Maize Stover in Relation to *Fusarium* Inoculum and Mycotoxins in Maize Grains of Two Agro-ecological Zones in Tanzania

Madege, R.R.

Department of Crop Science and Horticulture, Sokoine University of Agriculture

Correspondence: *rimadege@gmail.com*, Mobile +255 688248994, +255735288222

Abstract

Fusarium infection and the contamination of mycotoxins in maize are an important source of yield loss and deterioration of maize grain quality produced in Tanzania. This research aimed to establish information about which type of maize stover is the most important source of Fusarium inoculum. Three components of stover: straw, husks and litter (mix of silk, leaves and tassels) were randomly sampled in maize fields. Parallel to sampling maize stover, maize kernels were collected from the same fields. A molecular approach was employed to determine the toxigenic Fusarium species. This survey showed that the type of stover, the ecosystem and the mutual interaction strongly influenced the occurrence of F. verticillioides, F. graminearum and F. poae. Both F. verticillioides and F. graminearum were abundantly present in grains, husks and litter. However, F. poae occurred in lower frequencies. In regard to mycotoxins in maize kernels, Fumonisins (FBs) were predominantly present in almost 90% of the samples, the incidence of deoxynivalenol and zearalenone was rather low with 19% and less than 5%, respectively. Remarkably, fumonisin concentrations in maize from the Northern highlands exceeded legal threshold values (1000 µg/kg) more often than samples from Eastern lowland. Significant positive correlations between contamination of Fusarium in stover and maize grains were observed. Occurrence of Fusarium species in stover correlated positively with occurrence of fumonisins and deoxynivalenol in maize grains. These results showed convincingly that both ecosystem and stove type influence contamination of F. verticillioides, F. graminearum and F. poae. Appropriate management options for husks and litter in these areas are needed to minimize mycotoxin contamination.

Keywords: Maize stover, Inoculum source, *Fusarium*, mycotoxins

Introduction

Fusarium species are important pathogenic soil borne fungi that are responsible for high levels of maize yield loss in Tanzania. They are more important not only because of threat of food security but also they pose food safety problem as some species contaminate the grain with various mycotoxins. Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease and death in humans and other animals. Population of *Fusarium* species is primarily governed by environmental factors like temperature, moisture and agricultural practices like tillage. However, management of stover is known to play a fundamental role in *Fusarium* species population dynamics (Hofgaard *et al.,* 2016; Landschoot *et al.,* 2013). Appropriate handling of maize stover of the preceding crop to reduce the population of *Fusarium* species and subsequent pre-harvest infection in maize grains is one of the Good Agricultural Practices (GAP) recommended by FAO Codex alimentarius (Codex Alimentarius Commission, 2016).

Since *Fusarium* species can colonize different parts of maize plants including stover, it is important to understand the contribution of these residual components to the survival, population patterns and production of primary *Fusarium* inoculum. This knowledge is currently lacking in the agricultural settings of Tanzania. This study therefore aimed to achieve the following; the first is to establish the colonization pattern of *Fusarium* species in different stover materials. Secondly we aim to investigate the influence of

the environment on this colonization and thirdly are to quantify the occurrence of *Fusarium* species and related mycotoxins in grains. Lastly we aimed to investigate the relation between colonization in stover and maize grains from the same harvest season. Knowledge on the differential colonization of different pathogens on crop residues is essential for further optimization of preventive measures, such as crop rotation and tillage to reduce contamination of Fusarium mycotoxins in maize.

Materials and Methods

The study area

Sampling was carried in two ecosystems in Tanzania; the Northern highland (Manyara region Hanang' district) (4°S and 25°S and 84°E and 45°E, 1000-1500 m a.s.l.) and the Eastern lowland (Morogoro region, Kilosa district) (6°S and 8°S, and 36°30'E and 38°E; <900 m above sea level) between July and August 2013, the period during which the maize attains physiological maturity. Both zones are main maize growing areas.

