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Comprehensive analysis of multiple mycotoxins and *Aspergillus flavus* metabolites in maize from Kenyan households

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ABSTRACT

This study assessed the levels of mycotoxins in maize from Kenyan households. Further, local open pollinated maize varieties were compared with commercial hybrids to evaluate which variety is less susceptible to mycotoxin contamination. Four hundred and eighty ($n = 480$) maize samples were collected in the years 2018–2020 from households in Eastern, Western, Coastal and Lake Victoria regions of Kenya. Liquid chromatography coupled to tandem mass spectrometry was used to detect and quantify 22 mycotoxins, along with 31 *Aspergillus flavus* metabolites in the samples. Eastern Kenya had the highest aflatoxin (AF) contamination with 75% of samples having AF levels above the Kenyan regulatory limits (10 μg/kg), the highest concentration was 558.1 μg/kg. In Western Kenya, only 18% of samples had concentration levels above the Kenyan regulatory limits for AF with highest sample having 73.3 μg/kg. The Lake Victoria region had the most fumonisins (F) contamination, with 53% of the samples having fumonisin B_1 (FB_1) < 1000 μg/kg. However, only 20% of the samples surpassed the Kenyan regulatory limit for total fumonisins (2000 μg/kg) with the highest concentration being 13,022 μg/kg. In addition, 21.6% of samples from the Lake Victoria region had zearalenone (ZEN) and deoxynivalenol (DON) above regulatory limits for European countries (1000 μg/kg). Western region had the least *A. flavus* metabolites contamination (18%) while the Eastern region had the highest incidence of *A. flavus* metabolites (81%). Among the *A. flavus* metabolites, cyclopiazonic acid (CPA), beta-cyclopiazonic acid (β CPA), flavacol (FLV) and methylcitreo-isocoumarin (MIC) positively correlated with each other but negatively correlated with the other metabolites. Significant positive co-occurrence was also noted among *Fusarium* mycotoxins: nivalenol (NIV) positively correlated with DON ($r = 0.81$), fusarenon-X (FX) ($r = 0.81$) and ZEN ($r = 0.70$). Negative correlations were observed between *Aspergillus* and *Fusarium* mycotoxins: aflatoxin B_1 (AFB_1) negatively correlated with FB_1 ($r = -0.11$), FX ($r = -0.17$) and ZEN ($r = -0.20$). Local open-pollinated maize varieties (L-opv) were less susceptible to mycotoxin contamination compared to the commercial hybrids (C-hy). This study reveals that Kenyan maize is contaminated with multiple mycotoxins most of which are not regulated in Kenya despite being regulated in other parts of the world. A comprehensive legislation should therefore be put in place to protect the Kenyan public against chronic exposure to these mycotoxins. In addition to high yield, there is a need for commercial hybrid maize breeders to incorporate mycotoxin resistance as an important trait in germplasm improvement in seeds production.

1. Introduction

Mycotoxins are low molecular-weight secondary metabolites produced by some moulds. They comprise a chemically and structurally heterogenous assemblage of natural small molecules with varying toxicities to humans and animals (Zain, 2011). Food and feed can be colonized by mycotoxigenic fungi at any point along the value chain and therefore at risk of mycotoxin contamination from the farm to fork

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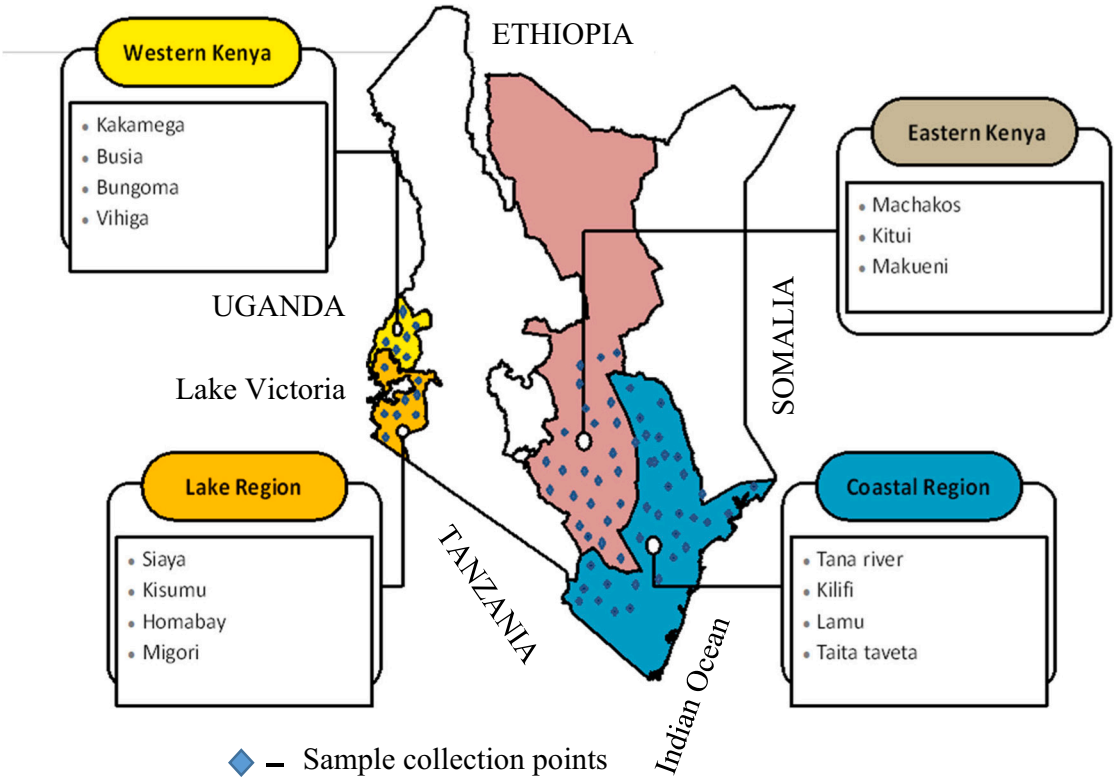

Fig. 1. Map of Kenya showing maize sample collection sites. Map courtesy of https://yourfreetemplates.com/free-kenya-editable-map/ 2021.

