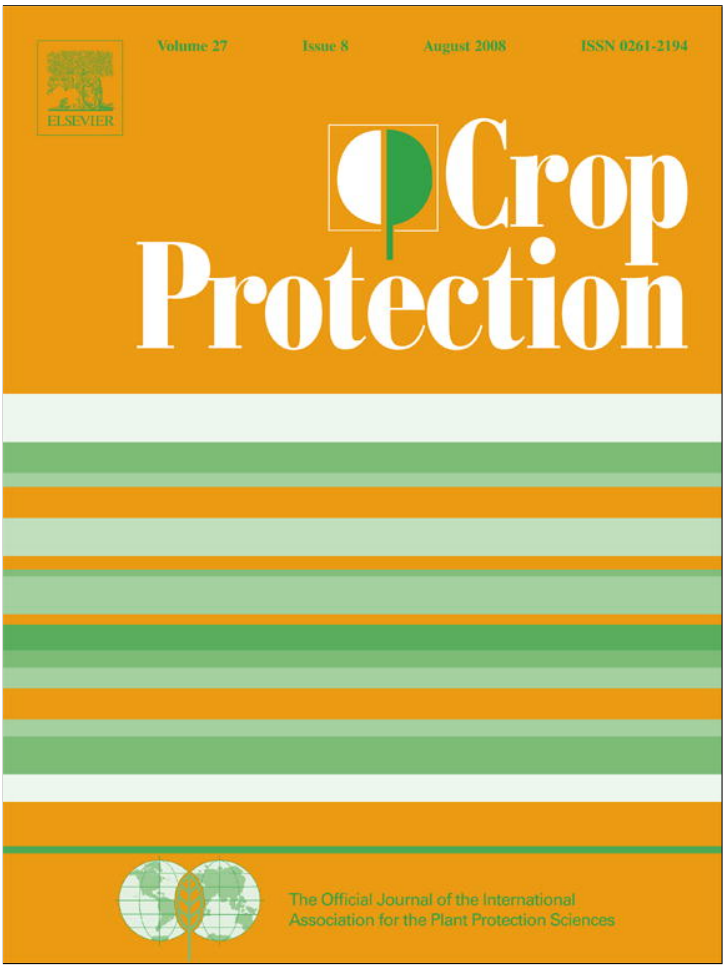

Crop Protection 27 (2008) 1215–1219

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

The occurrence of *Fusarium* species and mycotoxins in Kenyan wheat

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ARTICLE INFO

Article history:
Received 5 January 2007
Received in revised form
5 March 2008
Accepted 5 March 2008

Keywords:
Fusarium
Mycotoxins
Wheat
Head blight

ABSTRACT

Freshly harvested wheat grain samples were collected during the 2004 growing season to determine the presence of head blight-causing *Fusarium* species. Fungal contamination was determined by isolation on agar media, while mycotoxin analysis was by direct competitive enzyme-linked immunosorbent assay (ELISA). The wheat grain samples were highly contaminated with fungi, especially *Epicoccum*, *Alternaria* and *Fusarium* species. The mean *Fusarium* infection rate varied from 13% to 18%, with the major head blight-causing species being *Fusarium poae*, *Fusarium graminearum*, *Fusarium equiseti* and *Fusarium avenaceum*. *F. graminearum* isolates were found to be highly virulent (79% disease severity) and significantly reduced kernel weight. Most grain samples were contaminated with mycotoxins, with a mean incidence rate of up to 75% for deoxynivalenol (DON) and 86% for T-2 toxin. Other mycotoxins detected were zearalenone and aflatoxin B1. Co-occurrence of DON, T-2 toxin and zearalenone was found in up to 35% of the samples. The results suggested the presence of *Fusarium* head blight and associated mycotoxins in Kenya. The presence of several mycotoxins, even at such low levels, could pose chronic adverse health effects to human and livestock fed on the contaminated wheat products.

