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SUSCEPTIBILITY OF MYCOTOXIGENIC FUNGI TO COMMERCIAL FUNGICIDES, A POTENTIAL TOOL FOR MYCOTOXIN CONTROL IN MAIZE IN KENYA

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ABSTRACT

Mycotoxin contamination of food grains represents significant health and economic challenges in developing countries as well as the developed world. Mycotoxin-producing fungal species affecting maize mainly belong to the genera *Aspergillus*, *Fusarium*, and *Penicillium.*They pose serious phytopathological and mycotoxicological risks both at pre-harvest and post-harvest stages. Maize in Kenya has been associated with frequent outbreaks of aflatoxin contamination. A number of mycotoxin control strategies both chemical and biological have been developed as potential tools for mycotoxin control. A Laboratory based cross-sectional study was carried out in a Mycology Laboratory at the Center for Microbiology Research in Kenya Medical Research Institute, Nairobi, Kenya. A total of 138 maize samples obtained from Machakos, Nairobi, Mombasa, Kitale and Kisumu were subjected to mycological analysis. The samples were treated with the fungicides; Antracol (propineb), Milraz (propineb700g/kg and Cymoxanil60g/kg), Mistress (Cymoxanil 8% and Mancozeb 64%) and Victory (Metalaxy 80g/kg and Mancozeb 640g/kg.) before inoculation on Sabourauds dextrose agar (SDA). Infestation rates on fungicide- treated and non treated control maize kernels were scored. The susceptibility of the isolates to the four test fungicides was determined by disk diffusion technique. All the maize samples were infested by moulds and there was a significant difference in regional infestation rates ($p<0.05$). Maize from Mombasa had the lowest infestation of 72.5% while Nairobi was the highest with 99.1%. Fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium* were frequently isolated from the five regions. There was a significant reduction {$p<0.05$ (0.00)} of infestation rates on fungicide- treated maize compared to the untreated. Generally, 26% (n=35) and 34% (n=47) of maize samples treated with Mistress and Victory, respectively had 0% infestation while those treated with Milraz and Antracol were 10% (n=14) and 14% (n=19), respectively. Some mycotoxigenic isolates were found to be resistant to more than one of the test fungicides. However, their in- vitro antifungal activity is of great importance and could further be evaluated to determine their field efficacy for mycotoxin control in maize.

Key words: Maize, Mycotoxigenic fungi, Fungicides, Susceptibility

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INTRODUCTION

Mycotoxigenic fungi are often found as contaminants in agricultural products before or after harvest as well as during transportation and storage. Mycotoxins are produced by moulds as secondary metabolites which when ingested, adverse effects occur on humans and animals resulting in illnesses and economic losses [1]. Mycotoxigenic fungi are part of the microbial flora associated with many agronomic crops, including maize, peanuts, tree nuts, grapes, barley, coffee, cotton, wheat and other cereal grains [2]. Depending on the crop plant affected and the fungal species, mycotoxigenic moulds may cause plant disease, such as *Aspergillus* fruit rot of grapes, maize ear rots caused by *Aspergillus* and *Fusarium* species, and *Fusarium* head blight as well as seedling blight diseases on cereal crops [3]. Fumonisins are a group of mycotoxin contaminants found in food and feed products produced by *Fusarium* species. Fumonisin B1 primarily is of international, agroeconomic, and food safety concern. High doses of fumonisin B1-infested corn feed have been shown to cause pulmonary edema in swine, while lower doses lead to hepatic disease [4]. The mycotoxins are produced predominantly by toxigenic strains of *Fusarium verticillioides*. The fungus commonly proliferates in maize, causing stalk and ear rot diseases, in addition to mycotoxin contamination. Aflatoxins are another important group of mycotoxins which affect different food grains including maize and maize- based food. The most common aflatoxins are B1, B2, G1 and G2 which are potential carcinogens produced by *Aspergillusflavus* and *Aspergillus parasiticus* [5]. The height of human aflatoxicoses occurred in Eastern Kenya in 2004 where 317 infections and 125 deaths were reported resulting from acute hepatotoxicity [6].