(Kagot et al., 2019; Zain, 2011). On the global scale, it is estimated that between 60 and 80% of the food produced is contaminated with mycotoxins (Eskola et al., 2020; Kebede et al., 2020). Once food or feed are contaminated above regulatory limits, they are rejected by the market and condemned for destruction (Kagot et al., 2019). In sub-Sahara Africa, produce worth approximately $4 billion is lost annually due to mycotoxins contamination, while in the United States of America, the loss is between $ 0.5–1.5 billion annually (Cinar and Onbaşı, 2019; Kebede et al., 2020; Wielogorska et al., 2019).

Consumption of mycotoxin-contaminated food leads to mycotoxicosis, varying on severity depending on type of mycotoxin consumed, concentration of the mycotoxin, duration of the exposure, age and general health of the individual (Ayelign and De Saeger, 2020). Mycotoxicosis can either be acute or chronic, where chronic mycotoxicosis results from regular intake of small doses of mycotoxins over a prolonged period of time, usually asymptomatic, but with immunological repercussions and a cumulative effect on the risk of diseases *e.g.* cancer in the long run (Ayelign and De Saeger, 2020; Okoth, 2016). While acute mycotoxicosis occurs following sudden exposure to high levels of mycotoxins, characterized by the rapid onset of symptoms often leading to hospitalization and sometimes demise (Ayelign and De Saeger, 2020; Barac, 2019). In Kenya, cases of acute aflatoxicosis have been reported repeatedly. In 1981, 12 people died, while in 2004, 125 people lost their lives after consuming AF contaminated maize in Eastern Kenya (Mutegi et al., 2018). In 2006, 10 deaths were again reported, while between 2007 and 2008, 4 other people were reported dead as a result of ingesting AF contaminated maize (Mutegi et al., 2018). In 2010 cases of dogs dying in Nairobi associated with consumption of AF-contaminated feed was reported (Mutegi et al., 2018). Despite efforts to mitigate AF contamination in Kenyan staples, the mycotoxin is still persistent. In 2019, 7 peanut brands were banned by the Kenya Bureau of Standards (KEBS) for having AF levels above the regulatory limits in their products (Otieno, 2019). In January 2020, KEBS banned 17 maize-flour brands, over high AF levels (Asumba, 2020). In 2021, Kenya momentarily banned importation of maize from Tanzania and Uganda with regards to high AF contamination (Junior, 2021).

In addition to AF, *Aspergillus* has the ability to synthesize a range of other secondary metabolites which can be classified as emerging mycotoxins (Uka et al., 2019). Emerging mycotoxins are mycotoxins with suspected toxicity and are not regulated with legislation (Singh and Mehta, 2020). While some emerging mycotoxins could be toxicologically docile, others are potentially carcinogenic, genotoxic and immunotoxic (Fraeyman et al., 2017).

In Kenya, exposure to *A. flavus* metabolites should be of public health concern, more so since aflatoxigenic strains of *A. flavus* have been previously isolated from Kenyan soils (Dooso Oloo et al., 2019; Probst et al., 2007). Potentially toxic *A. flavus* metabolites are sterigmatocystin (STE), aspertoxin (ASPT), flavacol (FLV), cyclopiazonic acid (CPA), paspalinine (PASL), versiconol (VER), kojic acid (KA), aspergillic acid (AA), aflatrem (AFTR), aflavarin (AFLVR), aflavinine (AFLV), leporin C (LEP C), noranthrone (NOR) and speradine A (SPER) (Okoth et al., 2018). Other emerging mycotoxins, produced by other fungi include alternariol (AOH) produced by *Alternaria alternata* and roquefortine C (ROQ C) produced by *Penicillium roqueforti* among others. Gaps in knowledge about toxicities and prevalence of *A. flavus* metabolites and other emerging mycotoxins exist (Gruber-Dorninger et al., 2017). This study unravels *A. flavus* metabolites and other emerging mycotoxins present in Kenyan maize.

Despite the public health and economic challenges posed by mycotoxins, most low-and middle income countries have lean regulatory frameworks to tackle this problem (Matumba et al., 2017). In Kenya, only AF and F are currently regulated with a legislation set at 10 μg/kg for total AF, 5 μg/kg for AFB_1 and 2000 μg/kg for total F in maize (Kagot et al., 2019; KEBS, 2017; Mutegi et al., 2018). Regulatory authorities focus more in the formal market owing to the difficulty in regulating the informal market. The informal market is characterized by small-scale farmers who practice limited mechanized rain fed farming. Mostly, the farmers' plant maize on small family owned land parcels for consumption and surplus for sale. To sustain family needs, the surplus is sold in the informal local market unpackaged and available in small quantities

Table 1
Yearly and regional effect on mycotoxin concentration in Kenyan maize.

MYCOTOXIN	Yearly effect[a] (*P*- values)[c]	Regional effect[b] (*P*- values)[c]
Nivalenol (NIV)	0.719	0.000
Deoxynivalenol (DON)	0.022	0.000
Neosolaniol (NEO)	0.135	0.111
Fusarenon x (FX)	0.415	0.000
3 Acetyldeoxynivalenol (3 ADON)	0.096	0.000
15 Acetyldeoxynivalenol (15 ADON)	0.346	0.000
Aflatoxin G_2 (AFG_2)	0.000	0.000
Aflatoxin G_1 (AFG_1)	0.000	0.000
Aflatoxin B_2 (AFB_2)	0.000	0.000
Aflatoxin B_1 (AFB_1)	0.000	0.000
Diacetoxyscirpenol (DAS)	0.680	0.000
Alternariol (AOH)	0.674	0.000
HT2 toxin (HT_2)	0.306	0.000
Fumonisin B_1 (FB_1)	0.821	0.000
T2 toxin (T-2)	0.360	0.000
Fumonisin B_3 (FB_3)	0.869	0.000
Ochratoxin A (OTA)	0.426	0.000
Fumonisin B_2 (FB_2)	0.866	0.000
Alternariol monomethyl ether (AME)	0.584	0.000
Sterigmatocystin (STE)	0.000	0.000
Roquefortine C (ROQ C)	0.107	0.145
Zearalenone (ZEN)	0.011	0.000