1. Introduction

Fusarium head blight (FHB) has recently re-emerged as a devastating disease of wheat and other small-grain cereals throughout the world (McMullen et al., 1997; Windels, 2000). The significance of the disease in wheat production is attributed to both yield reduction and mycotoxin contamination of the grain harvested from the infected ears. Yield losses from FHB are due to sterility of the florets and formation of shrivelled, lightweight kernels. The disease is caused by different *Fusarium* species, including *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, *F. cerealis*, *F. sporotrichioides* and *Microdochium nivale* (Parry et al., 1995). *F. graminearum* and *F. culmorum* are the most virulent species. All the *Fusarium* species that infect cereals are capable of surviving saprophytically on crop debris (Parry et al., 1995; Jones, 2000). The most favorable conditions for infection are prolonged periods (48–72 h) of high humidity and warm temperatures (25–30 °C). Therefore, a combination of abundant inoculum, prolonged or repeated wet periods during flowering through kernel development and very susceptible cultivars lead to severe yield and quality loss (Obst et al., 1997).

Mycotoxins associated with grain affected by FHB include trichothecenes (deoxynivalenol (DON), nivalenol, T-2 toxin, HT-2 toxin) and zearalenone (Park et al., 1996; Doohan et al., 2003; Llorens et al., 2006). The main trichothecene-producing species are *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. poae* and *Fusarium crookwellense* (Marasas et al., 1984). The occurrence, amount and kind of mycotoxin is dependent on the environment, species of the fungus present, severity of infection and the cultivar or kind of crop (McMullen and Stack, 1999; Mentewab et al., 2000; Doohan et al., 2003).

Management strategies for FHB include crop rotation, seed treatment, planting different cultivars, fungicide application, appropriate use of fertilizers, irrigation, weed control, proper land preparation and timely harvesting (McMullen and Stack, 1999; Mathies and Buchenauer, 2000; Haidukowski et al., 2004). Use of fungicides in the management of FHB has been at most 77% and 89% effective in the reduction of disease severity and mycotoxin content, respectively (Haidukowski et al., 2004). Adjusting the combine to blow out the small, shrivelled kernels can help reduce mycotoxin levels. Currently there are no wheat cultivars with complete resistance to FHB, although some cultivars have significant levels of partial resistance that limit yield loss and mycotoxin accumulation (Pereyra and Dill-Macky, 2004; Wisniewska and Kowalczyk, 2005).

Harvested wheat grain from Kenya has been previously shown to be contaminated with mycotoxin-producing *Fusarium* species (Muthomi and Mutitu, 2003). However, mycotoxin levels of the naturally infected wheat grain have not been determined. Therefore, this study was carried out to determine the level of *Fusarium*

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0261-2194/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cropro.2008.03.001

contamination and associated mycotoxins in freshly harvested wheat from Nakuru and Nyandarua districts of Kenya.

2. Materials and methods

2.1. Survey and mycological analysis

A survey was carried out during the 2004 harvesting season in the wheat-growing districts of Nakuru and Nyandarua, Kenya. Five agroecological zones in Nakuru and four agroecological zones in Nyandarua were selected. The agroecological zones selected were upper highland 2 (UH2—very long cropping season), upper highland 3 (UH3—long cropping season), upper highland 4 (UH4—low rainfall and frost), lower highland 2 (LH2—long cropping season), lower highland 3 (LH3—long cropping season), lower highland 4 (LH4—short to medium cropping season) and upper midland 4 (UM4—medium cropping season) (Jaetzold and Schmidt, 1983). In each agroecological zone, 10 farms were randomly selected, giving a total of 89 farms. Wheat grain samples (1–2 kg per farmer) were collected for mycological and toxin analysis. The samples were stored at 4 °C until analyzed. Each sample was thoroughly mixed and a 100 g sub-sample taken randomly for mycological analysis. The seeds were surface sterilized in 5% sodium hypochlorite containing four drops of Tween 20 for 3 min, and then rinsed three times with distilled water. The kernels were plated on petri plates containing low strength potato dextrose agar (PDA) amended with mineral salts (0.005 g $CuSO_4 \cdot 5H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$) and antimicrobial agents (0.05 g chloramphenicol, 50 mg penicillin, 50 mg tetracycline, 50 mg streptomycin) to suppress growth of fast-growing fungi and bacteria (Muthomi, 2001). A total of 100 seeds were plated per sample. The plates were incubated at room temperature 20 ± 5 °C for 7–14 d, after which kernels showing fungal infection were recorded, and the different fungal colony types determined. The fungal genera growing on the kernels were identified based on cultural and morphological characteristics. The *Fusarium* isolates were identified to species level based on synoptic keys by Nelson et al. (1983). *Fusarium* colonies were subcultured on both synthetic nutrient agar (Nirenberg, 1981) and PDA. The cultures were incubated under near-UV light for 14–21 d to induce sporulation.

