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FUSARIUM SPECIES AND MYCOTOXINS IN WHEAT: RESULTS OF A TEN-YEAR FIELD MONITORING PROGRAMME

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RÉSUMÉ
L'étude menée au Luxembourg entre 2007 et 2017 a montré une forte variation de la distribution des espèces de *Fusarium* et de la teneur en mycotoxines. Les souches de *F. graminearum* de chémotype 15-ADON produisant la mycotoxine deoxynivalenol et/ou sa forme acétylée 15-ADON sont prédominantes par rapport au chémotype NIV produisant la mycotoxine nivalenol, qui reste très minoritaire. Malgré la prépondérance de *F. graminearum*, il existe de grandes disparités entre les années. L'abondance et la composition des espèces varient deux fois après deux années sèches consécutives lors de la floraison. Cependant, après la première période sèche, *F. culmorum* est majoritaire, tandis que *F. poae* domine après la seconde période sèche. Des concentrations élevées de mycotoxines (jusqu'à 9247,06 ng/g de DON) ont été détectées. Cette étude montre la nécessité de continuer à travailler sur des solutions durables pour limiter les risques de contamination et mieux comprendre les facteurs écologiques qui déterminent un déplacement de la population de Fusarium et de la composition des mycotoxines dans les céréales.

Mots-clés : Fusarium, chémotype, mycotoxines, deoxynivalenol, blé.

ABSTRACT
The 2007-2017 survey in Luxembourg showed a strong variation in the distribution of the *Fusarium* species and mycotoxin content when comparing different years. The *F. graminearum* strains of the 15-ADON chemotype were predominant over the entire population compared to the NIV chemotype, which remained very much in the minority. Despite the preponderance of *F. graminearum*, there were large disparities between different years. The abundance and composition of the species deviated strongly from the long-term average on two occasions after two dry years, respectively. In the first dry period, *F. culmorum* prevailed, while *F. poae* dominated at the end of the second dry period. High levels (up to 9247.06 ng/g of DON) of mycotoxins were found, except in dry conditions. This study shows the need to better understand the ecological factors determining shifts among *Fusarium* species and thus, mycotoxin composition in grains.

Keywords: Fusarium, chemotype, mycotoxins, deoxynivalenol, wheat.

INTRODUCTION

According to the STATEC (National Institute of Statistics and Economic Studies of the Grand Duchy of Luxembourg), in 2016, agricultural land accounted for 52.6% of the total land use in Luxembourg. The cropping area of cereals represented 22.3% of the total agricultural area used, of which 11% was wheat, one of the most important crops in Luxembourg. Wheat is, along with maize and rice, one of the most widely consumed cereals and is therefore a central component of human nutrition.

Fusarium head blight (FHB) is one of the world's major wheat diseases. However, it is known that the different *Fusarium* species causing fusarium head blight are able to produce secondary metabolites, such as mycotoxins. These are present in a whole series of food and feed products and their accumulation in feed and food can cause many diseases if consumed, such as skin irritations, nausea, suppression of the immune system, and the favouring of necrosis, estrogenic, mutagenic and hepatoxic effects. Fusarium species produce several types of mycotoxins, including trichothecenes, which can be type A or B depending on the presence or absence of a keto group at the C-8 position. Fusarium species are able to produce different types of trichothecenes according to the core trichothecene cluster. A variation in the cluster and gene activity defines the chemotype of the strain (Dejardin 2008). Nivalenol (NIV), deoxynivalenol (DON) and their acetylated derivates, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), are the main type B trichothecenes described to date by Ward et al. (2002). A new chemotype has recently been described, although it has not yet been found in Europe (Varga et al., 2014).

Due to their implications for human health, animal productivity and both national and international trade, mycotoxins often cause significant economic losses. According to FAO estimates, 25% of foodstuffs contain mycotoxins, this would represent losses of the order of 1000 million tons per year all over the world.
It is in this context that our study came into being in 2007, since wheat represents one of the major crops in Luxembourg. Before 2007, no data were available in Luxembourg regarding the *Fusarium* species and mycotoxin content in grains. Fusarium mycotoxins vary greatly from year to year (Vogelgsang et al., 2017). It is therefore important to assess the risks of *Fusarium* infections and mycotoxin contaminations over several years, as was the case in the present study. Our main objective is to understand the impact of meteorological conditions on the annual variation of *Fusarium* species in Luxembourg and their effect on mycotoxins.