[a]Effect of the different years of study on mycotoxins concentration: 2018,2019 and 2020.
[b]Effect of the different regions of sample collection on mycotoxins concentration. The regions were, Eastern, Western, Coastal and Lake Victoria regions of Kenya.
[c]P-values of Kruskal-Wallis tests. *P*-values < 0.05 indicate a significant effect at $\alpha = 0.05$.

from 250 g. The formal market on the other hand, is much organized with brands required to comply with set legislation, package and brand their products before they can be stocked in shops and chain stores. In the formal market, the maize is processed to flour and is available only from 1 kg or more. Consequently, food items in the informal market, are much cheaper compared to food in the formal market. Over 90% of the Kenyan food is transacted in the informal market (Mutegi et al., 2018; Okoth et al., 2012; Walker et al., 2018). This study evaluated the status of *A. flavus* metabolites and other mycotoxins in Kenyan households maize with the aim of contributing data required to support legislative decisions for mycotoxins control in Kenya. Further, local open pollinated maize varieties were compared with commercial hybrids to evaluate which variety is less susceptible to mycotoxin contamination.

2. Methodology

2.1. Study site and Sampling

Maize samples were obtained from household storage facilities from farmers in Eastern, Coastal, Western and Lake Victoria regions of Kenya (Fig. 1). Western and the Lake Victoria regions were selected for the study due to previous reported high levels of AF and fumonisins in maize and peanuts (Mutegi et al., 2009; Mutiga et al., 2015). Eastern and the Coastal regions were selected for the study due to previous reported high levels of AF in maize and cases of human exposure to AF, respectively (Mutiga et al., 2017; Yard et al., 2013). In addition these regions were selected for the study because they are dominated by small scale farmers who plant both local open pollinated maize varieties (L-opv) and commercial hybrids (C-hy) (Marechera et al., 2019).

In this study, L-opv represents the maize varieties where individual farmers select and save seeds from year to year, allowing for adoption to local conditions. While C-hy represent maize varieties where farmers have to buy branded seeds from the agro-dealers each planting season. From each region, 40 samples were collected annually in the years 2018, 2019 and 2020. Of the 40 samples collected from each region, 20 were C-hy and 20 were L-opv. In each year, 160 samples were collected 80 C-hy and 80 L-opv and 480 samples were collected cumulatively after the 3 years of study, 240 C-hy and 240 L-opv. So as to get a sample as representative as possible, maize samples were acquired from each bag using a 1.5 m long compartmentalized grain probe from Cimbria®. The grain probe was inserted once down the middle of the bag and twice along the sides and once along each diagonal. Based on the number of bags in the store, representative samples were retrieved from at least two-thirds of the bags. A composite sample was made by thoroughly mixing samples from each bag, from which a 2 kg subsample was secured in double Ziplock bags (Walker et al., 2018). Representative samples were retrieved from all the 90 kg bags in the farmers stores with $<$10 bags. In cases where farmers had more than 10bags, samples were acquired from 10 bags randomly selected (Cochran, 2007; Okoth et al., 2012). To avoid cross-contamination, from one farmer to the next, the grain probe and associated sampling equipment were sanitized with 1% sodium hypochlorite (NaOCl) (Orbit Africa products ltd, Nairobi, Kenya) and further rinsed with ethanol/water (70/30, v/v) then dried before use (Walker et al., 2018). The samples were then transported to the mycology laboratory at the University of Nairobi, where they were kept refrigerated at 4 °C until further processing.

