]	0.524[b]	**2.208**[e]	0.621[c]	0.159[b]
Waldorf	0.271[a]	0.537[a]	0.180[ab]	0.236[ab]	**1.433**[d]	0.455[b]	0.072[a]
Oakley	0.383[b]	0.374[a]	0.146[a]	**1.266**[d]	**1.882**[de]	0.244[a]	0.345[c]

Different letters per location point to differences in DON level between wheat varieties after non-parametric data analysis Kruskall-Wallis and Mann-Whitney test with a Bonferroni correction for multiple comparisons at a significance level of P=0.05/n with n the number of two-by-two comparisons. Bold values indicate exceeded European thresholds

ND not determined

exceeded the threshold level of 1.250 mg kg^{-1}. This location was characterised by very high amounts of rain during and after the period of anthesis in June 2007 (210 mm rain m^{-2}). Occasional significant differences in DON content were observed among varieties in each location; however these differences were not consistent throughout Flanders. Correlation analysis showed no relationship between DON content and disease severity (Fig. 2b). In addition, DON content was not correlated with the number of days with rainfall in June 2007 or with the rainfall in May 2007 (mm rain m^{-2}) (Table 3). A clear correlation was observed between DON content in the different varieties and the rainfall (mm rain m^{-2}) in June 2007 (Fig. 2a). Statistical analysis revealed a linear relation with DON = mm rain $m^{-2} \times 0.010–0.790$ (r= 0.709 and Pearson-chi square: 0.011, 2-sided). However, using a curve estimation method, a better fit was obtained using an exponential model with correlation coefficient r=0.728.

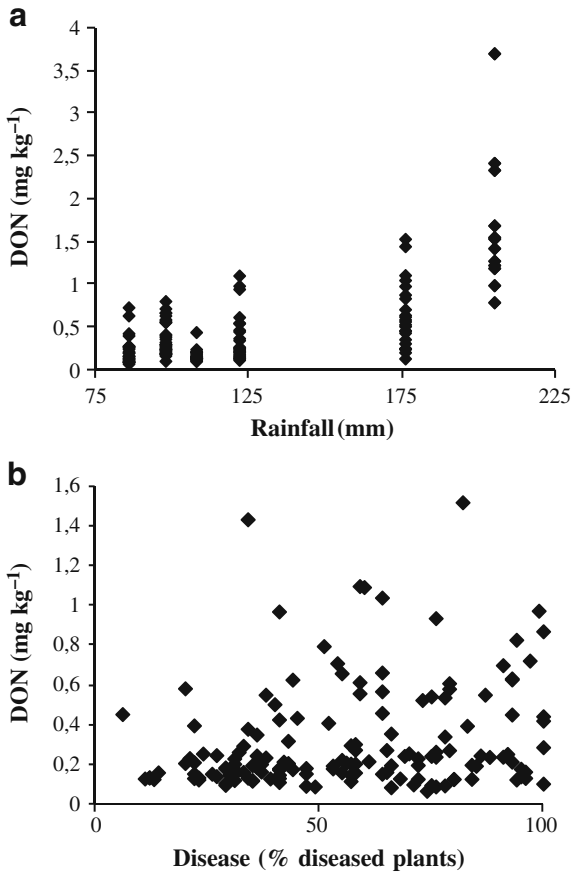

Fig. 2 Correlation between deoxynivalenol (DON) level and rainfall (mm m^{-2}) in June 2007 (**a**) and the percentage of diseased plants (**b**). The DON measuring points per location represent the values for all varieties in a location. Correlation was determined by a linear regression (r=0.709 and Pearson-chi square: 0.011 (2-sided)) and a curve estimation algorithm (r=0.728)

Similar to 2007, significant differences in DON content between varieties were not consistent throughout Flanders. In addition, the relation between DON content and rainfall as observed in June 2007 was not present in 2008. In June 2008, rain fell rather uniformly throughout Flanders with extremes only varying from 68 mm rain m^{-2} (Poperinge) to 104 mm rain m^{-2} (Verrebroek). Due to this small divergence, the exponential relationship between DON and rainfall in June as observed in 2007 was not present in 2008.

