Identification, Characterization and Distribution of *Aspergillus* and *Fusarium* Species Isolated from Maize Kernels from Western Part of Kenya.

Nyukuri, N. J.[1], Wagara, I. N.[1] and Matasyoh, J. C.[3]
[1]Department of Biological sciences, Laikipia University, [2]Department of Biological sciences, Laikipia University [3]Department of Chemistry, Egerton University
Corresponding Author: Email. Address: janetokumu@yahoo.ca.in

Abstract

Moulds destroy more than 30% of crop yields and produce potentially poisonous mycotoxins. The most prevalent on foods are *Aspergillus, Fusarium*, *Penicillium, Rhizopus* and *Mucor*. Kenya has experienced dramatic outbreaks of mycotoxin poisoning resulting in loss of lives. The aim of the study was, to isolate and characterize moulds associated with maize from Lake Victoria Basin (LVB). Thirty samples of unaffected maize and mouldy maize were collected from Trans-nzoia, Kakamega and Kuria districts to determine the mould's distribution. These areas are in mid altitude agroecological zones with warm and humid conditions which favors development of moulds and mycotoxins. *Aspergillus* and *Fusarium* were isolated and identified from these areas. Among the genus *Aspergillus,* twelve mycotoxigenic species and two atoxigenic species were identified and among the genus *Fusarium,* fourteen mycotoxigenic species were identified. In all the three districts, the most frequent *Aspergillus* and *Fusarium* species on maize were *A. flavus* at 23.1% and *F. proliferatum* at 20.3% frequency**.** The quantity of the moulds from mouldy and good maize was compared using T- test for each of the district but they were not significantly different.

Key words: Mycotoxins, moulds, maize, *Aspergillus* and *Fusarium*

Introduction

Moulds are opportunistic biological agents of ubiquitous nature (Ryan and Ray, 2004). Because of their powerful arsenal of hydrolytic enzymes, these microorganisms can cause a high degree of deterioration when present in foods and are responsible for considerable economic losses (Souza *et al*., 2005). They are known to destroy 10 to 30% of the total yield of crops and more than 30% of perishable crops in developing countries by reducing their quality and/or quantity (Matasyoh *et al*., 2009). Moulds cause extensive damage on foods, feeds and other agricultural commodities in the field, during transportation, storage and processing, leading to postharvest losses. The ubiquitous nature of moulds, their ability to colonize diverse substrates and lack of effective control measures has contributed to the high incidences of mould and mycotoxin contamination in foods and feeds. Acute mycotoxicoses epidemics occur in Africa leading to death of several hundred people. In 2004, an acute aflatoxicosis outbreak occurred in Kenya resulting in 317 cases and 125 deaths, while cases of esophageal cancer have been linked to high levels of fumonisins in Lake Victoria Basin (Azziz -Baumgartner *et al*., 2005). One of the available methods of controlling moulds is the use of synthetic chemical preservatives like sodium nitrate which have been the cause of appearance of resistant micro-organisms, leading to occurrences of emerging food borne diseases. Furthermore, other methods such as use of solar driers are expensive for the small scale farmers.

Generally when *Fusarium* species invade maize in the field they cause diseases like seedling blights, kernel, root, seed, stalk and ear rots, for example *F. subglutinans* and *F. moniliforme* causes Fusarium ear rot and stalk rots. *Fusarium graminearum* attack maize and cause stalk, cob and root rots. *Fusarium moniliforme* also causes many other diseases like kernel and root rot, seed rot and seedling blight (CIMMYT, 2004). Reports of surveys conducted in some African countries showed *F. moniliforme* as the most prevalent fungus on maize (Marasas *et al.*, 1988; Allah Fadi, 1998; Kedera *et al*., 1999).

Toxigenic fungi can attack maize prior to harvest and further decay the crop during storage. As a result, mycotoxins may form both during crop development and in storage. *Aspergillus, Fusarium, Penicillium* and *Cladosporium* are the predominant fungal genera associated with grain in storage. Mycotoxins are chemicals produced by fungi that are harmful to humans and domestic animals. These chemicals may contaminate staple foods and feeds worldwide, posing a number of significant food safety concerns (Schmale and Munkvold, 2009). They contaminate 25% of agricultural crops worldwide and are a source of morbidity and mortality throughout Africa, Asia and Latin America (Smith *et al*., 1994). They cause diseases referred to as mycotoxicoses in humans and animals (Agrios, 1997). Most mycotoxicoses are caused by the common and widespread moulds namely *Aspergillus, Penicillium* and *Fusarium.* They cause acute liver damage, liver cirrhosis, induction of tumours and attack on the central nervous system, skin disorders and hormonal effects (Erkekoglu *et al*., 2010).

