Eur J Plant Pathol (2011) 131:59–66
DOI 10.1007/s10658-011-9787-1

Biodiversity of *Fusarium* species in ears and stalks of maize plants in Belgium

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Accepted: 30 March 2011 / Published online: 21 April 2011

Abstract In order to investigate the pre-harvest contamination of maize plants by *Fusarium* species in Belgium, a three-year survey has been performed in five fields in which three hybrids differing in susceptibility to maize stalk rot were sampled at four different physiological stages. An extensive collection of 5,659 *Fusarium* isolates characterized at the species level was established during the 2005, 2006, and 2007 growing seasons, with a total of 23 different *Fusarium* species identified to occur on ears and stalks. A high number of plants was already contaminated by *Fusarium* spp. at the anthesis stage, although no symptoms were visible on ears or on stalks. As the season progressed, the incidence of *Fusarium*-infected maize plants reached 100% in several fields. At the end of the growing season, the most frequently isolated species in maize ears were *F. graminearum*, sometimes associated with *F. avenaceum*, *F. crookwellense*, *F. culmorum*, *F. poae*, and *F. temperatum*, a new species recently described on maize. The predominant *Fusarium* species detected in stalks at the end of the growing season were *F. graminearum* and *F. crookwellense*, often associated with *F. culmorum* and *F. temperatum*. Year-to-year variability observed for the incidence of *F. graminearum* can most likely be associated with differences in climatic conditions among the three years.

Keywords *Fusarium* spp. · Maize · Ear rot · Stalk rot · Multi-year survey

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Introduction

In Belgium, 9.2 million tons of maize plants are harvested annually from 247,000 hectares of farm land, mainly used as livestock feed (Anonymous 2010). However, crop quality is often reduced by ear rot and stalk rot due to a large number of *Fusarium* species, and potential mycotoxin contaminations caused by *Fusarium* species can pose a serious problem for both human and animal health (Wilson et al. 1990; Chu and Li 1994; Desjardins 2006).

Many *Fusarium* species have been associated with ear rot and stalk rot of maize in Europe. Gibberella ear

rot or red ear rot is caused by *Fusarium graminearum* Schwabe (Sutton 1982). Spores reach the silks mostly by splashing, wind dispersal or insect vectors. Infection usually starts from the tip of the ear and develop a reddish mould covering the ear extensively (Munkvold 2003b). *F. graminearum* is widely distributed in Europe and repeatedly associated with many additional *Fusarium* species, among which the most representative are *F. avenaceum* (Fries) Saccardo and *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (Logrieco et al. 2002). Fusarium ear rot or pink ear rot is caused by *F. verticillioides* (Saccardo) Nirenberg and *F. proliferatum* (Matsush.) Nirenberg. Infection by airborne conidia occurs through insect injuries or silks with a random kernel rot phase that develops pink mycelium under wet conditions (Munkvold et al. 1997; White 2000). Fusarium ear rot is mostly observed from southern to central European areas and is sometimes associated with *F. subglutinans* and *F. sporotrichioides* Sherbakoff (Bottalico 1998). The most commonly *Fusarium* species isolated from stalk rot of maize in Europe are *F. graminearum*, *F. culmorum* (W. G. Smith) Saccardo, which predominate in northern areas, and *F. verticillioides*, which is mainly recovered in southern areas. Other species usually associated with maize stalk rot in Europe are *F. subglutinans*, *F. crookwellense* Burgess, Nelson and Toussoun, *F. avenaceum* and *F. equiseti* (Corda) Saccardo (Lew et al. 1997).

Environmental conditions have a significant impact on incidence of ear rot and stalk rot of maize. Regarding climatic conditions, Gibberella ear rot has been associated with wet years while Fusarium ear rot is often associated with dry years (Vigier et al. 1997; Doohan et al. 2003). Geographical situation and cultural practices such as crop rotation, tillage, planting date or fertilization also influence the occurrence and prevalence of all *Fusarium* species (Cotten and Munkvold 1998; Munkvold 2003a). Selection of less susceptible maize hybrids may offer some possibilities to minimize *Fusarium* infestation and associated mycotoxin contamination (Hoenisch and Davis 1994; Ramirez et al. 1996; Warfield and Davis 1996).