Fungicides are mainly chemical compounds or live organisms used to eradicate or inhibit fungi and their spores [7]. Conventionally, the control of mycotoxigenic fungi was achieved using chemical fungicides, variation in cultural practices, and development of resistant cultivars. Additionally, postharvest sorting of contaminated yields, such as maize, wheat, peanuts and tree nuts, have been developed to reduce mycotoxin content [9, 10, 11]. Biological control methods have also been investigated for controlling mycotoxigenic fungi where several bacterial and fungal antagonists have been developed against the moulds [12, 13]. Several chemical control agents have also been developed. Quinone-outside inhibitor fungicides (qoi) were first labeled for use on maize in the year 2000. These fungicides are commonly referred to as strobilurin fungicides. They are now extensively marketed in corn production for management of both biotic and abiotic stresses [7]. These fungicides are applied as a preventive measure or as early as possible in a disease cycle. They are effective against spore germination and early mycelium growth but this has less or no effect when the fungus is already established [8]. The objective of the current study, therefore, was to determine the susceptibility of mycotoxigenic fungi to some commercial fungicides as a potential for the control of mycotoxin infestation in maize.

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MATERIALS AND METHODS

Methods

Study design and sample collection

This was a laboratory based cross-sectional study that involved the collection 138 maize samples distributed equally among five study sites in Kenya. The sites were Machakos, Nairobi, Mombasa, Kitale and Kisumu. These regions represent different agro-ecological zones where various maize varieties are grown. The samples were packaged and transported to Kenya Medical Research Institute, Centre for Microbiology Research, Mycology Laboratory where they were analyzed. Scientific approval for the study was granted by the Kenya Medical Research Institute scientific steering committee.

Test fungicides

Antracol WP 70

This is a broad spectrum protective fungicide effective against early and late blight on potatoes and tomatoes, various fungal diseases on vegetables, fruits and ornamentals. The active ingredient of antracol WP 70 propineb, belongs to a chemical class of dithiocarbamates. Propineb is a fungicide with multisite activity and kills conidia or germinating conidia by contact. It is used across the world for the control of various fungi, especially Oomycetes, Ascomycetes, Basidiomycetes and Fungi imperfecti. The fungicide is manufactured by Bayer Crop Science.

Mistress 72 WP

This is a systemic and contact fungicide for the control of early and late blight on tomatoes and potatoes. The fungicide is manufactured by Osho chemical industries. This fungicide has cymoxanil 8% and mancozeb 64%, cymoxanil has preventive and local systemic activity and also inhibits blight. Mancozeb has protective antifungicidal activity and works through translaminar action.

Milraz WP 76

This is a broad spectrum preventive fungicide. The active ingredients are propineb700g/kg a dithiocarbamate and Cymoxanil which is an ethyl urea 60g/kg. This fungicide is manufactured by Bayer Crop Science.

Victory Fungicide

This is a systemic and contact foliar fungicide for the control of foliar and root diseases of potatoes and tomatoes and downy mildew of ornamental crops. The active ingredients in this fungicide are Metalaxy 80g/kg and Mancozeb 640g/kg. This is manufactured by Victory Chemical Co., Ltd.

Inoculation of Maize samples and Culture of fungi

Maize samples were washed with the recommended concentration of each fungicide independently and inoculated on Sabourauds dextrose agar (SDA). Each SDA plate was inoculated with four maize kernels obtained from each sample for the four fungicides. A control for every sample was washed concurrently with sterile distilled water and inoculated on a different SDA plate. Every single sample from each site was inoculated

in five SDA plates, four for each test fungicide and one untreated control plate for the same sample. The plates were incubated at 30^{o}C for 72 hours to allow for fungal growth. Growth of fungi on the maize grains washed with the fungicides was scored to determine the percentage infestation and compared to their control plates. Where all the four maize kernels had visible growth of fungi in a plate, this was scored as 100% infestation and if three of the four kernels in that plate were infested, this was scored as 75% while two infested kernels was scored as 50% infestation rate. In a case where only one grain out of the four plated kernels was infested, the score was 25% infestation rate.