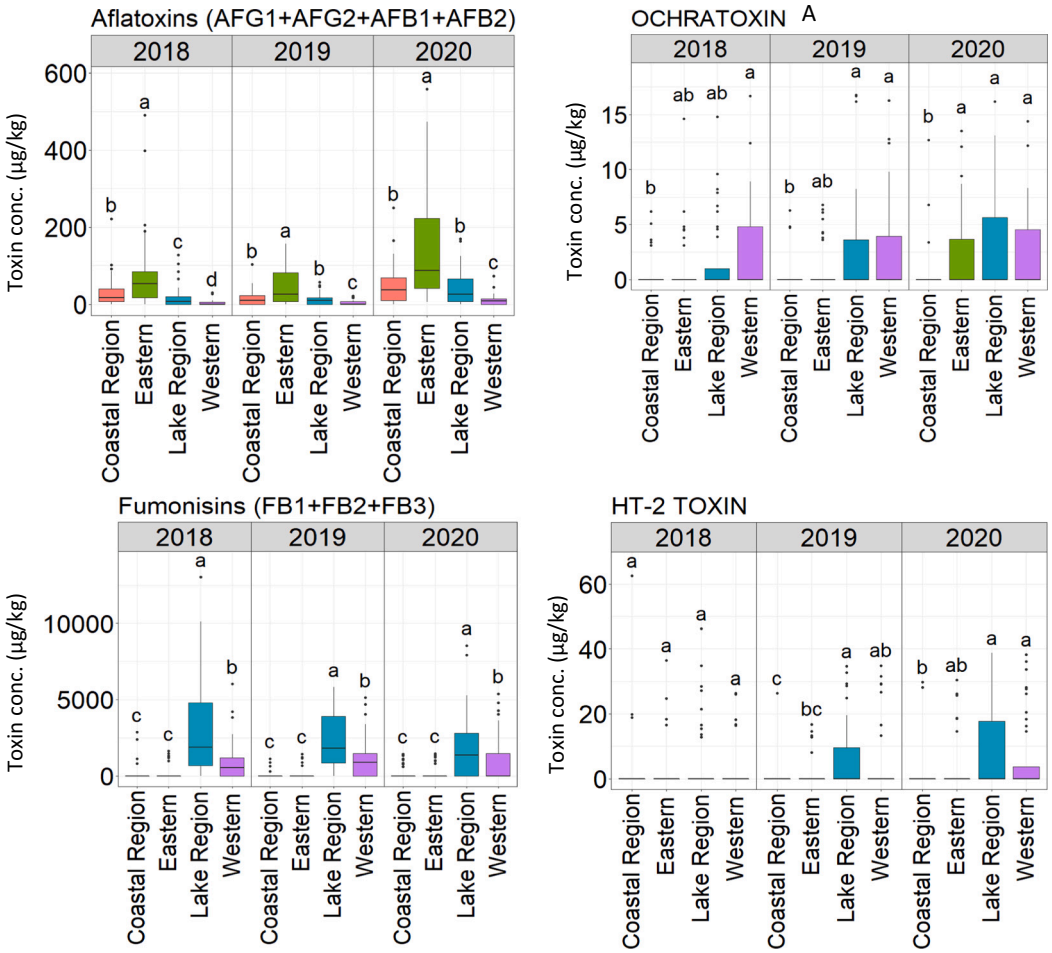

Fig. 2. Yearly and regional variation in mycotoxin concentration of total aflatoxins ($AFG_1 + AFG_2 + AFB_1 + AFB_2$), ochratoxin A, total fumonisins ($FB_1 + FB_2 + FB_3$) and HT-2 toxin in maize samples from Kenyan households. Within the box plots, a, b, c and d are alphabets obtained after a post-hoc Dunn test. Years and regions sharing the same alphabet do not significantly vary in toxin concentration.

2.2. Sample preparation

The maize kernels were ground using a laboratory mill. The samples were ground to flour of particulate size that can pass through a 150 µm sieve. The mill and associated equipment were sanitized with 1% NaOCl and further rinsed with ethanol/water (70/30, v/v) after every sample. The mill was dried using a blow dryer. The flour was homogenized and a 200 g subsample was packed and shipped to the Centre of Excellence in Mycotoxicology and Public Health (CEMPH) at Ghent University for mycotoxins analysis within 2 days.

2.3. Multiple mycotoxins' analysis

A Waters® Acquity UPLC system coupled with a Waters® Quattro Premier XE™ tandem quadrupole mass spectrometer equipped with MassLynx® for data processing was used to detect and quantify aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), T-2 toxin (T-2), HT-2 toxin (HT-2), nivalenol (NIV), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), diacetoxyscirpenol (DAS), fusarenon-X (F-X), neosolaniol (NEO), alternariol (AOH), alternariol monomethyl ether (AME), roquefortine-C (ROQ-C), and sterigmatocystin (STE) in all the samples using a Symmetry C18 column (5 µm, 150 × 2.1 mm) with a guard column (10 × 2.1 mm, Waters, Zellik, Belgium). Reagents, chemicals, sample preparation, solvents preparation and the LC-MS/MS analysis, settings and conditions were as described by (Monbaliu et al., 2010)), validated according to the European Commission Decision 2002/657/EC. The method was performed according to the standards of EN ISO 17025, where mycotoxin quantification began with construction of a calibration curve by spiking blank maize samples with known standards. Mycotoxins were extracted by adding 20 ml of acetonitrile/water/acetic acid (79/20/1, v/v/v) to 5 g of maize flour. The mixture was then agitated for 1 h prior to centrifugation. The supernatant was filtered through SPE columns and the filtrate defatted using hexane. Purification of the defatted extract was done using the Multisep® 226 columns after which the extract was evaporated by a gentle flow of pure dry nitrogen. The residue was then redissolved using the injection solvent ready for the LC-MS/MS analysis (Monbaliu et al., 2010).

2.4. Aspergillus flavus metabolites analysis

To detect the presence of AFB_1, AFB_2, AFG_1, AFG_2, STE, dihydro-*O*-methylsterigmatocystin (DHOMST), *O*-methylsterigmatocystin (OMST), dihydroxyl-*O*-methylsterigmatocystin (DHoxyHOMST), oxy-*O*-methylsterigmatocystin (OxyHOMST), AA, hydroxyneoaspergillic acid (HNAA), AFLV, dihydroxy-aflavinine (DHAFLV), AFLVR, aflavarin analogue$_1$ (AFLVR AN_1), aflavarin analogue$_2$ (AFLVR AN_2), AFTR, CPA, beta-cyclopiazonic acid (β CPA), $ASPT_1$, $ASPT_2$, PASL, VER, versiconol hemiacetal acid (VERHA), KA, FLV, methylcitreo-isocoumarin (MIC), LEP C, NOR, SPER and ditryptophenaline (DIRTYPTOPH), a Waters®