However, limited information is available concerning the *Fusarium* spp. ecology from maize fields in Belgium. Such information will help to develop strategies aiming to prevent ear rot and stalk rot, and their related contaminations with mycotoxins. Therefore, the objectives of this research were to investigate the pre-harvest contamination of maize ears and stalks by *Fusarium* species in fields with contrasted cultural practices, in order to: (a) establish an extensive collection of *Fusarium* isolates from maize in Belgium, characterized at the species level; (b) determine the incidence of *Fusarium*-infected plants in maize fields; and (c) analyze the diversity and the distribution of *Fusarium* spp. on ears and stalks during the growing season.

Materials and methods

Samples

Three *Zea mays* L. hybrids, differing in susceptibility to maize stalk rot, were planted in 2005, 2006 and 2007. The hybrid Lukas was rated as less susceptible, Baxxos as susceptible, and Sunday as highly susceptible to maize stalk rot (Renard et al. 2007). Five field plots under different tillage and crop rotations were located in the Brabant Wallon and Hainaut provinces of Belgium. Hybrids were planted in a randomized complete block design in two long-term continuous maize fields, with no-tillage and ploughed plots, in two wheat-maize rotation fields, with no-tillage and ploughed plots, and in one root chicory-maize rotation ploughed field. Maize kernels were sowed mechanically with 75 cm distance between the rows on 19–24 May 2005, 11–12 May 2006, and 27 April-3 May 2007. For each of the three block replicates, two 8-m rows per hybrid were dedicated for samplings and two 8-m rows per hybrid for harvest parameters evaluation.

The ear and the stalk of five maize plants were randomly sampled at four different physiological stages: R1-anthesis stage, R4-dough stage, R5-dent stage, and R6-maturity stage (Ritchie and Hanway 1982). Each year, a total of 900 ears (5 plants, 4 growing stages, 3 block replicates, 5 field plots, and 3 hybrids) and 900 stalks samples were analyzed for detection and identification of *Fusarium* species.

Isolation of *Fusarium* spp.

Three 5-mm stalk sections were collected at 5 cm, 10 cm, and 15 cm from the first internode above the brace roots of each stalk sample and a total of 9 kernels were selected at random from the bottom, the

middle and the top of each ear sample. Stalk sections and kernels were surface treated in 0.5% sodium hypochlorite for 2 min, rinsed extensively with sterilized water, drained, and placed by direct plating technique on chloramphenicol water agar 3% (0.2 g of chloramphenicol, and 30 g of agar l^{-1}), used for fungal isolation. In addition, for the R1-anthesis stage and the R4-dough stage, all maize silks of each collected ear were ground in buffered peptone water (10 g of peptone, 5 g of NaCl, 9 g of $Na_2HPO_4.12H_2O$, and 1.5 g of KH_2PO_4 l^{-1}, pH 7) and *Fusarium* species were screened by observation of 3 dilutions plating on a rich medium chloramphenicol malt extract agar 2% (20 g of malt extract, 0.05 g of chloramphenicol, and 15 g of agar l^{-1}). Petri dishes were maintained at room temperature (20–22°C) until *Fusarium* colonies were transferred to potato dextrose agar (PDA; Sharlau, Spain) for observation of the typical characters of colonies and macroconidia, and incubated at room temperature for 10 days. Sub-cultured monoconidial strains were cryopreserved and maintained in tubes on SNA (Leslie and Summerell 2006) under mineral sterile oil at the BCCMTM/MUCL collection. Information on the collecting dates, the GPS data and the exact sample position on the maize plant were recorded.

Identification of *Fusarium* strains

Cultural characters of *Fusarium* species were assessed morphologically by examination using a stereomicroscope (Olympus SZ60, Japan) and a light microscope (Nikon Optiphot, Japan), with references to Leslie and Summerell (2006) and Nelson et al. (1983). For assessment of the morphological identifications, isolates representative of the various morphotypes observed along the three years were identified molecularly by elongation factor (*EF-1α*) gene sequencing, known as one of the most pertinent gene for determining the species rank in the *Fusarium* genus (O'Donnell et al. 1998).