MATERIALS AND METHOD

METEOROLOGICAL DATA

Meteorological data are provided by the Agrometeorological Service of the Administration of Technical Agricultural Services (ASTA). The data are collected from an extensive network of meteorological stations throughout the territory. The network of stations, as well as the weather data, can be accessed via the AgriMeteo website.

PLANT MATERIALS

Despite its relatively small size, the Grand Duchy of Luxembourg is characterized by contrasting topoclimatic conditions, being approximately 200m above sea level in the south and 400m above sea level in the north, but also with rural structures in the north contrary to the urban structures in the south. Each year, between 15 and 20 sites are chosen to reasonably cover all districts of Luxembourg. Plant materials were sampled according to the procedure outlined in Beyer et al. (2014) and Giraud et al. (2010).

FUNGAL STRAIN ISOLATION AND MOLECULAR CHARACTERIZATION

DNA was extracted from mycelium grown in potato dextrose broth (PDB, BD Biosciences) at 150rpm and 22°C for six days in the dark. The mycelium was harvested by filtration through sterile miracloth paper (Merck), collected in a 2mL microtube (Eppendorf) containing two sterile steel balls and then conserved in liquid nitrogen. The mycelium was lyophilized for 24 hours before being crushed with an MM 400 mixer mill (Retsch GmbH, Germany). DNA was extracted using a Qiagen Plant DNA extraction kit according to the manufacturer's protocol. Species identification was

performed according to the protocol described by Dubos et al. (2011) based on the sequencing of the elongation factor 1 alpha (EF1alpha) gene.
Chemotype determination was done following the method described in Pasquali et al. (2011).

MYCOTOXIN ANALYSIS

For mycotoxin assays, 500g of wheat grains per field (two samples per field) were dried for 48 hours at 30°C for humidity standardization, and aliquots (200g) were obtained by milling with an RM 200 mortar grinder (Retsch GmbH, Germany).
5g of this flour were weighed in centrifuge tubes and 15mL of acetonitrile/water (80/20, v/v) were added. Tubes were vortexed and agitated for 10 minutes at 10Hz with an MM 400 Mixer Mill (Retsch GmbH, Germany). After centrifugation (4700rpm, 15min, 20°C , Multifuge X3R, Thermo), a supernatant aliquot was filtered through a 0.20 mm GHP membrane filter (PAL) and diluted 10 times in water. Prior to the analysis, extracts were stored for at least 1 hour at 4°C.
The mycotoxins were separated and detected via HPLC coupled with tandem mass spectrometry (LC-MS/MS, Dionex Ultimate 3000, Applied Biosystems API 3200) in multiple reaction monitoring (MRM), positive and negative mode. A Zorbax Eclipse Plus C18 column was used (2.1x150mm, Agilent Technologies) with a mobile phase consisting of methanol and water containing 2.5Mm ammonium acetate.

Until 2009, only DON and NIV were measured. We subsequently extended our analysis to 3-ADON, T-2 and ZON in 2009 and to 15-ADON and HT-2 in 2013).