2.2. Mycotoxin analysis

Mycotoxin content in the wheat grain was determined by direct competitive enzyme-linked immunosorbent assay (ELISA) (Association of Official Analytical Chemists (A.O.A.C), 1995). The antibodies were supplied by the Department of Veterinary Pathology, Microbiology and Parasitology, University of Nairobi. A total of 80 samples were analyzed for DON, T-2 toxin, zearalenone, and 41 samples for aflatoxin B1. Each sample was homogenized and 100 g ground to a fine powder. Five grams of the ground sample was extracted with 25 ml of methanol:water (50:50v/v) for aflatoxin, zearalenone and DON and 70:20 (v/v) for T-2 toxin. The extract was de-fatted with 10 ml hexane, and 4 ml of the methanolic layer was diluted to 10% using phosphate buffer solution. For T-2 toxin, the methanolic extract was diluted with an equal volume of distilled water. The 96-well microtitre polystyrene (Maxisorp®, Nunc, Denmark) plates were coated with 100 μl of anti-aflatoxin antiserum K147 (Gathumbi et al., 2001) for aflatoxin B1, anti-DON antiserum DON143/16 (Usleber et al., 1992) for DON and anti-serum ZEA A37 (Usleber et al., 1992) for zearalenone in bicarbonate buffer (pH 9.6) per well. For T-2 toxin, a commercial kit (Ridascreen, r-Biopharm, Germany) was used and the ELISA procedure performed following the manufacturer's recommendations. Absorbance was determined using the spectrophotometer Elisa reader (Uniskan II, Finland) at 450 nm. A calibration curve for the standards for each toxin dilution was plotted using $\log_{10}$ of standards concentration against the percentage inhibition of the standards.

2.3. Virulence of Fusarium species

Twenty isolates of different *Fusarium* species isolated from wheat kernels were inoculated onto spikes of 'Mbuni', a highly susceptible wheat cultivar (Muthomi et al., 2002) under greenhouse conditions. Each isolate was cultured separately at room temperature (22 ± 5 °C) on mung bean medium (Bai and Shaner, 1996) for 14 d. Conidial suspensions of each isolate was harvested and adjusted to 5×10^5 conidia ml^{-1} by counting the conidia using a hemocytometer and diluting them to the required concentration with sterile distilled water. Three drops (0.01%) of Tween 20 were added to ensure uniform conidia dispersion. Wheat ears were inoculated at 50% flowering (GS65, Zadoks et al., 1974) by spraying with a hand sprayer, exposing all spikelets to the inoculum. Controls were treated similarly with distilled water. After inoculation, the ears were incubated under polythene bags for 48 h to ensure high relative humidity for optimal infection. Each isolate was inoculated separately and replicated four times. Head blight severity was visually assessed using a 1–9 scale (Miedaner et al., 1996) as the proportion of bleached spikelets after every 5 d on 10 average sized ears per replicate until physiological maturity (GS92). Mean head blight severity and the area under disease progress curve (AUDPC; Shaner and Finney, 1977) were calculated from the disease severity data. The ears from each replicate of each isolate were harvested separately and threshed to determine grain weight.