Field sampling

Stratified random sampling was used, whereby ten villages were randomly selected from the list of maize growing villages of each district. From each village four households were selected and each represented one farm. Farm stover components were collected from each of the households. This biomass was comprised of structural components including straws, leaves, tassel, silks, husk, and cob (Moebius-Clune *et al.,* 2008). The litter component was made up of leaves, tassels and silk. To obtain a representative sample, four sampling sites were selected at the corners of each farm and a fifth sampling site in the centre. At each sampling site, ten maize plants were cut down into smaller fragments from which litter, straw, husk were collected to constitute about 25 g of each component making 100 g sample per component and per household. Maize cobs of unharvested maize were collected as previously described by Fandohan *et al.* (2005). At each sampling point, 20 maize cobs were sampled. The cobs of each sample were shelled by hand and mixed thoroughly to create a homogenous lot. Sampling of maize grains was carried out by dividing the homogenous lot into four parts from which 250 g was taken to constitute a 1 kg sample whereby 40 samples were collected per district. A total of 80 samples of each component of stover were collected and packed in paper bags, transported to the Plant Pathology Laboratory of Sokoine University of Agriculture where they were cut into smaller pieces before storing at 4°C until mycological analysis.

Mycological assays

Before plating, stover components were sliced into 5 mm pieces. Plating of triplicate samples of both maize grain and stover was done randomly by picking three stover pieces and grains and surface sterilized for 30 seconds in 1% NaOCl, washed for 30 seconds with 70% Ethanol, washed again with sterile distilled water, dried for five minutes, placed on PDA plates (potato dextrose agar, Oxoid Belgium, 39 g PDA/l) and subsequently purified as previously described in Landschoot *et al.* (2011).

Plates were incubated for seven days at 25°C with daily observations. After seven days, purification was done by differentiating colonies in terms of their colour and morphology before they were transferred to fresh PDA plates. Pure cultures were transferred into PDA slants and incubated at 25°C for seven days to re-establish before storage at 4°C. To determine the incidence of the species, the isolates were identified morphologically as well as using PCR assays. Morphological characteristics especially the macroscopic (colour, reverse colour and mycelium) and microscopic conidia and conidiophores shape (Leslie *et al.,* 2006) were used to distinguish the genus *Fusarium* from other fungi contaminating the grain.

To determine the amount of DNA for each species in maize, husks and litter, DNA was directly isolated from the plant fractions. DNA extraction from the plant materials was performed by using the extraction kit following the manufacturers' manual (Stratec Molecular, Berlin, Germany). Briefly, 10 g of the starting materials were milled by using laboratory grinder (Laboratory rotor mill pulveriser,

Belgium) into flour. Sixty milligrams of the ground materials were further homogenized in liquid nitrogen before the plant powder was transferred into 400 µl lysis buffer contained in 1.5 ml reaction tube. This step and the rest of the steps towards downstream DNA followed the extraction kit's manufacturers manual. The Q-PCR was carried out in a total volume of 12.5 µL consisting of 6.25 µL SYBR Green PCR Master Mix (Applied Biosystems), 250 nM of each primer, 0.5 µg/µL bovine serum albumin (BSA) and 2.5 µL template DNA. The Q-PCR was performed on a 7000 Sequence Detection System (SDS) (Applied Bioscience) using the following cycling protocol: 2 min at 50 °C; 95°C 10 min; 40 cycles of 95°C for 15 s and 62°C for 1 min followed by dissociation curve analysis at 60 to 95°C. A no template control and a dilution series of five known template concentrations (1^{-4} µg/mL – 1 µg/mL) were added to establish a standard curve.

Mycotoxin analysis

Mycotoxins were extracted from finely ground maize grains using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)-based approach (Anastassiades *et al.,* 2003; Frenich *et al.,* 2011; Rasmussen *et al.,* 2010; Rubert *et al.,* 2013). By means of ultra-high performance liquid chromatography (UHPLC)/time-of-flight mass spectrometry (TOFMS), the presence of FB1, FB2, DON and ZEN were established.