Genomic DNA preparation was performed as previously described (Scauflaire et al. 2010). Briefly, fungal isolates were grown in liquid culture in the dark at 25°C for 5 days, harvested mycelium was lyophilized and fungal DNA was extracted and purified using the Invisorb Spin Plant MiniKit (Invitek GmbH, Germany) according to the manufacturer's recommendations.

For gene sequencing, amplification of the *EF-1α* gene strains was carried out with PCR primers EF1 and EF2 and by using the amplification conditions of O'Donnell et al. (1998). The PCR products were sequenced in both directions in a 3,100 Genetic Analyser (Applied Biosystems, USA). Sequences were edited with Sequencher version 4.8 (Gene Codes Corporation, USA) and the most related sequence was obtained using GenBank Blast (NCBI-National Centre for Biotechnology Information).

Statistical analysis

Analysis of variance (ANOVA) for a randomized complete block design was conducted to assess the significance of parts of the plants, block replicates, years, and their interaction effects on *Fusarium* spp. colonization rates using XLSTAT 2008 version 7.01 (Addinsoft Inc., USA). The low number of samples per block replicate did not allow enough statistical accuracy to test the hybrid effect on the rate of infected plants and the field plot effect. Therefore, average infection percentages were pooled, based on 225 ears (5 plants, 3 block replicates, 5 field plots, and 3 hybrids) and on 225 stalks for each growing stage. Means of *Fusarium* spp. colonization rates were then compared using Chi-square statistics.

Results

Collection of *Fusarium* isolates

A total of 5,659 isolates was collected by sampling maize fields in the two provinces of Belgium during the 2005, 2006, and 2007 growing seasons. Twenty-three *Fusarium* species were collected and identified during this three-year survey of which *F. graminearum* (42.8%), *F. crookwellense* (16%), *F. avenaceum* (14.4%), *F. culmorum* (10.3%) and *F. temperatum* (4.9%) were the most abundant. The other *Fusarium* isolates of the collection are represented by *F. equiseti* (2.1%), *F. arthrosporioides* (1.8%), *F. poae* (1.4%), *F. heterosporum* (1.3%), *F. proliferatum* (1.1%), *F. tricinctum* (0.7%), *F. venenatum* (0.6%), *F. redolens* (0.6%), *F. oxysporum* (0.5%), *F. verticillioides* (0.4%), *F. sporotrichioides* (0.4%), *F. torulosum* (0.2%), *F. subglutinans* (0.2%), *F. lateritium* (0.1%), *F. sambucinum* (0.1%), one strain of *F. ramigenum*,

one strain of *F. flocciferum*, and one strain of *F. solani*. Molecular identification using gene sequencing of 1,805 selected isolates was in agreement with the morphological identification.

Incidence of *Fusarium*-infected maize plants

Over the three years, more than 62% of maize plants were already contaminated at the R1-anthesis stage, although no symptoms were visible on ears or on stalks yet. At the R6-maturity stage, up to 100% of maize plants were infected in 2005, in the long-term continuous maize fields without tillage and in the two wheat-maize rotation fields with and without tillage. Overall, more stalks than ears were infected by *Fusarium* spp., whatever the growing stage or the year. For the three years, the frequency of ears infected by *Fusarium* spp. significantly increased during the growing season (2005: df=3, F=25.4, P<0.001; 2006: df=3, F=13, P<0.001; 2007: df=3, F=24.8, P<0.001), from 15% at the R1-anthesis stage to 84% at the R6-maturity stage (Fig. 1a). Concerning stalk infection, the high level of *Fusarium* spp. colonization at the R1-anthesis stage ranged from 60% to 80% and *Fusarium*-infected stalks were collected with similar frequencies during the growing season, until the R6-maturity stage (Fig. 1b).