The most important mycotoxins are aflatoxins, deoxynivalenol, fumonisins, ochratoxins and zearalenones. Among these the most famous are the aflatoxins which are produced by *Aspergillus fl*a*vus, A. parasiticus* and several other species of *Aspergillus* in a wide variety of agricultural commodities including grains, legumes and nuts (Turner *et al*., 2003). Acute aflatoxicosis epidemics occur in several parts of Africa and Asia leading to death of several hundred people (Varga *et al*., 2009). Aflatoxins are known to be potent hepatocarcinogens in animals and humans. Ochratoxin A, which is has been experimentally shown to be teratogenic, a potent renal carcinogenic and immunosuppressive is largely produced by *A. ochraceus* and less frequently by *A. niger* (Nielsen *et al*., 2009). As an enzyme inhibitor, ochratoxin A affects lipid peroxidation and has been implicated in Balkan Nephropathy (BENO in humans (Atrosh *et al*., 2000).
Toxin-producing fungi may invade at pre-harvesting period, harvest-time, during post-harvest handling and in storage. Toxigenic fungi can be divided into three groups: (a) field fungi namely, genus *Fusarium,* e. g *F. moniliforme*, *F. roseus, F. trincinctum* and *F. nivale*; (b) storage fungi which include the genera *Aspergillus* and *Penicilium*, e.g *A. flavus*, and *A. parasiticus*; and (c) advanced deterioration fungi which normally do not infect intact grains but easily attack damaged ones and require high moisture content. Examples of the third group are *A. clavatus, A. fumigatus, Chaetomium, Scopulariopsis, Rhizopus, Mucor,* and *Absidia* (Makun *et al.*, 2009).

Materials and Methods

Sample collection

A total of 30 samples categorized as unaffected maize and mould damaged grain collected randomly from various rural households and markets in Trans-nzoia (Mean Temp: 23^{o}C, RH:37-

81%), Kakamega (Mean Temp: 26^{o}C, RH: 68-83%) and Kuria (Mean Temp: 16.5^{o}C, RH :above 80%). Sampling was done at random, having liaised with the local Agricultural officer to talk to farmers who were growing maize and willing to participate in the study. In Trans-nzoia, samples were collected from Kitale central and Kiminini divisions. In Kakamega, samples were collected from Kakamega municipality division whereas in Kuria district samples were collected from Kehancha and Masaba divisions. Most of these areas are in mid altitude agro ecological zones with warm and humid conditions which favours development of moulds and production of mycotoxins (Kaaya *et al.,* 2006). These areas have unpredictable rainfall patterns making it difficult for small scale farmers to efficiently dry their produce. Ten samples, each weighing half a kilogram were collected from each district in properly labeled khaki paper bags to minimize saprophytic fungal contamination and transported in a cool box to the laboratory for analysis. Each sample was divided into two portions; one was stored at 4^{o}C and the other at -20^{o}C to avoid further accumulation of mycotoxins.

Isolation and Identification of moulds associated with maize

Moulds were isolated from the samples using the direct plating technique. For each sample, 20 seeds were picked randomly and surface sterilized by soaking for 1 minute in 2.5% of sodium hypochlorite, and rinsed in three changes of sterile distilled water. The samples were blotted with sterile filter paper and plated (five seeds per plate) on the surface of malt extract agar (MEA) and potato dextrose agar (PDA) containing 7.5% sodium chloride and 33 mg/l of streptomycin sulphate powder. Addition of streptomycin sulphate is very effective in the inhibition of bacteria. Yet, it does not inhibit the growth of other mould species, including the mycotoxin producers. Thus, identification is not compromised (Diba *et al*., 2007). The plates were incubated at 25^{o}C and monitored daily for fungal growth for up to 14 days. Treatments were replicated four times and the experiment was laid down in a complete randomized design.