Limits of detection (LOD) and limits of quantification (LOQ) were determined based on the recovery experiments, but at 8 concentration levels (25-400 µg/kg for DON, FB1 and FB2, 0.5-8 µg/kg for aflatoxins, 2.5-40 µg/kg for HT-2, T-2, OTA and ZEA). LOD's were calculated using as LOD = (3sbl/a); where sbl is the standard deviation of the intercept and a is the slope of the respective Matrix-matched calibration curves (MMCC). The limit of quantification was calculated as 2 x LOD

Ground and homogenized blank maize samples were spiked with a multi-standard working solution at different concentration levels to evaluate the performance of an analytical procedure when testing a specific sample (matrix) type. The multi-standard stock solution and the spiked maize samples were prepared as described in Ortiz *et al.* (2013), using standards as solid pure extracts of FB1, FB2, DON and ZEN, supplied by Sigma Aldrich (St. Louis, MO, USA). Target Analysis TM software (Bruker Daltonics, Germany) was used to generating the extracted ion chromatograms. Identification of the ions was based on retention time deviation, mass accuracy and Sigma Fit TM algorithm, which is a rate for the agreement of the theoretical and measured isotopic.

Statistical analysis

Occurrence of fungal mycoflora and their associated mycotoxins were determined using an SPSS software version 18 (SPSS Inc, IL Chicago). Because conditions of normal distribution of data could not be met, non-parametric tests were used to compare occurrence of *Fusarium* species in the different types of stover. Proportion of positive samples with FBs, DON and ZEN which are above permissible limits based on European Union Scientific Committee on Food (van Egmond *et al.,* 2007) were calculated. Relationships between the species on maize stover, mycotoxins and main *Fusarium* species in maize grains were investigated using the Pearson correlation (p value <0.05).

Results

Occurrence of *Fusarium* fungi in two agro ecosystem and components of maize plant

Fig. 1 is presenting findings of the occurrence of genus *Fusarium* in maize samples from same fields and harvest season for which maize stover were collected. The results show that occurrence of genus *Fusarium* was independent of the agro ecosystem (p value = 0.113). About 65% and 45% of the grain samples from Northern highland and Eastern lowland respectively were infected with fungi of genus *Fusarium*. Genus *Fusarium* was differentially (p value <0.05) distributed in different components of maize plant. The genus was more abundant in maize kernels and least occurred in straws. There were no differences in occurrence of genus *Fusarium* in husks and litter.

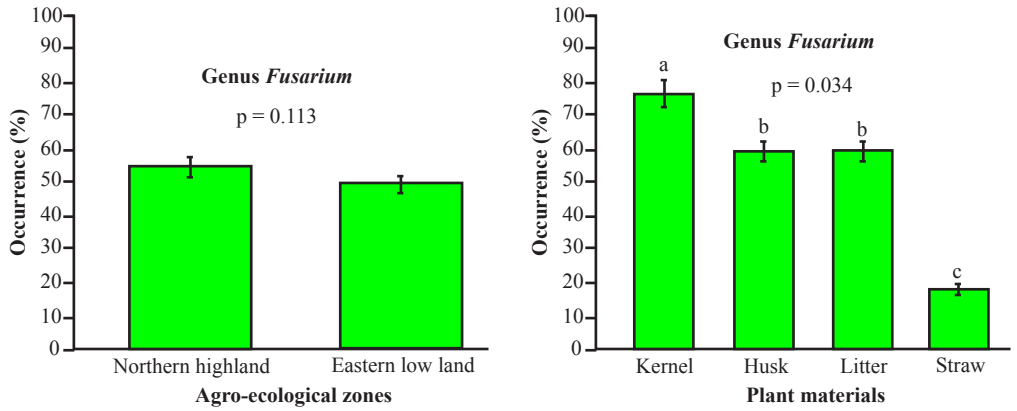

Figure 1: Occurrence of genus *Fusarium* in agro ecosystems and components of maize plant. *Bars with different letters represent means that are significantly different at p value<0.05*

In both Northern highland and Eastern lowland agro ecosystems, findings showed that the occurrence of *F. verticillioides* was higher than the occurrence of *F. graminearum* which was also higher than the occurrence of *F. poae*. Difference in occurrence of *Fusarium* species between agro ecosystems was significant (p-value<0.05). In Northern highland *F. verticillioides* was detected in 75% of the samples while detections of *F. graminearum* and *F. paoe* were 25% and 20% respectively. In Eastern lowland, *F. verticillioides* was recovered in 55% of the samples and 20% and 3% contained *F. graminearum* and *F. poae* in that order. Of the three components of maize plant (grain, husk and litter), *F. verticillioides* was distributed equally (p-value = 0.188) in the order of 45%, 38% and 35%. *F. graminearum*, colonized husks and litter differently (p-value <0.001). Highest (33%) incidence of *F. graminearum* was recorded in maize litter. Additionally, the incidences of *F. poae* in husks and litter components were not significantly different (Fig. 2).