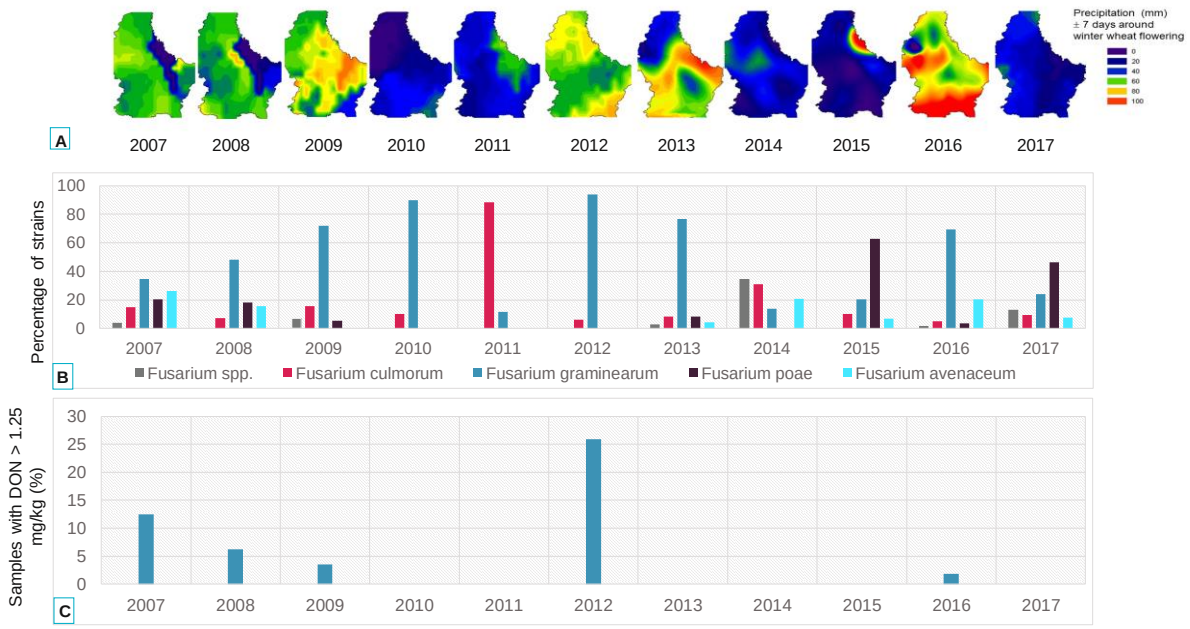
RESULTS

SPECIES AND CHEMOTYPE COMPOSITION

The number of strains isolated each year depended on climatic conditions and disease development. Over the past ten years, from 2007 to 2017, more than 1,250 strains have been isolated and characterized. After molecular identification, four species were mainly found in these ten years: *Fusarium graminearum* (46%), *Fusarium culmorum* (18%), *Fusarium avenaceum* (15%), *Fusarium poae* (17%). Rarer species detected were *F. tricinctum, F. sporotrichioides, F. equisiti and Microdochium nivale*.

Despite the preponderance of *Fusarium graminearum*, there are large disparities from year to year. In 2010 and 2012, *Fusarium graminearum* accounted for 90 and 94% of the isolated strains, respectively, while in 2011, 12% of the isolated strains were *Fusarium graminearum* and 88% were *F. culmorum*. In 2014 and 2015, 14% and 20% of the strains were *F. graminearum*. After a drought period similar to that of 2010/11 in the years 2014/15 (Fig. 1a), *F. poae* strains became the dominating species rather than *F. culmorum* strains. In the wet year 2016, *F. graminearum* once again became the dominating species (Fig. 1B).

Figure 1: Precipitation around the period of winter wheat anthesis (A), *Fusarium* species composition (B) and percentage of raw winter wheat samples with DON > 1.25 mg/kg (C) in Luxembourg between 2007 and 2017. Cumul de précipitations pour la période de +/-7 jours autour de la floraison (A), composition de la population de *Fusarium* (B), et pourcentage d'échantillons contaminés avec du DON au-dessus de la limite réglementaire de 1.25 mg/kg (C) au Luxembourg entre 2007 et 2017.