Diversity of *Fusarium* spp. on ears and silks

Twenty-two *Fusarium* species were isolated and some of them were identified as causal agents of maize ear rot. The order of prevalence of *Fusarium* spp. in ears throughout the growing season was very similar for the three years (Fig. 2). At the R1-anthesis stage, *F. graminearum* and *F. avenaceum* were the predominant species, although they were isolated from less than 15% of maize ears. With an increasing frequency along each season, *F. graminearum* was by far the most frequent species isolated from ears at the R6-maturity stage (2005: df=3, F=38.5, P<0.001; 2006: df=3, F=17.8, P<0.001; 2007: df=3, F=15.4, P<0.001). Incidences of all other species isolated in ears were constant along the growing seasons. *F. avenaceum*, *F. crookwellense*, *F. culmorum*, *F. temperatum*, and *F. poae* were detected in up to 15% of the maize ears. *F. arthrosporioides*, *F. proliferatum*, *F. tricinctum*, *F. equiseti*, *F. venenatum*, *F. verticillioides*, *F. heterosporum*, *F. oxysporum*, *F. torulosum*, *F. redolens*, *F. subglutinans*, *F. sporotrichioides*, *F. sambucinum*, and *F. lateritium* were infrequently encountered, at less than 5% in maize ears. One isolate of *F. ramigenum* and one isolate of *F. solani* were sampled from a maize ear in 2005 and 2006, respectively. *F. flocciferum* was never isolated from maize ears. As the season progressed, the number of species detected on individual ears significantly increased up to four (2005: df=3, F=79.1, P<0.001; 2006: df=3, F=28.9, P<0.001; 2007: df=3, F=79.2, P<0.001). Furthermore, *F. graminearum* was always observed among these four species, the most frequently in association with *F. avenaceum*, *F. crookwellense*, and *F. culmorum*, sometimes with *F. temperatum* and *F. poae*.

Concerning *Fusarium* species detected in maize silks at the R1-anthesis and R4-dough stages, *F. graminearum* and *F. avenaceum* were the predominant species along each season. For example, more than 30% of the ears had their silks infected by *F. avenaceum* at the beginning of the season 2006. During the R4-dough stage 2007, up to 40% of the ears had their silks infected by *F. graminearum*. *F.*

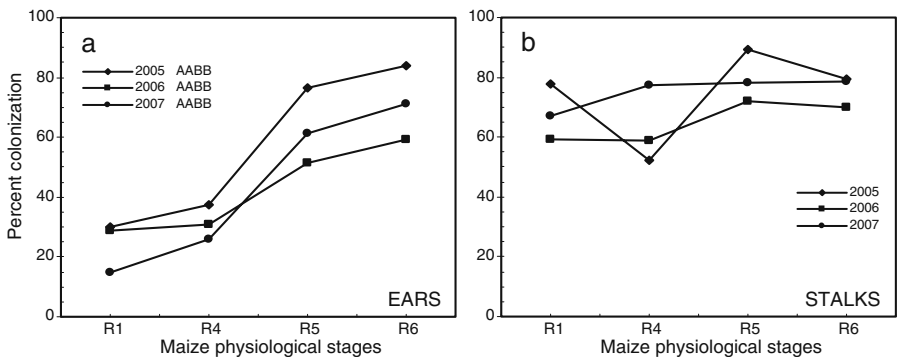

Fig. 1 Incidence of *Fusarium* species from 2005 to 2007: **a**, in ears and **b**, in stalks. For each year, colonization rates differing statistically between the maize physiological stages are followed by different letters mentioned in the legend (Newman-Keuls, 5% level). No letter when no statistical difference