Presence of *Fusarium* spp. in winter wheat

Table 5 shows the overall incidence of each FHB pathogen detected by species-specific PCR reaction in all tested locations. All *Fusarium* spp. and *M. nivale* isolates were equally distributed among varieties and there was no preference of any FHB pathogen to colonise a particular variety in the present two-year study. The prevalence of *Fusarium* spp. differed considerably between locations after analysis with a Pearson chi-square test (P<0.001). However, the marginal presence of *F. culmorum* was a general observation throughout Flanders both in 2007 and 2008.

In 2007 in Bottelare and Poperinge, the FHB population was composed respectively of *F. graminearum* and *F. poae*. In Lierde and Zuienkerke, *F. poae* and *F. avenaceum* were predominant and occurred often in multiple (two or more) -species associations. In Koksijde, *M. nivale* was abundantly present in the population together with *F. poae*. In particularly, the former species was present in all samples of Zwevegem in multiple-species associations.

In 2008, the absence of *F. avenaceum* at all locations was remarkable. The populations in Bottelare, Verrebroek, Zwevegem and Zuienkerke were dominated by *F. graminearum* and *F. poae*. In Lierde, the population consisted solely of *F. graminearum* and *M. nivale*. In Koksijde and Poperinge, *F. graminearum*, *F. poae* and *M. nivale* were the predominant species.

The incidence of multiple-species interactions varied between sampling years and locations (Table 6).

Table 5 Percentage of ears colonised by *F. graminearum (Fg), F. culmorum (Fc), F. avenaceum (Fa), F. poae (Fp)* and *M. nivale (Mn)* in seven locations throughout east and west Flanders (Belgium)

	Bottelare	Lierde	Verrebroek	Zwevegem	Koksijde	Poperinge	Zuienkerke
2007							
Fa	0	93	16	82	0	20	82
Fc	15	8	9	5	11	0	18
Fg	64	53	7	38	30	20	20
Fp	0	93	26	98	80	64	86
Mn	14	23	7	98	54	27	43
2008							
Fa	0	0	3	3	0	0	2
Fc	3	0	5	18	0	19	17
Fg	33	27	31	41	31	75	49
Fp	57	0	15	62	12	50	44
Mn	7	35	13	31	46	73	0

In the year 2007, under high infection pressure, multiple-species interactions were more prominently present than in 2008.

In 2007, only two locations (Bottelare and Verrebroek), showed FHB symptoms caused by single species. In most locations however, the FHB population was composed of multiple-species interactions. As such, in Poperinge and Koksijde the majority of the samples consisted of two-species interactions. In Lierde and Zwevegem, the majority of the population was composed of three-species interactions (Table 6). Using a log-linear model previously described by Xu et al. (2005), the multiple-species interactions were analysed. This analysis revealed no significant 4-way (P=0.998) or 5-way interactions (P=0.999). The K-way and higher order effects for two- and three-species interactions was 0.000 and 0.010 respectively, indicating multiple-species interactions. One significant 3-way interaction was observed between the species *F. graminearum*, *F. poae* and *M. nivale* (P=0.002). The two-way interactions are shown in Table 7. The majority of these interactions were not significant but in four out of ten two-way interactions, a clear association between *F. poae* and *F. avenaceum*, *F. graminearum* and *M. nivale* respectively, and between *F. graminearum* and *M. nivale* respectively, was observed. No higher-order associations were found with *F. culmorum* since this was an inferior species at all locations.

Table 6 Percentage of ears colonised by one or multiple-species complexes in seven locations throughout east and west Flanders

	2007					2008				
Number of species	1	2	3	4	5	1	2	3	4	5
Bottelare	72	38	0	0	0	71	39	0	0	0
Poperinge	37	49	14	0	0	19	64	17	0	0
Verrebroek	58	37	5	0	0	95	5	0	0	0
Koksijde	16	55	27	2	0	46	54	0	0	0
Lierde	3	27	62	8	0	82	18	0	0	0
Zwevegem	0	9	61	30	0	44	39	17	0	0
Zuienkerke	9	23	43	23	2	67	33	0	0	0

Table 7 Two-species interactions analysed by a simple log-linear model for the years 2007 and 2008