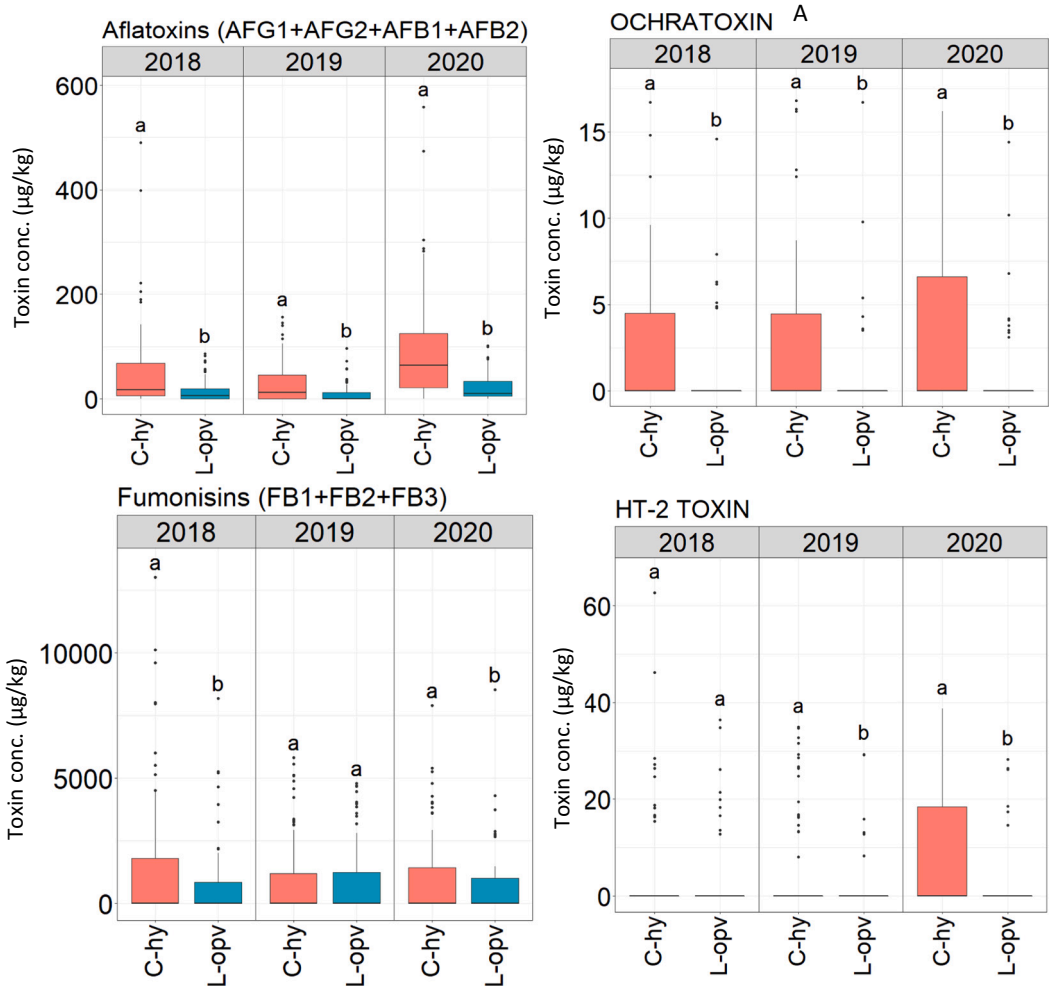

Fig. 3. Comparison of toxin contamination levels between local open pollinated (L-opv) maize varieties and commercial hybrids (C-hy) of total aflatoxins (AFG_1 + AFG_2 + AFB_1 + AFB_2), ochratoxin A, total fumonisins (FB_1 + FB_2 + FB_3) and HT-2 toxin. Within the boxplots, a and b are alphabets obtained after a post-hoc Dunn test. When the same alphabet is shared, then the toxin concentration do not significantly vary.

Acquity UPLC system coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA) equipped with MassLynx® software for data processing were used. A Zorbax Eclipse XDB C-18 column (1.8 µm, 100× 2.1 mm) was used. Reagents, chemicals, sample preparation, solvent preparation and the LC-MS/MS analysis settings and conditions were as described by (Okoth et al., 2018). The limit of detection (LOD) and limit of quantification (LOQ) were verified by the signal to noise ratio (s/n), which was greater than 3 and 10, respectively, agreeing with International Union of Pure and Applied Chemistry (IUPAC) guidelines. After constructing the standard curve, *A. flavus* metabolites were extracted by adding 5 ml acetonitrile to 5 g of maize flour, 10 ml of ultrapure water was then added followed by 20 ml of dichloromethane. The mixture was agitated for 45 min then centrifuged. Ten millilitres of the extract settled at the bottom of the extraction tube was retrieved, filtered then evaporated to dryness under a gentle flow of nitrogen. The residue was redissolved in the injection solvent then filtered through the centrifugal filters in readiness for LC-MS/MS analysis (Okoth et al., 2018).

2.5. Statistical analysis

Since the assumptions of normality and homoscedasticity for parametric analysis of variance (ANOVA) were not met, a non-parametric Kruskal-Wallis test was performed to test whether there was a significant effect on mycotoxin concentration as a result of maize variety, region and year. In cases where significant differences were observed (p-value <0.05) according to the Kruskal-Wallis test, a post-hoc Dunn test was done. This was so as to inspect where observed differences were situated. To test for correlations between the continuous toxin measurements, a Spearman correlations analysis was performed and correlograms developed where Spearman correlations between pairs of toxins could be observed. Statistical analyses were done in the R-studio version 4.0 (Wokorach et al., 2021).

3. Results

3.1. Regional and yearly differences in mycotoxin concentrations

Yearly variation in AFs, STE, ZEN and DON content in maize was significant with p-values of 0.000, 0.000, 0.011 and 0.022, respectively (Table 1). The Eastern region had the highest AF levels throughout the three years (Fig. 2). Fumonisins (F) levels, however, did not show yearly variation (p-values of 0.821, 0.866 and 0.869 for FB_1, FB_2 and FB_3, respectively) (Table 1). The fumonisins (F) levels, were highest in the Lake Victoria region and lowest in the Eastern region (Fig. 2). NEO and ROQ-C are the only mycotoxins that did not have any significant regional differences (p-values of 0.111 and 0.145, respectively) (Table 1).

Table 2
Comparison of mycotoxin contamination levels in local open pollinated (L-opv) maize varieties and in commercial hybrids (C-hy) maize varieties.