Identification and Characterization of the moulds

The resulting cultures of *Aspergillus* species were identified to species level based on cultural and morphological characteristics using taxonomic keys (Kozakiewtez, 1989; Klich, 2002) and *Fusarium* species were identified based on the criteria of Gerlach and Nirenberg (1982) and Leslie and Summerell (2006). Morphological features of moulds were studied and the major and remarkable macroscopic features that were looked at are colony diameter, colony colour on agar and reverse and colony texture. Microscopic characteristics that helped in the identification process were conidia heads, stipes, colour and length, vesicles shape and seriation, metula covering, conidia size, shape and roughness (Diba *et al*., 2007). *Aspergillus parasiticus* has green colonies but deeper in shade than *A. flavus*. *Aspergillus ochraceus has* ochre-coloured or buff colonies with submerged mycelium; conidiophores show shades of yellow in the outer layer of the wall which is rough or pitted.

For *Aspergillus niger,* the hyphae are septate and hyaline more or less yellow in colour. The colonies are black coloured and reverse usually colourless *Aspergillus versicolor's* cultures show considerable range of colour. Different strains may be variously pale green, grayish green, buff or, show patches of yellow. *Aspergillus nidulans has c*olonies of a clear green colour, developing

dirty white spots from the centre outwards. Conidiophores smooth walled, or more or less browned. The number of seeds contaminated by *Aspergillus* and *Fusarium* were counted and the infection rate (number of contaminated grain recorded as percentage) was determined and compared for each sample. Target moulds were sub-cultured to obtain pure cultures.

Data analysis

One way analysis of variance (ANOVA) was used to test whether the moulds affecting maize from the three districts are significantly different. Least significant difference (LSD) was used to discriminate which maize and from which district is highly contaminated with moulds. Students't-test was used to test if the two genera of moulds are statistically significant on the maize from the three districts. The statistical level of significance was fixed at $p < 0.05$ (95%).

Results

Moulds isolated from the maize and their incidence

Five different fungal genera; *Aspergillus, Fusarium, Penicillium, Rhizopus* and *Mucor* were isolated from the maize samples collected from Trans-nzoia, Kakamega and Kuria districts. *Aspergillus* was the most frequently isolated genus with a 63.3% frequency.

Aspergillus species identified

The moulds isolated and identified among the genus *Aspergillus* were 12 mycotoxigenic species which included *A. parasiticus, A. flavus, A. wentii, A. ustus, A. nidulans, A. ochraceus, A. tamarii, A. niger, A. versicolor, A. flavipes, A. terreus, A. fumigatus,* and two atoxigenic species, *A. humicola* and *A. sparsus* (Table 1). The resulting cultures were identified based on cultural and morphological characteristics using taxonomic keys (Kozakiewtez, 1989; Klich, 2002). Species of the genus *Aspergillus* are found almost everywhere on every conceivable type of substratum (Venkatesh, 2004).

Table 1 : *Aspergillus* and *Fusarium* species isolated from maize samples in the three districts

Maize sample	District/ Origin	Condition of sample	*Aspergillus* species isolated	*Fusarium* species isolated
KEKIM 01	Trans-nzoia	Good	*A. flavus, A. parasiticus, A. wentii, A. terreus*	*F. proliferatum*
KEKIM 02	Trans-nzoia	Bad	*A. flavus, A. parasiticus*	-
KEKIM 03	Trans-nzoia	Good	*A. flavus, A. ochraceus, A. parasiticus, A. niger*	*F. solani*
KEKIM 04	Trans-nzoia	Good	*A. flavus*	*F. solani*
KEKIM 05	Trans-nzoia	Bad	*A. flavus, A. ochraceus, A. wentii*	-
KEKIM 06	Trans-nzoia	Bad	*A. flavus, A. versicolor*	*F. solani, F. moniliforme*
KEKIM 07	Trans-nzoia	Bad	*A. versioclor*	*F. solani*
KEKIM 08	Trans-nzoia	Good	*A. ustus*	-
KEKIM 09	Trans-nzoia	Good	*A. parasiticus*	-
KEKIM 10	Trans-nzoia	Bad	*A. parasiticus*	*F. solani*