	Fa[a]	*Fc*	*Fg*	*Fp*	*Mn*
Fa	1	0.248	0.152	0.000**	0.134
		0.606	**0.115**	**0.621**	**0.264**
Fc	NR	1	0.163	0.179	0.971
			0.202	**0.743**	**0.791**
Fg	NR	NR	1	0.018*	0.000**
				0.013*	**0.935**
Fp	NR	NR	NR	1	0.000**
					0.090
Mn	NR	NR	NR	NR	1

Values from 2008 are indicated in bold

NR Not relevant

[a] For species key, see Table 5

**significant association at P=0.001

*significant association at P=0.05

In 2008, most of the *Fusarium* samples consisted of one single species or combinations of two species. Only in Poperinge and Zwevegem, the population complexity involved more complex three-species interactions. The association of *F. poae* with *F. graminearum* was significant (P=0.013).

Chemotypes of *F. graminearum* and *F. culmorum*

A total of 291 field isolates were identified as *F. graminearum* or *F. culmorum*. A PCR was conducted based on the *Tri13* gene to distinguish DON and NIV chemotypes (Fig. 3). The DON chemotypes were further characterised based on the *Tri3* gene distinguishing between 3- and 15-acetylated forms. In all locations, this PCR approach identified 64% to 100% of the chemotypes.

Both in 2007 and 2008, the *F. graminearum* population comprised mainly DON-chemotypes with percentages of, respectively, 72% and 87% while the NIV-chemotypes represented, respectively, 16% and 8% of the total *F. graminearum* population. Within the DON-producing *F. graminearum* population 15-ADON chemotypes (56%) and 3-ADON chemotypes (44%) were equally present in 2007. In 2008, there was a minor increase in abundance of 15-ADON chemotypes (69%) compared to the 3-ADON chemotypes (31%). The distribution of the DON chemotypes differed among locations. Both in 2007 and 2008, Bottelare, Koksijde, and Verrebroek were dominated by the 15-ADON chemotype. In Zuienkerke and Zwevegem, the 15-ADON chemotype and 3-ADON chemotype appeared in more or less equal amounts. Finally, in Poperinge the 3-ADON chemotype was predominantly present in 2007 but 15-ADON dominated the population in 2008.

For *F. culmorum* the majority of the isolates were of the NIV chemotype in 2007 (95%) and 2008 (88%) while the DON chemotypes were only marginally present.

Discussion

This study focuses on the presence of *Fusarium* spp. and *M. nivale* in commercial winter wheat in Flanders during the growing seasons of 2007 and 2008. Since existing information on the population composition and complexity in a small region such as Flanders was only fragmentary and restricted to morphological characterisation of predominant species (Isebaert et al. 2009), a PCR method was applied to detect and distinguish major FHB pathogens and to analyse their chemotypes. In addition, DON content was determined to elucidate eventual relationships between DON content and population composition, weather and FHB species present. Results show a clear and consistent differential resistance response of the different varieties to natural FHB infection. It is known that wheat varieties differ in their susceptibility to FHB (Brennan et al. 2003; Lemmens et al. 2004; Mesterhazy et al. 1999; Xu et al. 2004). These studies were mainly conducted using artificial infections to eliminate side effects such as differences in