2.4. Data analysis

Data were subjected to analysis of variance (ANOVA) using the PROC ANOVA procedure of Genstat (Lawes Agricultural Trust Rothamsted Experimental Station, 1998, version 8). Where a treatment effect was significant, pair-wise treatment mean differences were determined by Tukey least significant difference test at 95% confidence limit.

3. Results

The major fungal genera isolated from wheat grain samples were *Epicoccum*, *Alternaria*, *Fusarium*, *Aspergillus* and *Penicillium* (Table 1(a) and (b)). The mean kernel infection rate was 98.4%, but the level of contamination varied in different agro-ecological zones. The highest total *Fusarium* infection was observed in Nyandarua district where rainfall was higher and better distributed than in Nakuru district (Table 2). Agro-ecological zone LH4 had the highest *Fusarium* infection rates. However, agro-ecological zones with high *Epicoccum* and *Alternaria* infection rates had lower levels of *Fusarium* in both districts. *Fusarium* species isolated were *F. poae*, *F. chlamydosporum*, *F. oxysporum*, *F. graminearum*, *F. equiseti*, *F. verticillioides*, *F. avenaceum*, *F. semitectum*, *F. cerealis*, *F. lateratium*, *F. sporotrichioides*, *F. scirpi*, *F. sambucinum* and *F. solani* (Table 3). *Fusarium poae*, *F. chlamydosporum* and *F. oxysporum* were the most prevalent *Fusarium* spp. in all the agro-ecological zones, while *F. graminearum* was isolated in six out of the nine zones. The least prevalent species were *F. semitectum*, *F. scirpi*, *F. solani* and *F. sporotrichioides*.

Table 1
Proportion (%) of fungi isolated from wheat kernels from different agro-ecological zones

	Fusarium	*Epicoccum*	*Alternaria*	*Aspergillus*	*Penicillium*
(a) *Nakuru district, Kenya, during the 2004 cropping season*					
LH4	18.8_a	17.6_e	62.2_a	2.0_d	0_b
LH3	16.1_b	29.2_d	48.8_d	5.6_a	0.3_a
LH2	11.6_c	31.0_b	54.5_c	2.7_c	0.1_b
UM4	9.0_d	30.1_c	58.6_b	2.2_d	0.1_b
UH2	9.0_d	58.7_a	27.3_e	5.0_b	0_b
Mean	12.9	33.32	50.29	3.49	0.09
LSD	0.32	0.35	0.33	0.27	0.12
(b) *Nyandarua district, Kenya, during the 2004 cropping season*					
LH3	15.3_c	16.2_d	42.2_a	10.9_a	14.6_a
LH4	24.3_a	29.7_c	41.3_b	4.2_d	0.3_d
UH3	14.1_d	53.3_a	22.9_d	7.6_c	2.0_b
UH4	18.4_b	36.2_b	34.4_c	10.6_b	0.4_c
Mean	18.02	33.83	35.16	8.35	4.32
LSD	0.32	0.35	0.33	0.27	0.12

Values followed by different letters within columns are significantly different ($P \leqslant 0.05$).
UH2—upper highland 2; UH3—upper highland 3; UH4—upper highland 4; LH2—lower highland 2; LH3—lower highland 3; LH4—lower highland 4; UM4—upper midland 4.

Table 2
Rainfall amounts and number of rainfall days for Nakuru and Nyandarua districts during the 2004 growing season

	Nakuru		Nyandarua	
	Amount (mm)	Number of rainy days	Amount (mm)	Number of rainy days
January	129.5	5	118.5	13
February	15.5	4	22.9	8
March	53	3	80.5	6
April	209.5	20	185.4	20
May	113	9	182.8	18
June	60.4	4	44.5	7
July	92	14	146	14
August	119.9	17	94.3	17
September	12.3	5	55	12
October	48.3	13	71.9	18
November	116.6	15	63.1	17
December	91.2	11	51.2	6
Total	1061.2	120	1116.1	156