KEKAM 18	Kakamega	Good	*A. versicolor*	*F. scirpi*
KEKAM 19	Kakamega	Good	*A. flavipes*	-
KEKAM 20	Kakamega	Good	*A. flavus, A. niger*	*F. chlamydosporum*
KEKAM 21	Kakamega	Good	*A.flavus, A. niger, A. fumigatus*	*F. graminearum*
KEKAM 22	Kakamega	Good	*A. wentii*	-
KEKAM 23	Kakamega	Good	*A. ustus*	*F. trincinctum, F. chlamydosporum, F. semitectun, F. oxysporum*
KEKAM 24	Kakamega	Bad	*A. nidulans, A. terreus*	*F. nivale, F. proliferatum, F.chlamydosporum*
KEKAM 25	Kakamega	Good	-	*F. graminearum*
KEKAM 26	Kakamega	Good	-	*F. avenaceum, F. subglutinans*
KEKAM 27	Kakamega	Bad	*A. flavus, A. parasiticus*	-
KEKUM 31	Kuria	Bad	*A. flavus, A. versicolor*	*F. merismoides, F. culmorum*
KEKUM 32	Kuria	Good	*A. flavus, A. ochraceus*	*F.crookwellence*
KEKUM 33	Kuria	Good	*A. ustus, A. wentii, A. parasiticus, A. sparsus*	*F. proliferatum, F. nivale*
KEKUM 34	Kuria	Good	*A. flavus, A. niger*	*F. proliferatum*
KEKUM 35	Kuria	Good	*A. humicola*	*F. sporotrichioides*
KEKUM 36	Kuria	Bad	*A. niger, A. nidulans*	*F. merismoides*
KEKUM 37	Kuria	Bad	*A. niger*	*F. merismoides*
KEKUM 38	Kuria	Good	*A. flavus, A. tamari, A. sparsus, A. ochraceus*	*F. chlamydosporum, F. merismoides*
KEKUM39	Kuria	Bad	*A. flavus, A. nidulans*	*F. oxysporum*
KEKUM 40	Kuria	Good	*A. flavus, A. nidulans, A. versicolor, A. fumigatus*	*F. proliferatum, F. solani*

Legend for Maize Sample Codes

KEKIM: KE-Kenya	**KI- Kitale**	**M-Maize**
KEKAM: KE-Kenya	KA- Kakamega	M-Maize
KEKUM: KE-Kenya	KU- Kuria	M-Maize

Occurrence of *Aspergillus* species

The percentage occurrence of *Aspergillus* population was determined by comparing the number of seeds showing each type of mould growth for each sample. When the *Aspergillus* population was separated according to geographical areas of Western Kenya, the predominant *Aspergillus* species in Trans-nzoia was *A. flavus* (23.1%) followed by *A. parasiticus* (15.4%), *A. wentii* (7.5%), *A. ochraceus* (7.5%), *A. versicolor* (7.5%), *A. ustus* and *A. sparsus* at 7.5%. *Aspergillus flavipes, A. nidulans, A. niger* and *A. terreus* were the least isolated species at a frequency of 3.8%, while *A. humicola* and *A. fumigatus* were at 3.6%. In Kakamega district, the most prevalent *Aspergillus* species were *A. flavus, A. niger* and *A. versicolor* at 15%, followed by *A.*

parasiticus, A. flavipes, A. nidulans and *A. fumigatus* at 10%, *A. ochraceus, A. humicola* and *A. sparsus* at 5%. In Kuria district, the most frequent *Aspergillus* species isolated were *A. flavus* (20%), followed by *A. niger* (15%) and *A. parasiticus, A. wentii, A. sparsus,* and *A. humicola* were isolated at 8 %.