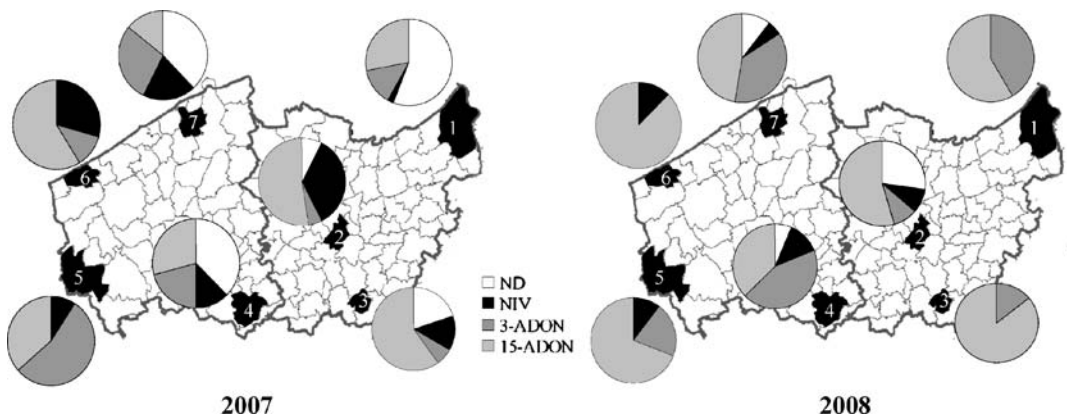

Fig. 3 Presence of NIV, 3-ADON and 15-ADON chemotypes of *F. graminearum* throughout west and east Flanders characterised by PCR in 2007 and 2008. 1: Verrebroek; 2: Bottelare; 3: Lierde; 4: Zwevegem; 5: Poperinge; 6: Koksijde; 7: Zuienkerke. ND: Not determined

timing of anthesis and environmental variations in rain or temperature (Doohan et al. 2003). In the present study, varietal susceptibility to FHB was evaluated at seven different locations in Flanders using natural field infection. Although the infection pressure varied significantly between the seven locations in this study, the overall varietal ranking with respect to FHB resistance was the same at all locations and thus exceeded the influence of environmental fluctuations, bias in period of anthesis or local cultural practices. This is a useful conclusion for further variety trials, eliminating the necessity for artificial infections to assess resistance to *Fusarium* spp. Finally, the ranking of the five common wheat varieties in the two-year survey was similar, with Lexus and Toisondor as the most susceptible and Limes as the most resistant variety.

Due to the experimental set-up of natural infection it was not possible to distinguish between the two main types of FHB resistance i.e. type I (penetration) and type II (spread within an ear) as previously described in the literature (Ban and Suenaga 2000). However, it was remarkable that in locations with high disease pressure, resistant varieties displaying scores 3 and 4, showed mainly multiple individual florets with symptoms not spreading within the ear, indicating numerous successful infections being prevented from spreading throughout the ears, whereas susceptible varieties showed continuous areas of ear bleaching. It is tempting to speculate that the former observation in resistant varieties is a consequence of repetitive successful penetrations of fungi of the FHB complex consequently stopped by a type II mechanism of resistance. These repetitive penetrations could originate from polycyclic disease development under conducive conditions.

No consistent significant differences in DON content were observed between varieties in either of the sampling years, indicating that DON accumulation is not variety-specific. This was also observed in a study by Brennan et al. (2007). In contrast to the 4-year study of Xu et al. (2008), no link between DON content and disease severity was seen in the present study. Some explanations can be suggested for this discrepancy. Firstly, the FHB populations in Flanders appeared to be complex with numerous multiple-species interactions. In these interactions, DON-producing species are not always dominant within the population. Secondly, when DON-producing species occur together with other fungi from the disease complex, both their growth and DON production can easily be affected (Simpson et al. 2004; Xu et al. 2007). Thirdly, chemotype analysis revealed that the majority of the *F. culmorum* isolates had the NIV chemotype. Finally, the presence of *Fusarium* spp. was verified in a qualitative way. It is possible that quantitatively, DON-producing species are only marginally present. The use of a quantitative PCR approach could confirm this hypothesis.

An exponential correlation was present between DON content and the rainfall in June 2007. A similar exponential relationship was reported previously (Hooker et al. 2002). Since June 2007 was characterised by abundant and continuous rainfall, it is likely that this exponential relationship was the result of polycyclic disease development resulting from continuous rain-splash spore dispersal. The significant and negative correlation between the number of days with rainfall in May 2007 and DON content was remarkable and unexpected. We hypothesise that this is an artefact originating from the fact that the number of days with rainfall in May 2007 was quite high and hardly fluctuated between 15 and 20 throughout Flanders, resulting in weak evidence for this apparent negative correlation. The absence of this correlation in 2008 confirms the artefact hypothesis.

All five pathogens included in the PCR analysis were detected in Flanders. *Fusarium culmorum* was the less common species in both sampling years while *F. poae* was the most dominant followed by *F. avenaceum* and *M. nivale* in 2007 and by *F. graminearum* in 2008. The marginal presence of *F. culmorum* agrees with observations by Xu et al. (2005) in a pan-European study.