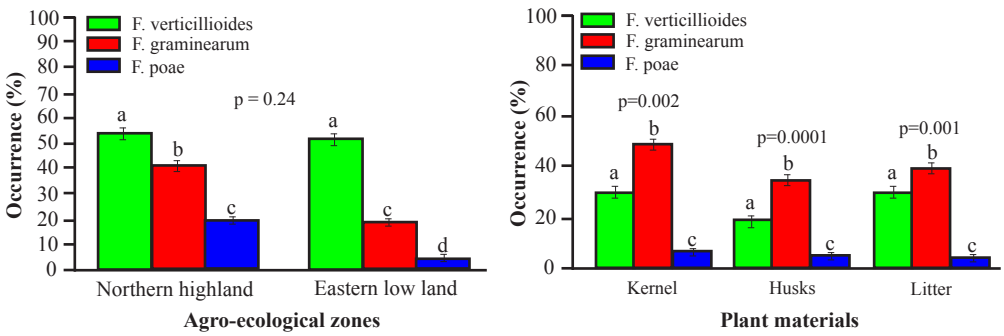

Figure 2: Occurrence of *Fusarium* species in agro ecosystems and components of maize plant. *Bars with different letters represent means that are significantly different at p value<0.05*

The content of *F. verticillioides* in grains, husk and litter samples from Eastern lowland was not different (p-value >0.05) (Fig. 3).

Co-occurrence of *Fusarium* species in plant materials

This study established that plant materials of both Northern highland and Eastern lowland were hosting more than one *Fusarium* species at significantly different (p-value<0.05) proportions (Fig. 4). *F. graminearum*, *F. verticillioides* and *F. poae* co-occurred in more than 20% and 10% of plant materials from Northern highland and Eastern lowland, respectively. Plant materials from the eastern lowland agro ecosystems had a more complex

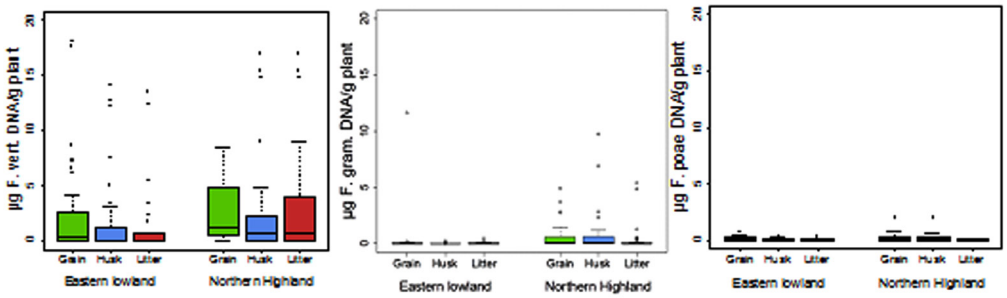

Figure 3: DNA content of *Fusarium* species in components of maize plant from two agro ecosystems. *F.vert: F. verticillioides, F.gram: F. graminearum*. No significant difference at p<0.05 according to Dunnet test.