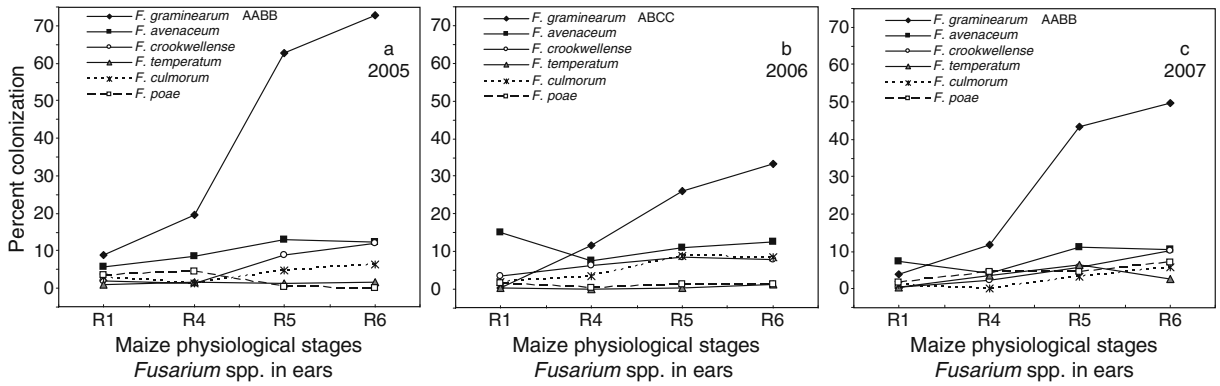

Fig. 2 Incidence of *F. graminearum, F. avenaceum, F. crookwellense, F. temperatum, F. culmorum,* and *F. poae* in ears: **a**, in 2005; **b**, in 2006; and **c**, in 2007. For each species, colonization rates differing statistically between the maize physiological stages are followed by different letters mentioned in the legend (Newman-Keuls, 5% level). No letter when no statistical difference

subglutinans, *F. flocciferum* and *F. solani* were never observed in silks. Incidences of the 18 other *Fusarium* species were low, at less than 5% of the ears.

Diversity of *Fusarium* spp. on stalks

A total of twenty-one *Fusarium* species occurred in infected stalks during the three-year survey. Compared to ears, the prevalence of the different species detected and their evolution along the growing season were more variable. At the R1-anthesis stage, the incidence of *F. graminearum*, *F. avenaceum*, *F culmorum*, *F. equiseti*, and *F. crookwellense* was greater than 10% of the maize stalks examined, regardless of the year (Fig. 3). The detection frequency of *F. graminearum* isolated from stalks increased significantly during the growing seasons 2005 and 2006 (2005: df=3, F=6.3, P<0.01; 2006: df=3, F=6.3, P<0.01; 2007: df=3, F=1.1, P>0.05), while incidence of *F. crookwellense* increased significantly in stalks for the three years (2005: df=3, F=10.4, P<0.001; 2006: df=3, F=4.6, P<0.05; 2007: df=3, F=3.7, P<0.05). At the R6-maturity stage, *F. graminearum* was detected in up to 65% of the stalk samples in 2005 and *F. crookwellense* was detected in up to 44% of the stalks in 2007. In addition, *F. culmorum*, and *F. temperatum* were detected in up to 18% of the maize stalks. *F. arthrosporioides*, *F. proliferatum*, *F. redolens*, *F. venenatum*, *F. oxysporum*, *F. heterosporum*, *F. verticillioides*, *F. tricinctum*, *F. torulosum*, *F. sporotrichioides*, *F. poae*, *F. subglutinans*, and *F. sambucinum* occurred less frequently in stalks at the R6-maturity stage. One

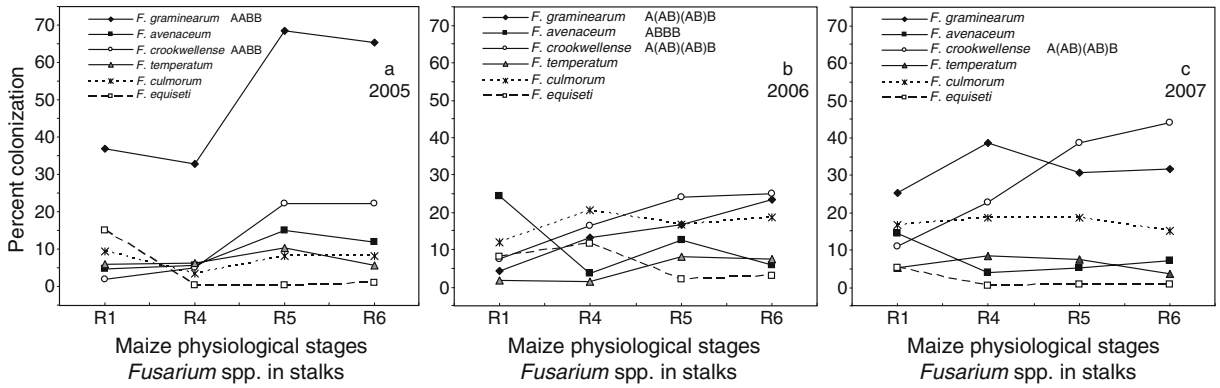

Fig. 3 Incidence of *F. graminearum, F. avenaceum, F. crookwellense, F. temperatum, F. culmorum,* and *F. equiseti* in stalks: **a**, in 2005; **b**, in 2006; and **c**, in 2007. For each species, colonization rates differing statistically between the maize physiological stages are followed by different letters mentioned in the legend (Newman-Keuls, 5% level). No letter when no statistical difference

isolate of *F. lateritium* and one isolate of *F. flocciferum* were recovered from two different maize stalks in 2005. *F. ramigenum* was not recovered from stalks.