MYCOTOXIN	Comparison of mycotoxins concentration in L-opv and in C-hy maize varieties (*P*- values)[a]
Nivalenol (NIV)	0.056
Deoxynivalenol (DON)	0.031
Neosolaniol (NEO)	0.157
Fusarenon X (FX)	0.040
3 Acetyldeoxynivalenol (3 ADON)	0.073
15 Acetyldeoxynivalenol (15 ADON)	0.053
Aflatoxin G_2 (AFG_2)	0.000
Aflatoxin G_1 (AFG_1)	0.000
Aflatoxin B_2 (AFB_2)	0.000
Aflatoxin B_1 (AFB_1)	0.000
Diacetoxyscirpenol (DAS)	0.000
Alternariol (AOH)	0.000
HT-2 toxin (HT-2)	0.000
Fumonisin B_1 (FB_1)	0.007
T-2 toxin (T-2)	0.000
Fumonisin B_3 (FB_3)	0.008
Ochratoxin (OTA)	0.000
Fumonisin B_2 (FB_2)	0.007
Alternariol monomethyl ether (AME)	0.000
Sterigmatocystin (STERIG)	0.000
Roquefortine C (ROQ C)	0.709
Zearalenone (ZEN)	0.179

[a]P-values of Kruskal-Wallis tests. P-values < 0.05 indicate a significant difference in mycotoxin contamination between the two maize varieties at $\alpha = 0.05$.

3.2. Mycotoxin resistance of local open pollinated maize varieties compared to commercial hybrids

Local open pollinated maize varieties (L-opv) were less susceptible to mycotoxin contamination in comparison to the commercial hybrids (C-hy) (Fig. 3). Apart from NIV, NEO, 3ADON, 15ADON, ROQC and ZEN, the L-opvs had significantly lower mycotoxin levels compared to the C-hy (Table 2). This effect was consistent in all the three years of the study (Table 2 & Fig. 3).

3.3. Positive and negative correlations of 22 mycotoxins quantified in Kenyan maize

Positive correlations were observed between the trichothecenes (Fig. 4). NIV was positively correlated with DON ($r = 0.81$), 3-ADON ($r = 0.81$), 15-ADON ($r = 0.80$), FX ($r = 0.81$) and ZEN ($r = 0.70$). In contrast, AFB_1 negatively correlated with FB_3 ($r = -0.12$), AOH ($r = -0.23$), and ZEN ($r = -0.20$) (Fig. 4). Notably, AFs exhibited a negative correlation with all the other mycotoxins, except for STE which is also an *Aspergillus* produced mycotoxin (Fig. 4).

3.4. Aspergillus flavus metabolites and other emerging mycotoxins

Emerging mycotoxins were present in all the regions (Fig. 5). Over 75% of the samples from the Lake region and Western regions were contaminated with *Alternaria* mycotoxins compared to only 25% of the samples from Eastern and 48% of samples from the Coastal region (Fig. 5). Eighty percent of samples from Eastern were contaminated with STE while only 35% from Western were contaminated with STE (Fig. 5). Over 25% of all the samples were contaminated with ROQ C (Fig. 5).

The *A. flavus* metabolites profile differed from region to region and also varied in the 3 years of study (Figs. 6–8). Western region had the least *A. flavus* metabolites contamination (18%) while the Eastern region had the highest incidence of *A. flavus* metabolites (81%). The *A. flavus* metabolites incidence in Kenyan maize is represented by heatmaps in Figs. 6–8 below. On the vertical axis of the heatmaps, different sample codes are shown and on the horizontal axis the different *A. flavus* metabolites are shown. The dendrogram on top of the graph clusters the *A. flavus* metabolites and the dendrogram on the left side clusters the samples. In the heatmaps, dark grey color indicates presence of a metabolite, while light grey indicates the absence of a metabolite. Samples originating from the same location have the same color in the color bar on the left. On the color bar, Eastern region is denoted by green, Western region is denoted by yellow, Lake region is denoted by blue and red represents the Coastal region (Figs. 6-8).

CPA, β CPA, FLV and MIC positively correlated with each other but negatively correlated with the other metabolites (Fig. 9).

3.5. Mycotoxin incidence above regulatory standards

Seventy five percent of the samples from Eastern region had total aflatoxins levels above the Kenyan regulatory limits. The most contaminated sample had a concentration of 558 μg/kg. In the Western region, only 18% of samples had total aflatoxins above the regulatory limit with the most contaminated sample from Western Kenya having a concentration of 73 μg/kg. The Lake Victoria region had the most fumonisins contamination, with 52.5% of the samples having $FB_1 <$ 1000 μg/kg. However, only 20% of the samples surpassed the Kenyan regulatory limit for total fumonisins with most contaminated sample having a concentration of 13,022 μg/kg. In addition, 25% samples from the Lake Victoria region also had DON $>$ 1000 μg/kg (Fig. 10).

4. Discussion

4.1. Maize harvest handling and processing

The general farmer practise of harvest handling and processing entails sun drying of the cobs either by placing the maize cobs directly on the ground/soil or on a tarpaulin for 5–7 days. Threshing is then done by hand for the majority of the farmers, however, a few farmers seek the services of commercial maize threshers. After threshing, the farmers further sun-dry the kernels for another 5–7 days, by placing their kernels on mats, plastered areas, plastic sheets or on a tarpaulin. Farmers estimate the moisture content of the kernels using the sound made by kernels when agitated by hand or the difficulty level in biting through the kernel. Usually, 5–7 days drying period after threshing is sufficient to adequately dry the maize and make it ready for storage. In most cases, the moisture content under the farmer's drying practices is approximately 15% (Walker et al., 2018) . Farmers do not have moisture meters therefore 15% moisture content is generally acceptable in the informal market where the subsistence farmers transact their trade in maize (Mutegi et al., 2018; Walker et al., 2018). Since the small holder farmers store small volumes, the moisture content of the grain equilibrates with the environment when storage is done in non-hermetic devices hence maize can go in to storage at 15% moisture content without ramifications. For hermetic storage, further drying is necessary (Walker et al., 2018). In this study the average moisture content for maize from Eastern Kenya was 16.5%. For the Lake Victoria region the average moisture content was15.8%. For the Coastal region and Western region, the average moisture content was 14.6% and 13.7% respectively. This is a possible explanation to the observed mycotoxin distribution. Notably, the small holder farmers cited maize weevil (*Sitophilus zeamais*), Angoumois grain moth (*Gelechiidae* sp), larger grain borer (*Prostephanus truncatus*) and rats as their major post-harvest storage concern. Presence