It was remarkable that all samples from 2007 were identified without exception whereas a small fraction of the population remained unidentified in 2008 at the locations Koksijde, Verrebroek and Lierde. In view of interseasonal population dynamics it is possible that a new *Fusarium* species was introduced in the population in 2008. One of the possible candidates is *F. langsethiae*, a *Fusarium* species firstly described in 1999 (Torp and Langseth 1999) that has recently been reported by several authors as progressing in Eastern Europe (Hudec and Rohacik 2009; Lukanowski et al. 2008; Torp and Nirenberg 2004).

A previous study by Isebaert et al. (2009) carried out in 2001 to 2004 at the same locations as in the

present study, clearly illustrates the dynamics of the FHB population over a period as short as three growing seasons. Isebaert et al. (2009) reported the dominance of *F. culmorum* and *F. graminearum* whereas *F. poae* was a rather marginal species surviving latently. These apparent interseasonal shifts within the Flanders FHB population agree with observations by Waalwijk et al. (2003) who reported a comparable shift in the FHB population from *F. culmorum* in the late 1990s to *F. graminearum* in 2001 to 2002 in The Netherlands. The main causal factor for this shift previously discussed by Waalwijk et al. (2003) was an increase in maize production, especially corn maize; in Flemish agriculture this crop contributes to the proliferation of *F. graminearum* since the pathogen has the capacity to survive on maize stubble. Additionally, the homothallic nature of *F. graminearum* allows the production of large masses of ascospores that can play a role in the epidemiology of the disease.

The dominance of *F. poae* in the present study was remarkable but consistent throughout the sampled area and present both in 2007 and 2008. In some other European countries, an increasing importance of *F. poae* in the FHB disease complex was also observed (Xu et al. 2005). There is no obvious explanation for this dominance. However, it is tempting to speculate on the origin of the *F. poae* infections as a driving force for this dominance. It is likely that *F. poae*, described as a moderately virulent pathogen (Brennan et al. 2003), merely acts as a secondary invader colonising the weakened ears already infected by other more aggressive FHB pathogens such as *F. graminearum*. This hypothesis agrees with the observation that statistically significant two-pathogen associations were mainly observed between *F. poae* and the other FHB pathogens *F. avenaceum*, *F. graminearum* and *M. nivale*. The only other significant two-way interaction was between *F. graminearum* and *F. avenaceum*. The fact that the dominance of *F. poae* is more prominent in 2007 with very high infection pressure compared with lower infection pressure (2008), would be a consequence of its nature as a secondary pathogen. Secondly, the increasing occurrence of *F. poae* could be explained by its sporulation strategy. This fungus produces very considerable amounts of microconidia in a dry powdery form that can easily invade ears.

Regarding chemotypes of *F. graminearum* and *F. culmorum* present in Flanders, it was obvious that the *F. culmorum* population contained mainly NIV producers while the *F. graminearum* population comprised mainly DON producers. The dominance of NIV chemotypes in the *F. culmorum* population in Europe is comparable to observations by other workers throughout Europe (Jennings et al. 2004a, b; Miedaner et al. 2000; Waalwijk et al. 2003). However, the proportion of NIV producers within the *F. graminearum* lineage (6%) was considerably lower than observed in neighbouring countries such as the UK and The Netherlands (Jennings et al. 2004a; Waalwijk et al. 2003).

In conclusion, this study has clearly demonstrated differences in the resistance of commercial wheat varieties to FHB using natural infection. DON production was clearly correlated with rainfall during and after anthesis but not with the presence of typical DON producers such as *F. graminearum*. The FHB population is characterised by large variability and complexity, with numerous multiple-species associations and differential composition at a species level at each location. In two-way pathogen interactions, *F. poae* was always present. In comparison with FHB survey data of Flanders during previous years, *F. poae* dominance is remarkable but an explanation for this observation is not obvious. *Fusarium poae* is an efficient secondary pathogen and is favoured in years with high infection pressure; this is one of the possible explanations for its dominance.

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