Each year, more than 20% of the maize stalks were infected simultaneously by up to four different *Fusarium* species, whatever the growing stage. In contrast to the situation on ears, *F. graminearum* was not always observed in these multiple infections. Nevertheless, the most frequent species association was represented by *F. graminearum*-*F. crookwellense*-*F. culmorum*.

Species distribution on the plant

Statistically, three main results were obtained. Firstly, each year, *F. graminearum* was more often found in stalks than in ears during the R1-anthesis stage and the R4-dough stage, whereas it was found more frequently in ears than in stalks during R5-dent stage and the R6-maturity stage. Secondly, *F. crookwellense*, *F. temperatum*, *F. culmorum*, and *F. equiseti* were recovered with significantly higher percentages on stalks than on ears, whatever the growing stage and the year. Thirdly, there were no significant differences between incidence of ear and stalk infections for the other *Fusarium* species encountered during this three-year survey. Nevertheless, there was a trend that each year, *F. avenaceum* and *F. poae* were detected more frequently in ears than in stalks, whereas *F. venenatum*, *F. oxysporum*, and *F. subglutinans* infected more stalks than ears.

Discussion

Ear rot and stalk rot are two major diseases of maize and are responsible for severe crop damage and mycotoxin contaminations (Charmley et al. 1995). Both diseases are caused by a complex of *Fusarium* species (Kommedahl et al. 1979). In the present study, we have demonstrated that maize grown in the southern part of Belgium hosted a wide range of *Fusarium* species and we have analyzed the diversity and the distribution of the *Fusarium* spp. on ears and stalks during the growing season. An extensive collection of *Fusarium* isolates from maize in Belgium was set up, and strains were characterized at the species level.

During the 2005, 2006, and 2007 growing seasons, twenty-three *Fusarium* species, which are represented by 5,659 isolates, were identified from ears and stalks of three maize hybrids grown in five fields under different tillage and crop rotations in Belgium. Stalk rot symptoms recorded at the end of the growing seasons showed expected differences of susceptibility among the Lukas, Baxxos, and Sunday hybrids (data not shown), although raw data analyses showed no differences between these hybrids when considering the number of plants colonized by the different *Fusarium* species. This underlines the ability of the different hybrids to express their reduced susceptibility to *Fusarium* development and, subsequently, to limit the rot symptoms development. Furthermore, in several fields where the three hybrids presented high incidences of *Fusarium*-infected plants, the final level of stalk rot symptoms was surprisingly low. Correlations between rot diseases and *Fusarium* spp. infection are not always clear cut, suggesting that final ear and stalk rots are not only dependant of the level of resistance to *Fusarium* spp., but also by stress subsequently imposed on the plant (Munkvold 2003a).

Year-to-year variability was mostly observed for the incidence of *F. graminearum*, which might be easily explained by differences in climatic conditions among the three years. Indeed, the growing season 2005 was characterized by moderate precipitations and warm temperatures from the early stages of maize development until the end of anthesis, while drought and very high temperatures occurred from end of June to end of July in 2006. In 2007, moderate temperatures and prevalent rainfalls during the entire plant development occurred. According to Doohan et al. (2003), high rates of infected ears and stalks by *F. graminearum* were observed when warm and moderately wet weather appeared at the anthesis stage. These optimal climatic conditions occurred in 2005 and to a lesser extent in 2007.

In Belgium, the most frequently encountered species in maize ears were very similar to that found in North/Center Europe according to previous studies (Bottalico 1998). At the end of the growing season, the most frequently species in ears was *F. graminearum*, sometimes associated with *F. avenaceum*, *F. crookwellense*, *F. culmorum*, *F. temperatum*, and *F. poae*. In 2005, 70% of the *F. graminearum*-infected ears presenting rot symptoms were not colonized by any other *Fusarium* spp., confirming its ability to infect the ear and cause disease symptoms without any association (Reid et al. 1999; Carter et al. 2002).