Fusarium species identified

Maize was found to be the host of an extremely wide range of *Fusarium* species under natural infection at the sample sites. Sixteen mycotoxigenic species were identified and these included *F. solani, F. proliferatum, F. sporotrichioides, F. moniliforme, F. scirpi, F. chlamydosporum, F. trincinctum, F. oxysporum, F. subglutinans, F. nivale, F. avenaceum, F. graminearum, F. culmorum* and *F. crookwellence.* In Trans-nzoia district, the most frequent *Fusarium* species was *F. solani* at 71.4%, followed by *F. proliferatum, F. avenaceum, F. sporotrichoides,* and *F. moninliforme* at 7.15% as shown in Table 3. In Kakamega district, *F. chlamydosporum* was the highest at 25.1% followed by *F. graminearum* at 16.7%, *F. oxysporum, F. nivale, F. proliferatum, F. subglutinans, F. semitectum,* and *F. trincinctum* at 8.3%, *F. crookwellence* was at 4.3% and *F. scirpi* the least isolated at 4%. The most prevalent *Fusarium* in Kuria district was *F. merismoides* at 36.4% followed by *F. proliferatum* at 27.3%, *F. oxysporum, F. solani* and *F. nivale* at 9.1%, *F. chlamydopsorum* at 5.1% and *F. culmorum* at 4.3% . Generally, the relative level of *Fusarium* distribution within and between the districts ranged from 0% to 71.4%. However, the distribution of the moulds isolated and identified from the three districts were not significantly different at $P \leq 0.05$ as shown in Table 4. When the data of the moulds isolated in the three districts was analyzed separately using Tukey HSD, there was no significant ($P \leq 0.05$) difference in the number of the moulds in the three districts. The mean difference in the number of the moulds between Trans-nzoia and Kakamega was 0.365, between Trans-nzoia and Kuria was 0.335 and between Kuria and Kakamega was 0.030 (Table 5).

Table 2: Analysis of Variance for Significance of moulds distribution within and between the three districts

Number of moulds					
Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.014	2	1.007	.846	.434
Within Groups	80.944	68	1.190		
Total	82.958	70			

df=degree of freedom, F= Frequency, Sig=Significance

Table 3: Comparison of the moulds in the three districts

Multiple Comparisons Turkey HSD

(I) Sampling place	(J) Sampling place	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Kakamega	Trans-nzoia	-.365	.325	.502	-1.14	.41
	Kuria	-.030	.327	.995	-.81	.75
Trans-nzoia	Kakamega	.365	.325	.502	-.41	1.14
	Kuria	.335	.306	.519	-.40	1.07
Kuria	Kakamega	.030	.327	.995	-.75	.81
	Trans-nzoia	-.335	.306	.519	-1.07	.40

Discussion

The following section discusses the results based on the objectives of the study. This section begins by discussing objective one which aimed at identifying and quantifying the moulds associated with maize from Trans-nzoia, Kakamega and Kuria counties.

The frequency of *Aspergillus* and *Fusarium* species

The high occurrence of moulds in the samples can be attributed to the fact that the three districts are found in warm and humid region which predisposes the maize to the moulds in the field and also after being harvested. These findings were also expected because of the relatively high temperature and relative humidity in Lake Basin region, which was optimum for growth of *Aspergillus* species. In Trans-nzoia, the mean temperature is 23^{o}C annually and relative humidity range is 37-81%. Kakamega has mean temperature of 26^{o}C and relative humidity range of 68-83%. Kuria experience mean tempeature of 16.5^{o}C and relative humidity above 80%. In all the three districts, the most frequently isolated *Aspergillus* species on maize was *A. flavus,* followed by *A. niger, A. parasiticus* and *A. versicolor* (Table 2). Overall, infection of genus *Aspergillus* on the maize seeds in Trans-nzoia district was substantially higher than those collected from Kuria and Kakamega district. The less prevalent *Aspergillus* species throughout the three districts were *A. fumigatus, A. sparsus, A. tamarii, A. ustus, A. humicola*, and *A. terreus.*

The ubiquitous nature of the moulds, ability to colonize diverse substrates and lack of effective control measures could have contributed to the high incidences of the moulds in maize isolated from these regions (Souza *et al*., 2005). *Aspergillus* species are more commonly associated with cereals during drying and storage. *Aspergillus flavus* and *A. parasiticus* have a particular affinity for cereals and can be recognized by yellow-green or grey green colour on corn kernels in the field and in storage (Varga *et al*., 2011). Pre-harvest invasion is partly dependent on insect damage to cobs, but the fungi can also invade down the silks of developing ears and they cause diseases like maize ear and kernel rot. Invasion is primarily due to inadequate drying and improper storage (Pitt, 2000). There is a possibility that since invasion starts in the field and continues in storage, the maize samples collected from markets and various rural households had already accumulated high levels of the moulds especially the *A. flavus* followed by *A. parasiticus* because they commonly attack maize both in the field and in storage.