Identification of Isolates

Fungi growing on the maize after were identified by morphologic characteristics for both microscopic and macroscopic features. The colonial morphology shapes and types of conidia produced by the mould were used to key out the identity of the individual fungi [14].

Bioactivity of the Fungicides

The activity of the fungicides against fungal isolates was determined by disk diffusion technique. Briefly, absorbent Watman filter paper disks of 6 mm were impregnated with 20µl of the test concentration of the fungicides independently. A pure culture of each fungal isolate was inoculated on an SDA plate and the impregnated disks containing the fungicide were placed aseptically onto the SDA plate using a sterile forceps. The plates were incubated at 30^{o}C for 72 hours and thereafter, the zones of inhibition around the disks were measured and expressed in millimeters (mm). A standard azole antifungal drug (fluconazole) was used as a reference for susceptibility or resistance to the four fungicides and interpreted according to CLSI breakpoints.

Data Analysis

Data generated in this study were analysed using the Statistical Software SPSS Version 17. Comparison of infestation rates on fungicide treated maize with controls and infestation per region was done using (ANOVA).

RESULTS

All fungicide-treated maize samples had a reduced infestation by fungi compared to the untreated controls (Figure 1). Maize from all the five regions that were treated with Mistress had low infestation where the lowest value of 28% was from Mombasa (Figure 1). The Infestation rate on untreated control samples was also low in samples from Mombasa with 72.5%. Control samples obtained from Nairobi and Kitale had the highest infestation of 99.1% and 98.2%, respectively. Maize samples treated with Milraz had a slightly higher infestation compared to the other three fungicides used. In Kitale and Kisumu, infestation on Milraz treated maize was highest with 76.8% and 81% respectively. This was also seen in Mombasa where the infestation was high (59.4%) in maize treated with Milraz compared to Mistress (28%), Victory (37.5%) and Antracol (50%). Nevertheless, maize samples from Nairobi treated with Antracol had the highest infestation of 92%, while those from Kisumu treated with Milraz were also more infested (81%) compared to all the fungicide treated maize kernels from all the regions.

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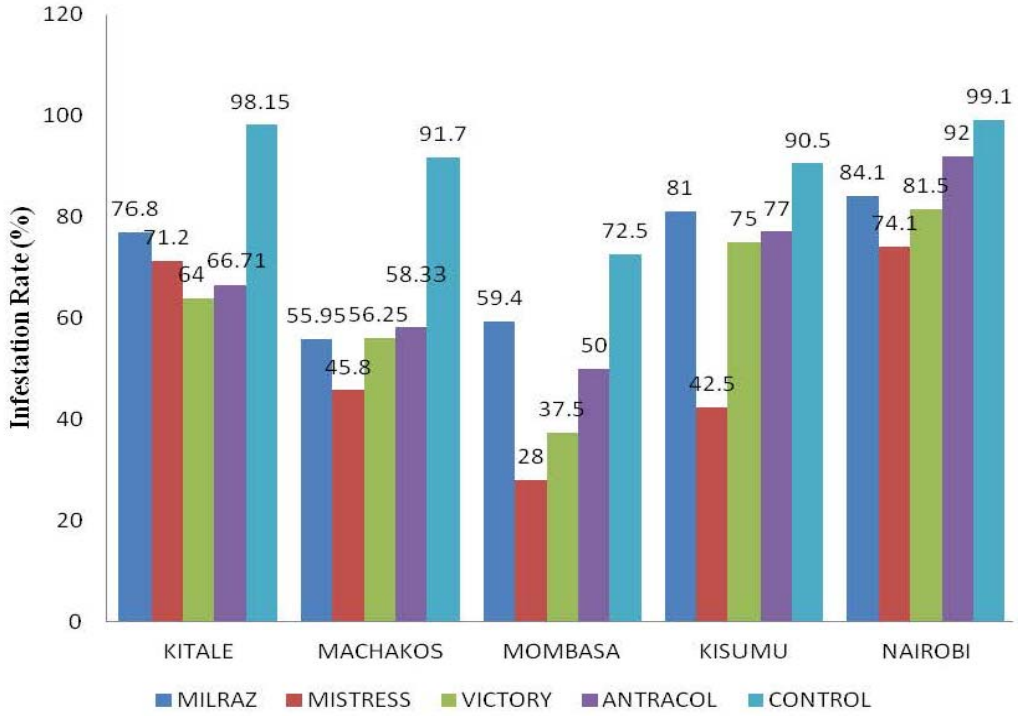