The incidence and levels of mycotoxins varied in samples from different agro-ecological zones. The most prevalent was T-2 toxin, followed by DON, zearalenone and aflatoxin B1 (Table 4). However, DON was detected at concentrations of up to 302 $\mu g\,kg^{-1}$, while aflatoxin B1 was only detected at low levels to a maximum of 6.9 $\mu g\,kg^{-1}$. Co-occurrence of DON, zearalenone and T-2 toxin occurred in 35% of the samples analyzed. Samples from Nakuru district had higher mean levels of DON (132.65 $\mu g\,kg^{-1}$), while samples from Nyandarua contained higher mean levels of T-2 toxin (29.8 $\mu g\,kg^{-1}$) and zearalenone (7.1 $\mu g\,kg^{-1}$). High variation in mycotoxin content was also observed among wheat grain samples from different agro-ecological zones in both districts. Samples with a high total *Fusarium* infection contained more DON and T-2 toxin.

The various *Fusarium* species differed significantly ($p \leqslant 0.05$) in the severity of head blight symptoms they induced, AUDPC and corresponding kernel weight (Table 5). Based on these three attributes, the isolates of the different *Fusarium* species could be grouped in four distinct categories: highly virulent (*F. graminearum*), moderately virulent (*F. avenaceum*, *F. verticillioides*, *F. poae* and *F. sporotrichioides*), low virulence (*F. oxysporum*, *F. solani*, *F. equiseti*, *F. sambucinum*, *F. chlamydosporum* and *F. semitectum*) and mildly virulent (*F. scirpi*, *F. lateratium* and *F. cerealis*). Only the highly and moderately virulent isolates caused a significant reduction in grain yield.

4. Discussion

The wheat samples were highly contaminated with *Epicoccum*, *Alternaria* and *Fusarium* species. Agroecological zones with high infection levels of *Fusarium* species had low levels of *Epicoccum* and *Alternaria*, suggesting antagonistic effect among *Epicoccum*, *Alternaria* and *Fusarium*. The multiple contamination of wheat with different fungi indicates a potential risk of contamination of the grain with different mycotoxins like trichothecenes, T-2 toxin, zearalenone, fumonisins, moniliformin, alternariol monomethyl ether, altenuene, diacetoxyscirpenol, aflatoxins and ochratoxins (Placinta et al., 1999; Conkova et al., 2006).

Pathogenic and mycotoxin-producing *Fusarium* species such as *F. graminearum* and *F. poae* were isolated at high frequency from the wheat grain samples. The results correspond with findings of Muthomi and Mutitu (2003) who isolated *Fusarium* species at high frequencies from five wheat-growing areas in Kenya. The incidence of infection in the field could be higher than what the results suggest because severely infected kernels are light enough to be expelled with chaff during harvest (Tuite et al., 1990).

Over 10 *Fusarium* species were isolated from the wheat samples, indicating that head blight in the two districts is due to a complex of *Fusarium* species. Species isolated at high frequencies were *F. poae*, *F. oxysporum*, *F. graminearum* and *F. chlamydosporum*. Inoculation studies showed that only *F. graminearum* was highly virulent, suggesting that this species could be implicated in the possible head blight of wheat in Kenya. Other species including *F. poae*, *F. avenaceum*, *F. equiseti* and *F. sporotrichioides* do not blight the heads, but sometimes cause damage to the spikelets, resulting in low disease severity and latent seed infection (Parry et al., 1995). Despite its higher virulence, *F. graminearum* was less frequently isolated than *F. poae* and *F. chlamydosporum*. This could be due to the lower competitive capacity of *F. graminearum* in the presence of other *Fusarium* species. According to Marasas (1991), *F. equiseti*,

Table 3
Mean percentage isolation of different *Fusarium* species from wheat samples collected from different agroecological zones of Nakuru district during the 2004 cropping season