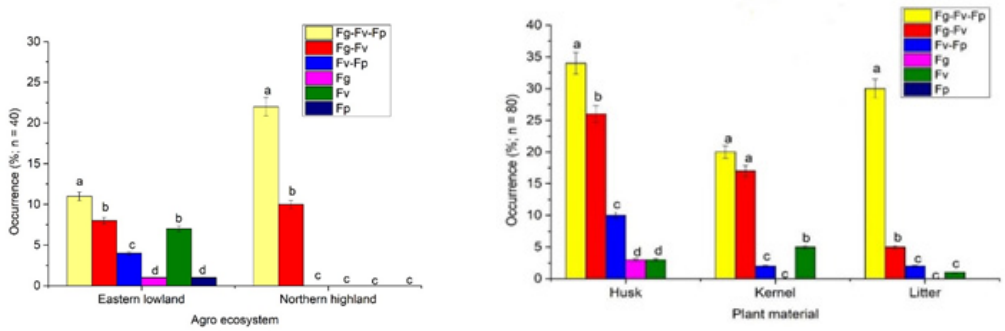

Figure 4: Co-occurrence of *Fusarium* species in plant materials from Northern highland and Eastern lowland and within different types of plant materials (Fg: *F. graminearum*, Fv: *F. verticillioides*, Fp: *F. poae*). *Bars with different letters means significantly different at p value <0.05*

pattern of *Fusarium* species co-occurrence with less than 5% plant materials hosting one species at a time. No *Fusarium* species was observed to occur in isolation in any type of plant materials of Northern highland agro ecosystem. Significant differences (p-value <0.05) were observed in the co-occurrence of *Fusarium* species within each type of plant material. Highest co-occurrence of *F. graminearum*, *F. verticillioides* and *F. poae* was observed in husk (33%), litter (31%) and kernel (20%). Co-occurrence of *F. verticillioides* and *F. poae* was the lowest observed in husk (14%), kernel (2%) and litter (2%).

Occurrence of *Fusarium* mycotoxins in maize kernels from two agro ecosystems

Using the same grain samples which were used for mycological assays FBs, DON and ZEN were quantified to determine occurrence and toxin content in these grains. With respect to the occurrence of FB1 FB2, DON and ZEN, the Northern highland agro ecosystem had the highest frequencies of samples contaminated with these toxins. In Northern highland ecosystem, the grains were contaminated with FB1, FB2, deoxynivalenol and ZEN at incidences of 95, 45, 36 and 2.5%, respectively (Fig. 5). Similarly, the Eastern lowland had a larger number of samples were contaminated with FB1 (78.65%) compared to FB2 (42.23%) and DON (1.5%). The occurrence of FBs did not vary between agro ecosystems while DON and ZEN occurred only in Northern highlands with significant differences in their occurrence.

The findings in Fig. 6 demonstrate that the grains from Northern highlands had the greatest content of FB1 (0-43540 µg/kg) and FB2 (0-32263 µg/kg). A range of 0 to 25651 µg/kg DON was recovered from maize grains

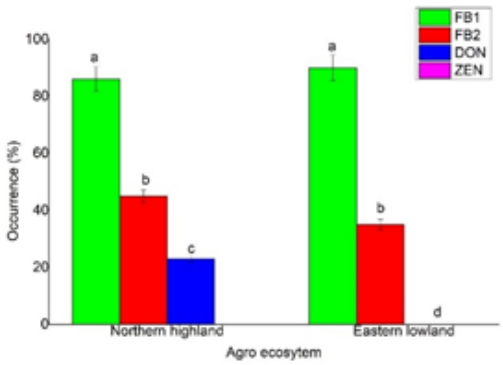

Figure 5: Occurrence of mycotoxins in grains from two agro-ecosystem zones. *Bars with different letters represent means that are significantly different at p value <0.05*

collected from Northern highland. ZEN was detected in Northern highland maize ecosystem (33 – 20131 µg/kg) but not in Eastern lowland ecosystem.

The analysis presented in Fig. 7 revealed that

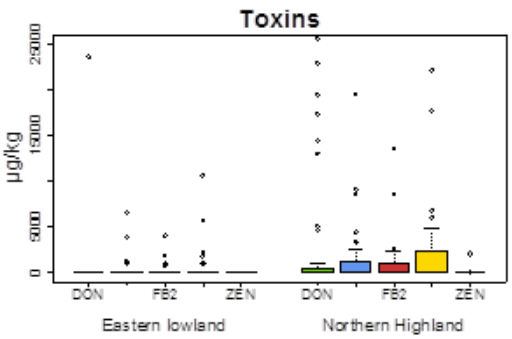

Figure 6: Concentration of DON (DON), fumonisin B1 (FB1), fumonisin B2 (FB2), total fumonisin (FBtot) and ZEN (ZEN) in maize grains from two agro ecosystems. *Bars with different letters represent means that are significantly different at p value <0.05*