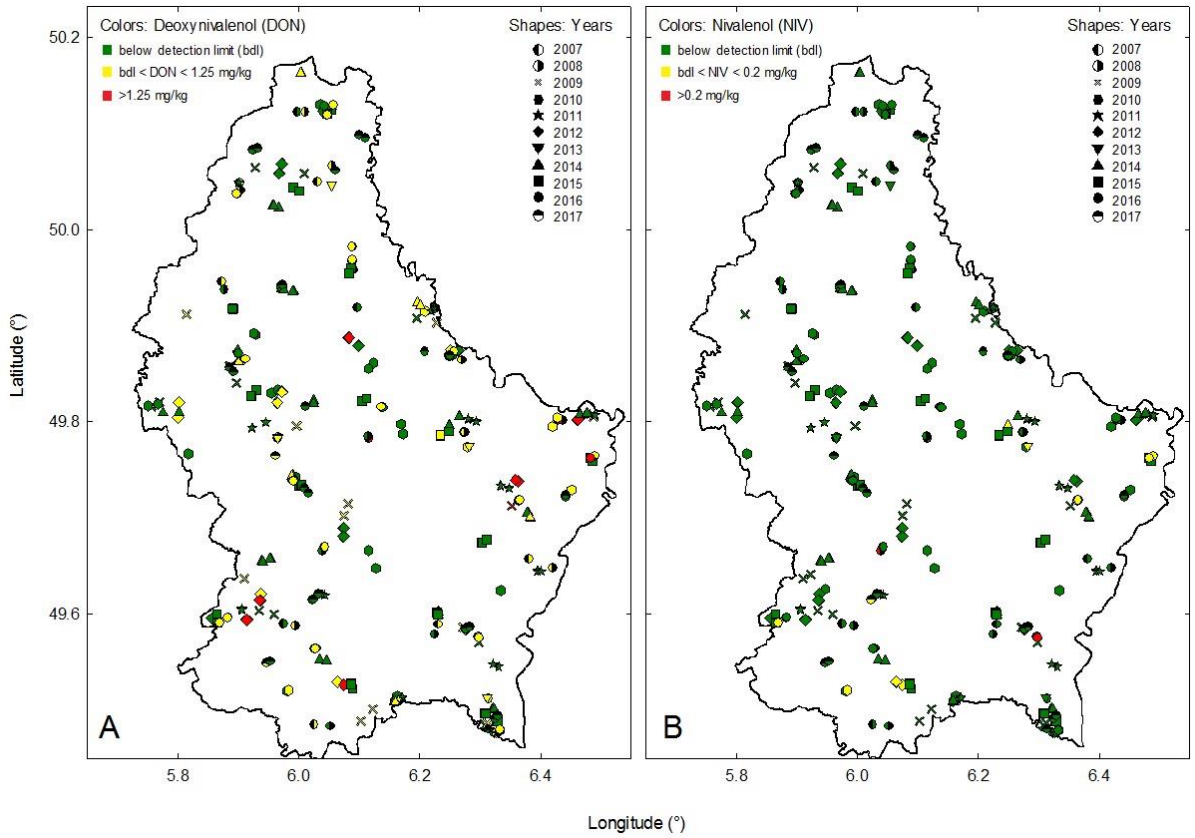


MYCOTOXIN DYNAMICS

Over the ten years of the study, 575 wheat grain samples were analysed. DON, NIV, 3-ADON and ZON were detected in the analysed samples.

Chemical analysis by LC-MS/MS revealed that 28.7% of the samples analysed were contaminated with deoxynivalenol (range 0.73 (limit of detection) - 9247.06 ng/g), 2.6% were contaminated with 3 acetyl deoxynivalenol (range 0.45 (limit of detection) - 408 ng/g), 2.8% were contaminated with nivalenol (range of 0.73 (limit of detection) - 293 ng/g) and 0.7% were contaminated with zearalenone (range of 0.73 (limit of detection) – 232.4 ng/g). DON levels above the EU limit of 1.25 mg/kg in raw grain were found in the years 2007, 2008, 2009, 2012 and 2016, while DON levels were consistently lower in the other years of the monitoring campaign (Fig. 1C). In 3.5% of the samples, DON content was > 1.25 mg/kg.

Figure 2: Deoxynivalenol (DON) content (A) and nivalenol (NIV) content (B) in raw winter wheat samples in Luxembourg between 2007 and 2017. Teneur en deoxynivalenol (DON) et nivalenol (NIV) d'échantillons de blé tendre d'hiver au Luxembourg entre 2007 et 2017.



Samples with high DON levels were more commonly found in the South (Fig. 2A) during years with wet periods around the wheat flowering time (2007, 2008, 2009, 2012 and 2016). NIV levels were rarely > 0.2 mg/kg. NIV levels above the limit of quantification were also found more commonly in the South (Fig. 2B). ZON was detected in 2017 in untreated control fields in one location at concentrations of between 0.73 (limit of detection) and 232.4 ng/g.
High DON and NIV values were loosely clustered in the south-west and east-south-east with a gap in between (Fig. 2). The gap, however, is the location of the capital city where little wheat is grown.

DISCUSSION

Our work aimed to identify potential correlations between the variation in climatic factors and the prevalence of toxins and species in Luxembourgish fields.