Figure 1: Percentage fungal infestation of maize from different regions in Kenya following treatment of grains with fungicides

Table 1 shows the statistical comparison of infestation rates that occurred in the five regions, the general mean difference is statistically significant $p< 0.05$. However, there was a non statistically significant difference in the infestation rates between Kitale and Nairobi $p>0.05$ ($p=0.66$) as well as Kitale and Kisumu $p>0.05$ ($p=0.189$). The same observation was noted in the infestation rates between Machakos and Mombasa where $p>0.05$ ($p=0.621$). Apart from the above similarities, the differences in the infestation rates between Machakos, Kitale, Nairobi and Kisumu were highly significant {$p<0.05$ ($p= 0.00$)}.

Table 2 shows the distribution of control and fungicide treated maize samples in each category of infestation. From Table 2, most of the control samples were heavily infested by moulds having 75% (n=11) and 100% (n=115) infestation compared to the treated samples. Many samples treated with Antracol and Millraz had 100% infestation (n=54 and n=55, respectively) compared to those treated with Mistress and Victory which were 30 and 29, respectively. In addition, more samples treated with Mistress and Victory had no infestation (n=35 and n=47, respectively) compared to Milraz and Antracol (n=14 and n=35, respectively).

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a. Control plates with *A. niger (top) and* Milraz treated maize (bottom)

b. A. flavus infested maize (bottom) and Mistress (top right) and Victory (top left) treated maize

c. Control plate with *Fusarium spp& A .flavus infestation*

d. Control (top right) and maize treated with Mistress (left) and Victory (bottom right) fungicide

Figure 2: Sabourauds dextrose agar plates showing infestations by different mycotoxigenic fungi and fungicide treated maize

Few isolates from the various regions were resistant to the test fungicides by disk diffusion. *Aspergillus flavus* isolates from Kisumu were more resistant to Milraz compared to the other fungicides where there was no zone of inhibition on SDA plates. Among the resistant fungi, *A. Flavus* and *Fusarium spp* isolates were more resistant than the *Penicillium spp* and *Rhizopus spp.* On the other hand, most of these isolates were susceptible to Mistress and Antracol while being resistant to one or the other fungicides.

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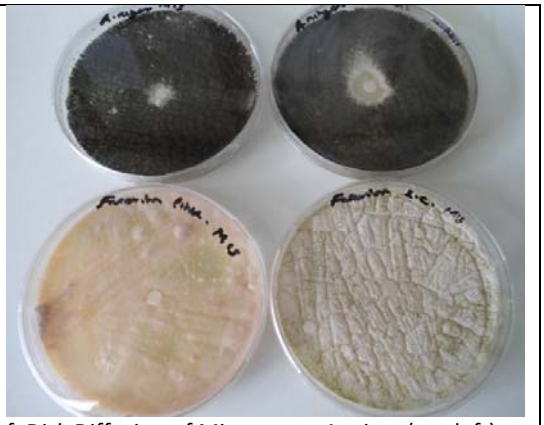
e. Disk diffusion of Victory on *Aspergillus spp* (bottom left*)*, Milraz on *Penicillium spp* (top left), Victory on *Fusarium spp* (top right) and *Penicillium spp* (bottom right*)*.