many samples had mycotoxins levels which were above tolerable limits recommended by the European Union (EU). European Union commission provides limit levels (µg/kg) of mycotoxins in maize intended for direct human consumption as 1000, 750 and 100 for FBs, DON and ZEN respectively. Based on European Union (EU) legal limits for cereals meant for human consumption (Van Egmond *et al.,* 2007), maize grains from Northern highland ecosystem had the highest number of samples exceeding the EU legal limits. For FB1, FB2, DON and ZEN, 30, 25, 22.4 and 2.5 % of the samples respectively in Northern highland exceeded the EU legal limit. In Eastern lowland, 20, 17, 5 and 2.5% of the samples had the FBtot, FB1, FB2 and DON respectively which were above EU limits. Frequency (2.25 %) of contamination with ZEN in both ecosystems were above legal limits proposed by the European Union Scientific committee on food (European Commission, 2006).

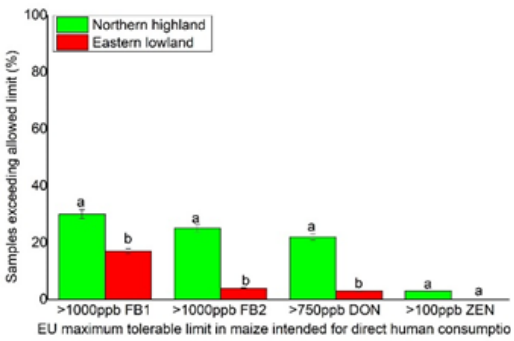

Figure 7: Percentage of samples with toxin concentrations (µg/kg) of FB1, FB2, DON and ZEN above EU limits. *Bars with different letters represent means that are significantly different at p value <0.05*

Linkage between incidence of *Fusarium* species in stover and grains and the occurrence of mycotoxins in maize grains

Both positive and negative correlations were observed between the contamination of *Fusarium* species in stover and the prevalence of the same species in maize grains (Table 1). The relationship between the occurrence of *F. verticillioides* in litter and husks with the occurrence of these species in grains was significant at P<0.05 and P<0.01 respectively. Similarly, the occurrence *F. graminearum* in litter was positively correlated with its occurrence in maize grains (P<0.01). Contamination of *F. verticillioides* in both husks

and litter correlated positively with fumonisin (B1 and B2) levels in grains. Similarly, contamination of *F. graminearum* in litter and husks had a significant (P≤0.05) positive relationship with the DON level in maize grains. The correlation between occurrence of DON in grains and *F. graminearum* in litter was positive and significant (P<0.05). The correlation between *F. verticillioides* in litter materials and the content of the same in husks and grains were highly correlated (P=0.01). The incidence of *F. graminearum* in litter and husk were both significantly correlated (P=0.05 and 0.01 respectively).

and maize grains collected in Tanzania. The observation that FB and DON content in maize grains was higher in Northern highland than Eastern lowland, the same way with the DNA content of *F. verticillioides* and *F. graminearum* in maize grains and maize stover suggest a close link between the DNA content of these fungal species in maize stover.

However, the quantity of each type of stover present in a particular location and time can be another factor governing the amount of inoculum that can infect the next crop. This is because maize residue are the most important host of mycotoxigenic *Fusarium* species

Table 1: Correlation matrix for *Fusarium* species and mycotoxins contamination in maize grain and the occurrence (%) of the *Fusarium* species in components of a maize plant

G	Fv										
	-0.09	Fg									
	0.03	-0.03	Fp								
	0.14	0.21	0.15	FB1							
	0.23	0.22	0.11	0.46*	FB2						
	0.14	0.84**	0.11	0.21	0.19	DON					
H	0.56*	0.18	-0.25	0.87**	0.44*	0.19	Fv				
	-0.12	0.72*	0.13	0.07	0.14	0.25	0.21	Fg			
	0.15	0.18	0.59*	0.06	0.15	0.16	0.17	-0.19	Fp		
L	0.46*	0.20	0.15	0.51	0.49*	0.18	0.44	0.07	0.11	Fv	
	0.06	0.72*	-0.17	0.12	0.14	0.34	0.20	0.29	0.13	0.20	Fg
	0.02	0.05	0.68**	0.13	0.16	0.21	0.14	0.23	0.51*	0.23	-0.46*