	Nakuru						Nyandarua				
	LH4	LH3	LH2	UM4	UH2	Mean	LH4	UH4	LH3	UH3	Mean
FPOA	5.9_c	37.5_a	31.3_a	50.0_a	22.0_b	29.3	31.3_a	9.1_c	11.1_c	21.4_b	18.2
FOXY	23.6_a	18.8_b	25.0_b	10.0_c	0_d	15.5	6.3_d	9.1_c	5.5_d	21.4_b	10.6
FGRA	11.8_b	18.8_b	12.5_c	10.0_c	0_d	10.6	18.8_b	13.6_b	0_e	0_e	8.1
FCHL	11.8_b	12.5_c	6.3_d	0_d	11.0_c	8.3	12.5_c	27.0_a	38.9_a	28.6_a	26.7
FVER	11.8_b	6.3_d	0_e	20.0_b	0_d	7.6	6.3_d	9.1_c	0_e	0_e	3.8
FEQU	23.6_a	0_f	12.5_c	0_d	0_d	7.2	12.5_c	4.5_d	16.7_b	7.14_d	10.2
FAVE	0_d	0_f	0_e	0_d	33.0_a	6.6	0_e	13.6_b	5.5_d	7.14_d	6.6
FLAT	0_d	3.1_e	0_e	10_c	11.0_c	4.8	0_e	0_e	0_e	0_e	0
FSPO	0_d	0_f	6.3_d	0_d	11.0_c	3.5	0_e	0_e	0_e	0_e	0
FSEM	0_d	3.1_e	0_e	0_d	11.0_c	2.8	0_e	4.5_d	0_e	0_e	1.1
FSCI	0_d	0_f	6.3_d	0_d	0_d	1.3	0_e	0_e	0_e	0_e	0
FCER	0_e	0_e	0_e	0_e	0_e	0	6.3_d	9.1_c	0_e	14.3_c	7.4
FSOL	0_e	0_e	0_e	0_e	0_e	0	0_e	0_e	5.5_d	0_e	1.4
FSAM	0_e	0_e	0_e	0_e	0_e	0	6.3_d	0_e	0_e	0_e	1.6

Values followed by different letters within columns are significantly different ($P \leqslant 0.05$).
FOXY = *F. oxysporum*; *FGRA* = *F. graminearum*; *FCHL* = *F. chlamydosporum*; *FVER* = *F. verticillioides*; *FAVE* = *F. avenaceum*; *FSPO* = *F. sporotrichioides*; *FSEM* = *F. semitectum*; *FSCI* = *F. sambucinum*; *FCER* = *F. cerealis*; *FSOL* = *F. solani*; *FSAM* = *F. sambucinum*.
UH2—upper highland 2; UH3—upper highland 3; UH4—upper highland 4; LH2—lower highland 2; LH3—lower highland 3; LH4—lower highland 4; UM4—upper midland 4.

Table 4
Percent incidence, range and mean of deoxynivalenol, zearalenone, T-2 and aflatoxin B1 content ($\mu g\,kg^{-1}$) in wheat grain samples from Nakuru district during the 2004 cropping season

	Nakuru				Nyandarua			
	DON	ZEA	T-2	$AflB_1$	DON	ZEA	T-2	Afl B1
Samples size	48	48	44	27	34	34	36	23
% Incidence	75	60	68	41	59	53	86	52
Range	105–303	1.6–35	20–60	0–7	105–289	1–96	20–66	2–7
Mean[a]	132.7	3.8	22.7	1.7	113	7.1	29.5	2.2

DON = deoxynivalenol; ZEA = zearalenone; T-2 = T-2 toxin; AFLB1 = aflatoxin B_1.
[a] Indicates the mean mycotoxin concentration in the all the samples analyzed.