f. Disk Diffusion of Mistress on *A. niger* (top left), *Fusarium spp* (bottom left), *A. flavus* (bottom right) and Victory fungicide on *A. niger* (top right)

Figure 3: Disc diffusion susceptibility testing of mycotoxigenic fungi

DISCUSSION

A variety of agricultural produce are contaminated by various mycotoxin producing moulds. These include the *Aspergillus flavus*, which was frequently isolated from maize samples analysed in this study. Though the presence of the *Aspergillus* mould does not essentially designate aflatoxin contamination, there is definitely an increased risk for the occurrence of these toxins under stressful conditions for the mould [15]. Maize samples from the five regions showed gross contamination with mycotoxin producing moulds of the genera *Aspergillus spp*. *Fusarium spp* and *Penicillium spp*. *Rhizopus spp* was frequently isolated from the maize. The infestation rate per region for the untreated control maize samples was high with 99.1% and 98.15% for Nairobi and Kitale, respectively (Figure. 1). Maize samples from the five regions were also infested by the non mycotoxigenic *Rhizopus* species.

There was a significant difference $p<0.05$ in the activity of the four fungicides as shown on Table 2. All control samples were infested by different fungal species. Generally, a huge percentage of maize treated with Mistress and Victory (n=35) and (n=47), respectively were less infested. The two fungicides were more effective in reducing the mycoflora on the treated maize samples. In general, (n=55) and (n=54) of samples treated with Milraz and Antracol, respectively had 100% infestation rate compared to those treated with Victory and Mistress Fungicides (n=29) and (n=30), respectively. This shows that more fungi could survive in the presence of Milraz and Antracol as opposed to Victory and Mistress. However, the control samples (n=115) had 100% infestation rate which was higher than that of all treated samples. All the four test fungicides reduced the resident mycoflora in the respective treated samples to a great extent. The least number (n=29) of samples with 100% infestation were those treated with Victory fungicide while

the highest number of samples (n=55) with 100% infestation were those treated with Milraz. This indicates that Victory fungicide could prevent heavy infestation of the maize grains compared to the other fungicides.

A number of bioagents and different fungicides have been found to reduce mycoflora on seed while enhancing germination as well as vigour Index of various grains such as cowpea [17]. In a study which tested the efficacy of Benomyl, Dithane M-45 75% WP (Manganese ethylene bis dithio carbamate plus zinc) and Bavistin 50% WP (Methyl-H-benzimidazole-2ylcarbamate) on seed mycoflora of cowpea, variations in their infestation was observed depending on the respective treatments. Dithane M-45 and Bavistin were found to be effective in reducing seed-borne infection of *Fusarium* spp. [17]. This is consistent with the effect of the fungicides tested in this study where the fungicides significantly reduced the infestation on treated maize kernels. In another study that tested different concentrations of fungicides against various pathogens, Dithane M-45 and Bavistin were found to be effective in reducing seed-borne infection of *Fusarium* spp. on maize seeds [18]. *In vitro* studies have found Captan, Dithane M-45, Bavistin, and Vitavax effective in controlling *Fusarium* species on cereal grains [19]. Another study reported the reduction of wilt disease on treatment with Mancozeb M-45, Bavistin and Vitavax. In addition, the germination of fungicide treated seeds was enhanced compared to controls. Untreated seeds had the highest seed mycoflora infestation of 68% and lowest seed germination of 80% [19, 20]. High infestation of untreated maize kernels was also seen in this study.

During the years of commercial use of a fungicide, there can arise populations of the target pathogen that are no longer sufficiently sensitive to be controlled adequately [21]. Some isolates of *A. flavus*, *Fusarium spp* and *Penicillium spp* exhibited resistance to at least one of the test fungicides. This was shown by the overgrowth of the mould on fungicide impregnated disks with no zone of inhibition (plates e and f). This is, therefore, indicative of possible resistance and the ability to thrive and producce mycotoxins in the presence of the test fungicides. However, definitive susceptibility profiles for the moulds need to be ascertained using other techniques such as minimum inhibitory concentration method [16]. The use of chemical inhibitors which suppress spore germination of fungi and the development of the fungal mycelium is one of the most effective ways of controlling the problems caused by aflatoxin contamination in a susceptible product such as maize [24]. Fungicide in agriculture could also lead to resistance of human fungal pathogens to antifungal agents due to similarities in the mode of action of fungicides and antifungal agents. Environmental soil isolates of *Aspergillus fumigatus* from Tanzania were found harboring azole antifungal resistance mutations which are of clinical significance [25].