Damaged maize also favours the growth of *A. flavus* compared to any other *Aspergillus* species and this fact explains why the most frequently isolated *Aspergillus* species in the three districts, was *A. flavus* followed by *A. parasiticus* and other *Aspergillus* species. According to Fandohan *et al*. (2003), postharvest handling favourably and unfavourably affects fungal infection and mycotoxin production in maize. Mechanical damage during and after harvest may also offer entry to the fungal spores either in maize cobs or grains. This possibly explains why very high quantities of the moulds were isolated from the maize samples because some of them had damaged grains that maybe predisposed the grains to the fungus infection.

Fields that vary in cropping history, tillage practices, planting dates, soil types or hybrids can differ greatly in mould and aflatoxin contamination (Munkvold *et al*., 2009). There is likelihood that cropping history, tillage practices, soil types, planting dates and hybrids planted in the three districts are different. These factors may explain the differences in the quantity and type of the moulds isolated from the three districts. That is probably why the overall infection of the

Aspergillus on the grains was substantially higher in Trans-nzoia compared to Kuria and Kakamega districts.

All the *Fusarium* species isolated and identified are considered to be pathogenic to maize. This is in line with observations of Munkvold (2003) and the statement of Leslie and Summerell (2006) that *Fusarium* species is the most common pathogen on maize cobs. *Fusarium* species are destructive pathogens on cereal crops and other commodities, and produce mycotoxins before, or immediately after, harvest (Pitt, 2000). They are commonly considered as field fungi invading more than 50% of maize grains before harvest (Robleda-Robleda, 1991).

Sixteen *Fusarium* species were isolated from the three districts within the Lake Basin region (Table 3). The high number of species isolated could be due to interaction among fungi in maize, which constitutes an important factor influencing fungal infection and subsequent mycotoxins production. Mechanical damage during and after harvest may also offer entry to the fungal spores either in maize cob or grains. Grain damage is a common occurrence in maize especially during postharvest handling of the grains. This possibly predisposes the maize to *Fusarium* attack within the region (Fandohan *et al*., 2003). Another factor that could explain the high level of the *Fusarium* within the three districts is insect invasion. Insects also play an important role in infection of maize by *Fusarium* species. They can act as wounding agents or as vectors spreading the fungus from origin of inocula to plants. Insects are a great havoc to maize in the field as well as in the store and many pests and parasites attack maize during the storage period. According to Gwinner *et al*. (1996), insects are most often considered as the principal cause of grain losses. This therefore means that fungi always invade wherever insects have attacked maize and it justifies the high quantities of *Fusarium* species isolated from the three districts. The occurrence, prevalence and diversity of *Fusarium* species did not vary substantially between the samples.

Relatively high quantities of the moulds were detected from each of the districts sampled. These findings were expected because of the relatively high temperature and relative humidity in Lake Victoria Basin, which predisposes the maize to high infections of the moulds. There was a significant difference in the quantity of moulds between the three districts. In addition, differences were observed in the types of moulds occurring in the three districts probably due to use of different maize varieties by farmers in the respective districts. This is because maize hybrids vary in their resistance to the various types of ear molds (Munkvold *et al*., 2009). Some hybrids are more susceptible to ear mold than others and most probably the maize varieties planted in Trans-nzoia are different from those planted in Kakamega and Kuria. This therefore explains the difference in the type and quantity of moulds in the three districts. Environmental conditions also lead to localized or widespread outbreaks of ear molds that can produce mycotoxins contamination. Factors that affect ear mold development can vary from one portion of a field to another and include previous crop, tillage practices and even small temperature, moisture and humidity differences due to differences in elevation, slope and air movement (Munkvold *et al*., 2009). These three environmental factors determine the direction to which the spores of the ear mold will be blown and their mass. This could have a direct effect on the accumulation of the fungus on the grain. Farmers, therefore, need to be trained on improved pre-harvest and post-harvest practices to decrease the likelihood of mould infection. From the results obtained, there was no difference in the quantity of moulds in the mouldy and good maize indicating that maize which appears good is also contaminated with moulds.

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