Recently, *F. temperatum* Scauflaire and Munaut was described as a new biological species closely related to *F. subglutinans*. In Belgian fields, the *F. temperatum* : *F. subglutinans* ratio was very high (276:9), suggesting that *F. temperatum* competes with *F. subglutinans* (Scauflaire et al. 2010).

This study also demonstrated a high incidence of *Fusarium* species in the first internode-stalk. Since these contaminated internodes usually remain in the field, they will represent a threatening inoculum source for the following crop (Xu 2003; Maiorano et al. 2008). Furthermore, systemic infection suggested for various *Fusarium* species (Kedera et al. 1994; di Menna et al. 1997; Wagacha and Muthomi 2007) could lead to infection of the upper internodes and to subsequent silage toxic contaminations. Although the level of *Fusarium* spp. colonization in the bottom of the stalks ranged from 60% to 80% at the R6-maturity stage (Fig. 1b), no lodging was observed in the fields during the 3-year survey. Concerning *Fusarium* species detected in stalks at the end of the growing season, *F. graminearum* and *F. crookwellense* were the predominant species, often associated with *F. culmorum* and *F. temperatum*. In 2007, *F. crookwellense* was the major species isolated in stalks for the two last growing stages. Surprisingly, more than half of these *F. crookwellense*-infected stalks presenting rot symptoms were not colonized by any other *Fusarium* species, although *F. crookwellense* is usually not considered in literature as closely associated with disease symptoms in this host (Leslie and Summerell 2006). It suggests that *F. crookwellense* could represent an emergent pathogen in maize culture.

Most of the 23 *Fusarium* species identified in this study are able to produce a wide range of harmful metabolites that have a strong impact on the health of animals and humans (Logrieco et al. 2002; Leslie and Summerell 2006). Regarding the order of prevalence of *Fusarium* spp. in the fields throughout the three growing seasons, the following major associated mycotoxins might be produced : type B trichothecenes (produced by *F. graminearum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, and *F. poae*), zearalenones (produced by *F. graminearum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, and *F. sporotrichioides*), fusarins (produced by *F. graminearum*, *F. crookwellense*, *F. culmorum*, *F. avenaceum*, *F. poae*, *F. proliferatum*, *F. tricinctum*, and *F. verticillioides*), moniliformin (produced by *F. avenaceum*, *F. culmorum*, *F. temperatum*, *F. proliferatum*, *F. tricinctum*, *F. oxysporum*, *F. sporotrichioides*, and *F. subglutinans*), beauvericin (produced by *F. avenaceum*, *F. temperatum*, *F. poae*, *F. proliferatum*, *F. oxysporum*, and *F. sambucinum*), enniatins (produced by *F. avenaceum*, *F. temperatum*, *F. tricinctum*, *F. oxysporum*, *F. torulosum*, *F. lateritium*, and *F. sambucinum*), and type A trichothecenes (*F. equiseti*, *F. poae*, *F. venenatum*, *F. sporotrichioides*, and *F. sambucinum*). According to this study, fumonisin producers (*F. proliferatum* and *F. verticillioides*) occurred less frequently in Belgian fields, compared to other European countries (Brygoo and Gautier 2007; Goertz et al. 2008; Dorn et al. 2009).

In conclusion, results of this 3-year investigation revealed a wide range of *Fusarium* spp. present in maize fields in Belgium. Furthermore, most of them have already been detected on plants from the R1-anthesis stage. This raises the question of a possible underestimated contribution of several species, e.g. *F. temperatum* and *F. crookwellense*, to the final ear rot or stalk rot symptoms observed on plants at harvest. Currently we are testing the virulence of those *Fusarium* species and we are analyzing their mycotoxin profiles to allow a better understanding of their involvement in the pathogenic complex.

Acknowledgments This research was founded by the Direction Générale de l'Agriculture, Direction de la Recherche, ref. 1217, in the framework of the project *Caractérisation et dynamique des fusarioses sur maïs en Région Wallonne*. We thank the CIPF and the CARAH for providing the fields for the research and for scientific and technical assistance. We are grateful to the Mycothèque de l'Université catholique de Louvain (BCCM™/MUCL) for scientific support.

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