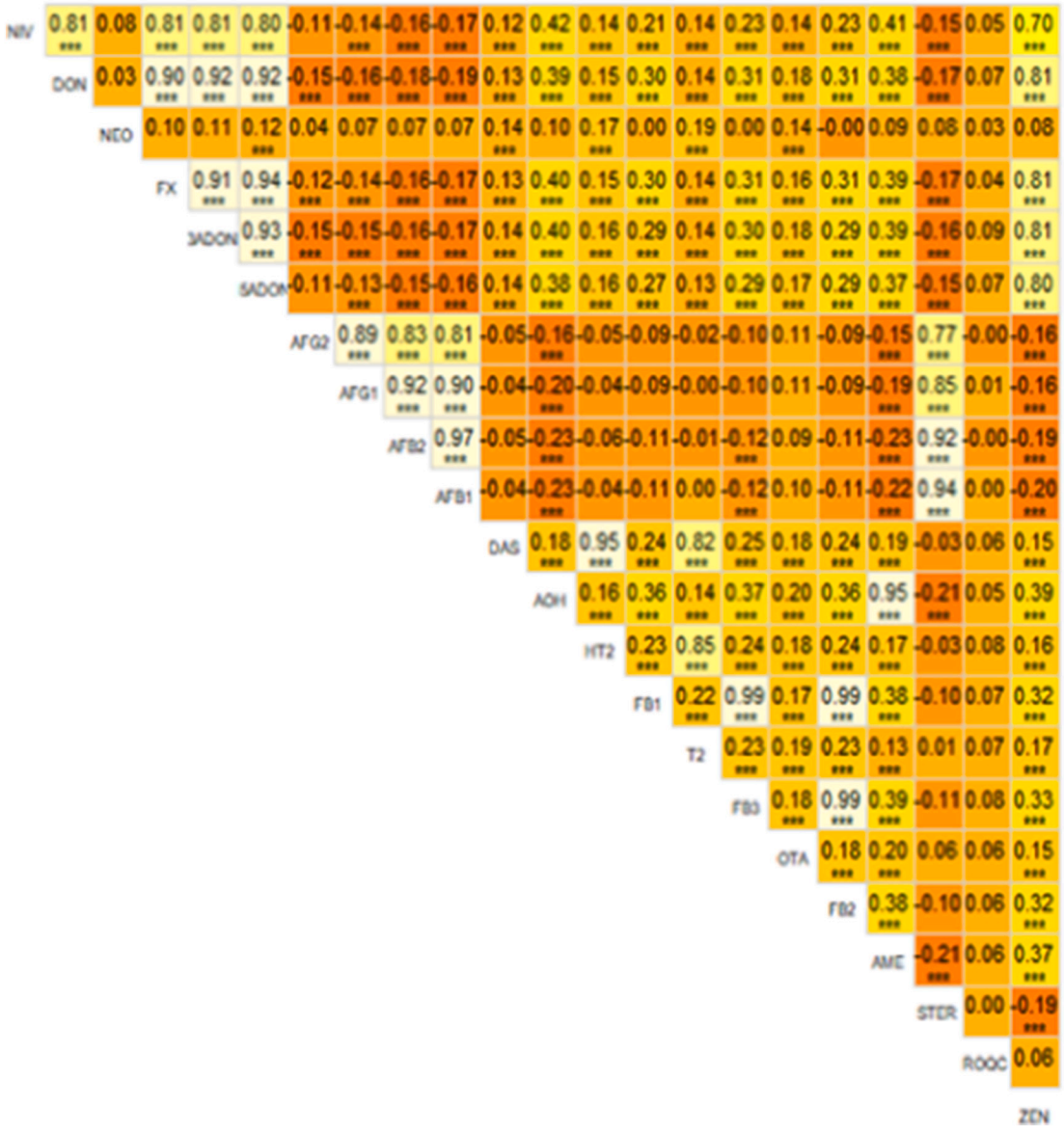

Fig. 4. Positive and negative correlation between mycotoxins recovered from Kenyan maize. The mycotoxins are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), deoxynivalenol (DON),), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), T-2 toxin (T-2), HT-2 toxin (HT-2), nivalenol (NIV), 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), diacetoxyscirpenol (DAS), fusarenon-X (F-X), neosolaniol (NEO), alternariol (AOH), alternariol monomethyl ether (AME), roquefortine-C (ROQ-C), and sterigmatocystin (STE). The colors range from white to yellow (positive correlation) and from orange to red (negative correlation) where white denotes high positive correlations whereas red denotes high negative correlation and orange intermediate *** indicates significant correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of storage pests contributes to mycotoxin proliferation in stored grain (Walker et al., 2018). For many small holder farmers, mycotoxins contamination is not considered a problem since it is invisible and does not affect marketability and sale of the grain (Mutungi et al., 2019; Walker et al., 2018). Therefore offering a special price for mycotoxin free maize can offer the necessary impetus for farmers to consider investing in mycotoxin mitigation technologies.