**, ** correlation coefficient significant at α ≤ 0.05 and α ≤ 0.01, respectively.*

G, H, L; Grains, husk and kernel respectively. Fv: F. verticillioides, Fg: F. graminearum, Fp: F. poae, FB: Fumonisin, DON: Deoxynivalenol

Discussion

The non-proportional distribution of *Fusarium* pathogens in different parts of maize plants in maize based ecosystems of Tanzania is for the first time confirming the results of Schollenberger *et al.* (2012) that *Fusarium* fungi can be present in all parts of maize plants, but unevenly distributed. The current study shows that with exception of *F. poae*, the occurrence of *F. graminearum* and *F. verticillioides* were unevenly distributed among litter, husks compared to other plants of the grass family (Landschoot *et al.,* 2011). Similar to previous studies (Atehnkeng *et al.,* 2008; Çepni and Gürel, 2013; Schollenberger *et al.,* 2012), the current study elucidated that agro ecological zones had notable influence on population of *F. graminearum* and *F. poae* but had no noticeable influence on *F. verticillioides*. The observation can be ascribed to the adaptability of *F. verticillioides* to different climatic conditions as opposed to *F. graminearum* and *F. poae* which

are more adapted to cool climate environment (Picot *et al.,* 2010; Bernhoft *et al.,* 2012). The study also clearly linked the occurrence of *F. verticillioides* with FBs, highest levels of fumonisin B1 and FB2 were observed in the samples from the Northern highland ecosystem although the Eastern lowland had equally high occurrence of *F. verticillioides*. This explains the fact that the level of fungal contamination in maize grain does not necessarily result into high levels of associated mycotoxins, but the interaction triangle of environment (temperature and moisture), host (susceptibility) and the pathogen (virulence) together are necessary to create optimal or suboptimal conditions for high or low disease severity as well as biosynthesis of mycotoxins (Scholthof, 2007).

The highest level of FB contamination detected in the Northern highland was twice as high as the highest contamination previously reported for Rungwe district in the southern highland zone of Tanzania (Kamala *et al.,* 2015) and three times the contaminations of the same in Cameroon (Ngoko *et al.,* 2001). The variation in FB contamination across locations can be explained by the differences in climate leading to different fungal proliferation rate, maize maturity and harvest times as also reported in the past (Bush *et al.,* 2004). Other factors could be replacement of new varieties with different susceptibility to *Fusarium* fungi and insect pests (van de Wouw *et al.,* 2010).

Apart from ear rot, *F. graminearum* in maize is associated with the production of both DON and ZEN (Audenaert *et al.,* 2009) both of which were detected in grains. Presence of F. poae, in the different types of stover and maize grains implies that, the grains could as well be contaminated with mycotoxin other than DON and ZEN. *F. poae* has been reported to produce type A trichothecenes; diacetoxyscipenol, monoacetoxyscipenol, scirpentriol, and neosolaniol, as well as the type B trichothecenes like NIV and fusarenone-X. Although previously, *F. poae* was associated with DON (Stenglein, 2009), recent investigations do not confirm this capacity (Stenglein *et al.,* 2014; Vanheule *et al.,* 2016).

Conclusion

As a potential source of primary inoculum of toxigenic *Fusarium* for the next crop the research has established that there is close association of *Fusarium* species that are occurring in maize stover with the toxigenic *Fusarium* species and their mycotoxins that are present in maize grains. Furthermore the findings in this research suggest that the different types of maize stover play different roles as source of potential Fusarium inoculum for the next crop. This implies that different management strategies can be foreseen to reduce the inoculum load in maize fields. The findings that *F. verticillioides* was most abundant in maize husks while F. graminearum dominated in litter components imply that in one hand maize husks are possibly the most important source of inoculum of *F. verticillioides* which contaminate maize grains with fumonisin mycotoxins. On the other hand, litter materials are the most important source of inoculum of *F. graminearum* which is the producer of various trechotecene mycotoxins.

Acknowledgement and declaration of conflict of interest

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