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CONCLUSION AND RECOMENDATIONS

Several methods for mycotoxin control have been investigated and these have included physical, chemical and biological methods [22, 23]. The present study revealed that maize samples collected from five regions of Kenya were heavily infested with mycotoxigenic fungal species. The results in this study have also demonstrated the ability of the four test fungicides to significantly reduce the infestation of maize by mycotoxigenic fungi {$p<0.05$ (0.00)}. A large number of the isolates particularly *Aspergillus* spp, *Fusarium* spp and *Pencillium* spp were susceptible to the test fungicides while few were resistant. Field studies may also be conducted to ascertain their efficacy for use in controlling infestation of maize for public food safety. On the other hand, erroneous use of fungicides for agriculture should be avoided due to the possible development of antifungal resistant mutants which are of great clinical significance.

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Table 1: Multiple comparison of the differences in infestation rates per region

Multiple Comparisons of regional infestation rates

(I) region	(J) region	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
KITALE	NAIROBI	-1.916	4.359	0.66	-10.48	6.65
	MACHAKOS	35.677*	4.359	0	27.11	44.24
	KISUMU	7.769	5.903	0.189	-3.83	19.37
	MOMBASA	32.769*	5.903	0	21.17	44.37
NAIROBI	KITALE	1.916	4.359	0.66	-6.65	10.48
	MACHAKOS	37.593*	4.318	0	29.11	46.08
	KISUMU	9.685	5.873	0.1	-1.85	21.22
	MOMBASA	34.685*	5.873	0	23.15	46.22
MACHAKOS	KITALE	-35.677*	4.359	0	-44.24	-27.11
	NAIROBI	-37.593*	4.318	0	-46.08	-29.11
	KISUMU	-27.907*	5.873	0	-39.45	-16.37
	MOMBASA	-2.907	5.873	0.621	-14.45	8.63
KISUMU	KITALE	-7.769	5.903	0.189	-19.37	3.83
	NAIROBI	-9.685	5.873	0.1	-21.22	1.85
	MACHAKOS	27.907*	5.873	0	16.37	39.45
	MOMBASA	25.000*	7.095	0	11.06	38.94
MOMBASA	KITALE	-32.769*	5.903	0	-44.37	-21.17
	NAIROBI	-34.685*	5.873	0	-46.22	-23.15
	MACHAKOS	2.907	5.873	0.621	-8.63	14.45
	KISUMU	-25.000*	7.095	0	-38.94	-11.06

The mean difference is significant at the 0.05 level (Fisher's Significant Difference Test)

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Table 2: General infestation of fungicide treated maize and controls

Infestation			**Type of fungicide used**				
rates on maize	Categories		MILRAZ	MISTRESS	VICTORY	ANTRACOL	CONTROL
	0	% within type of fungicide	10% n=14	26%	34%	14%	0%
		used		N=35	N=47	N=19	N=0
	25	% within type of fungicide	21%	27%	19%	24%	4%
		used	N=29	N=37	N=26	N=33	N=5
	50	% within type of fungicide	12%	12%	18%	15%	5%
		used	N=17	N=17	N=25	N=21	N=7
	75	% within type of fungicide	17%	14%	8%	8%	8%
		used	N=23	N=19	N=11	N=11	N=11
	100	% within type of fungicide	40%	22%	21%	39%	83%
		used	N=55	N=30	N=29	N=54	N=115
Total		% within type of fungicide	100%	100%	100%	100%	100%
		used	n=138	N=138	N=138	N=138	n=138

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