The co-occurrence of different Fusarium species in the field has been highlighted (Logrieco et al., 2007). The composition of the species belonging to the Fusarium community is dynamic depending on climatic factors and farming practices (Logrieco et al., 2002; Köhl et al., 2007; Xu et al., 2008). Indeed, several parameters, such as rain during anthesis, tillage, crop rotation, and cultivars are known to influence outbreaks of FHB (Doohan et al., 2003, Beyer et al., 2006, Giraud et al., 2010). Aspects of agronomic practices such as crop rotation, tillage systems or cultivars change slowly and are therefore unlikely to be responsible for the rather drastic reversible annual differences observed in the present study. However, rainfall varies greatly between the years and seems therefore a promising predictor for drastic annual changes in the species' composition and toxin levels.

For this reason, we are interested in the climatic conditions and in particular, the precipitation during the period before/after the flowering to +/- 7 days. 2010, 2011, 2014, 2015 and 2017 saw dry conditions during flowering with a rainfall lower than 50mm. In dry conditions, all *Fusarium* species were inhibited, as evidenced by low disease levels (Beyer et al. 2014) and low toxin levels (Figs 1C, 2). Strain isolation is much more difficult in dry years because the number of infected ears is lower. Nevertheless, the inhibitory effect of drought seems less pronounced for *F. culmorum* (Beyer et al., 2014) and *F. poae* strains than for *F. graminearum* strains.

After molecular identification, it was *Fusarium graminearum* (46%), *Fusarium culmorum* (18%), *Fusarium avenaceum* (15%) and *Fusarium poae* (17%) that were mainly found over these ten years. These results are in line with what has been observed in other European countries, particularly in the north-western part of Europe where *F. graminearum* is the dominant species in warm and wet conditions (Talas et al., 2011; Boutigny et al., 2014; Pasquali et al., 2016).
At the chemotype level, we have shown that the strains of *F. graminearum* of 15-ADON chemotype are more dominant in the population than the NIV chemotype. The distribution between the 3-ADON and NIV chemotypes for *F. culmorum* strains is similar except during the dry years 2014 and 2015, where 3-ADON is predominant.
In terms of overall results over the last ten years, what we have seen is in general agreement with the European data. If we look at the study done in 2016 at the European level, which includes data from Luxembourg but also from other countries, the analysis of the chemotype distribution confirms the dominance of the 15-ADON chemotype in western, southern and central Europe in *F. graminearum*. The 3-ADON chemotype is found in northern Europe. *F. culmorum* of chemotype NIV is more commonly found in western Europe.

Concerning the proportion of contaminated samples per year, we notice that the change in species' composition is not necessarily accompanied by a change in mycotoxin composition. In wet years, deoxynivalenol is the most common mycotoxin linked to the predominance of *F. graminearum*. On the other hand, in a dry year, the disease incidence, and consequently, the rate of mycotoxins is low, whatever the species present.

The reason for the clustering of relatively high DON and NIV levels in Southern Luxembourg is not entirely clear at present. Since one field with pre-crop maize and one field with another pre-crop (usually rapeseed) was selected at each sampling location, the role of previous crops is unlikely to be the cause. From Figure 1A, it can be concluded that the south is not systematically wetter during winter wheat anthesis when compared with the north. Thus, regionally different rainfall around anthesis also seems unlikely as a cause for the elevated frequency of relatively high DON levels in the south. Furthermore, when considering long-term average precipitation between the years 1971 and 2000, the hotspots of high mycotoxin occurrence were not related to the regions with the highest traditional annual precipitation. On the contrary, the mycotoxin hotspot in the east is among the driest regions in the country (Pfister et al. 2013). It may be speculated that the susceptibilities of particular cultivars or fungicide use differed between the north and the south, but at present, evidence for supporting one or the other hypotheses is lacking.

Information on the origin, year of isolation, host plant species and cultivar, and preceding crop is available for most of the fungal strains used for the present study in the Luxembourg Microbial Culture Collection (Piec et al., 2016)

CONCLUSION

In dry years, there is clearly a population change but it is necessary to continue investigations to understand which parameter favours which species.
Moreover, our data suggest that co-contamination with DON and other type B tricothecenes, such as NIV, can easily occur. Since NIV is more toxic than DON (Gutleb et al., 2002) even not yet regulated, it could also be worth regulating the co-contamination of toxins to better protect consumers.

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