Table 5
Disease severity (% ear bleached), area under disease progress curve (AUDPC) and 10-ear kernel weight of wheat ears inoculated with different *Fusarium* species

Fusarium species/isolate	Disease severity	AUDPC	10 ear seed wt.
F. graminearum 60.1	87.5	1031.8	8.4
F. graminearum 68.1	85.1	891.3	6.6
F. graminearum 58.4	84.9	719.6	7.7
F. avenaceum 45.4	41.0	487.5	10.0
F. avenaceum 69.7	43.8	248.2	11.1
F. verticillioides	32.9	206.8	14.1
F. poae 60.9	26.3	202.3	13.2
F. poae 8.10	17.3	140.1	11.9
F. poae 36D1	12.8	87.3	12.9
F. sporotrichioides	17.7	112.3	10.7
F. oxysporum	10.5	74.0	15.5
F. solani	16.9	73.7	14.6
F. equiseti 65.10	12.0	68.6	14.3
F. equiseti 10.10	8.3	32.9	14.2
F. sambucinum	9.0	44.8	11.8
F. chlamydosporum	7.7	44.1	14.7
F. semitectum	11.3	29.4	14.2
F. scirpi	4.3	23.0	13.6
F. lateratium	2.8	8.8	12.9
F. cerealis	1.8	4.4	14.9
Control	0.0	0.0	15.9
LSD ($P \leqslant 0.05$)	5.6	118.0	2.4

AUDPC = area under disease progress curve.

F. graminearum, *F. poae*, *F. verticillioides* and *F. sporotrichioides* are considered the most toxic *Fusarium* species. *F. graminearum* is the most important producer of DON and zearalenone.

The incidence of *Fusarium* mycotoxins DON, zearalenone and T-2 toxin varied, ranging between 68%, 57% and 76%, respectively. Although many *Fusarium* species are associated with FHB only a few contribute significantly to contamination of grain with mycotoxins. These are *F. graminearum* (DON, zearalenone and nivalenol), *F. culmorum* (DON, zearalenone and nivalenol), *F. avenaceum* (moniliformin), *F. poae* (nivalenol and T-2 toxin) and *F. sporotrichioides* (T-2 toxin and HT-2 toxin) (Chelkowski, 1989; Marasas, 1991; Miller and Trenholm, 1994). The production of the highly toxic type A trichothecenes like T-2 and HT-2 toxins by *F. poae* and *F. sporotrichioides* demonstrates their relevance in food and feed production. Other *Fusarium* species are likely to contribute to mycotoxin contamination too, but their toxicological relevance is not yet known. Some wheat samples were contaminated with aflatoxin B1 well above the $2\,\mu g\,kg^{-1}$ limit set by the European Commission (EC, 2006).

Although the mycotoxin content in grain was low, most of the wheat samples were contaminated with more than one of the four mycotoxins analyzed implying that Kenyan wheat products could be concurrently contaminated with low but significant levels of DON, zearalenone, T-2 toxin and aflatoxin B1. The co-occurrence of different mycotoxins in grain has been reported (Miller, 1991; Muller and Schwadorf, 1993; Stratton et al., 1993; Park et al., 1996). The co-occurrence of mycotoxins can affect both the level of mycotoxin production and the toxicology of the contaminated grain resulting in additive and synergistic effects (Muller and Schwadorf, 1993). Many countries have set maximum tolerance levels for different mycotoxins (EC, 2006; Van Egmond, 1999, 2002). Developing countries generally have less stringent mycotoxin standards, which would affect grain trade opportunities.

Attempts to exploit the resistance to mycotoxin production through either the inhibition of synthesis or chemical degradation may hold the most potential because of the limited number of genes which control this process (Bai et al., 1999; Snijders, 1990). More studies are required to determine actual disease levels in farmers fields in Kenya and the possibility of cross infection between wheat and maize by the causative *Fusarium* species.

Maize is the staple grain in Kenya and it is grown alongside wheat by most farmers. Since *Epicoccum* and *Alternaria* were found at high levels in most samples, there is a need to determine their effect on head blight and to confirm if there are any antagonistic effects against *Fusarium* species.

Acknowledgment

This research was funded by the International Foundation for Science (IFS) Grant no. E/3654-1.

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