4.2. Regional and yearly differences in mycotoxins

Eastern Kenya is an AF-hotspot having reported on numerous occasions, aflatoxicosis-related loss of life (Mutegi et al., 2018; Okoth, 2016; Okoth et al., 2018; Probst et al., 2012). This study confirmed these events with Eastern region recording highest AF-levels compared to the other regions. Fumonisins (F) and other *Fusarium* mycotoxins were more prevalent in the Lake Victoria and Western regions, a trend previously reported in a study by (Mutiga et al., 2015), where 87% of samples from Western and Lake regions of Kenya had detectable levels of F. In this study, over 25% and over 20% of the samples from the lake Victoria region had DON above 1000 μg/kg and total F above 2000 μg/kg, respectively. Regional variation in mycotoxin contamination can be attributed to region-specific microbial biodiversity and prevailing environmental conditions. Indeed, aflatoxin overproducing strains of *A. flavus* are known to habitate the Eastern Kenya soils a possible contributing factor the observed high AF prevalence in Eastern (Dooso Oloo et al., 2019; Okoth et al., 2018; Probst et al., 2012). Differences in rainfall and prevailing temperatures were responsible for the varying total AF, OTA and HT-2 toxin quantities in the different years. High levels of these mycotoxins were observed in 2020, compared to the other years. According to data from the Kenya metrological department

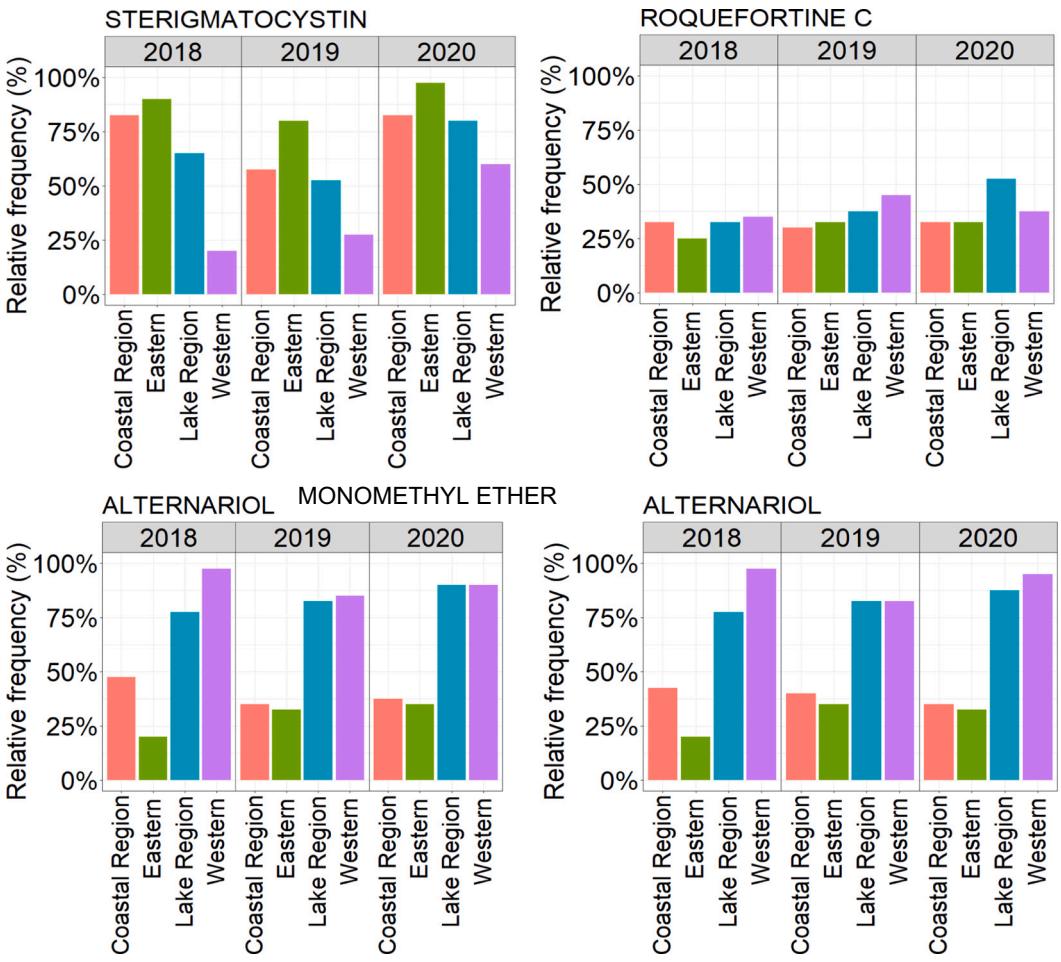

Fig. 5. Prevalence of sterigmatocystin (STE), roquefortine C (ROQ C), alternariol monomethyl ether (AME) and alternariol (AOH) in Coastal, Eastern, Western and Lake Victoria regions of Kenya.

(KMD), in 2020, farmers experienced heavy rainfall during the harvesting season coupled with moderate temperatures for several days (KMD, 2021). With the annual average temperature of 19.7 °C and 130/365 total days with rain (KMD, 2021), prevailing environmental conditions were ideal for fungal growth and mycotoxin production (Cotty and Jaime-Garcia, 2007; KMD, 2021). On the contrary, in 2019, cyclone *Idai* lead to a delayed onset of the long rains season all over Kenya giving farmers ample time to dry their grains potentially resulting to a reduced mycotoxin incidence (Wainwright et al., 2021). This suggests that in Kenya, most fungal contamination and mycotoxin production occurs post-harvest during produce handling, processing and in storage. With the changing climate, concern is growing since high temperatures characterized by low precipitation during the growing season and high rainfall during the harvesting season is becoming the norm. These unusual environmental factors are creating favourable conditions for fungal attack and mycotoxin production as was the case in 2020 (Assunção et al., 2018; Mutiga et al., 2015).

4.3. Mycotoxin resistance of the local open pollinated maize varieties

Local open-pollinated maize varieties (L-opv) demonstrated lower susceptibility to mycotoxin accumulation compared to the commercial hybrids (C-hy). This was observed for both *Aspergillus* and *Fusarium* mycotoxins. The double resistance is attributable to the fact that the quantitative trait loci associated with *Fusarium* and *Aspergillus* resistance are clustered in the same chromosome region in the maize genome (Okoth et al., 2017; Rose et al., 2017). Most of the open-pollinated varieties were flint and early maturing compared to the commercial hybrids which were mostly late maturing and dent. Flint maize kernels are more resistant to mycotoxins, pests and diseases due to a harder pericarp which confers more protection (Mutiga et al., 2017; Suleiman et al., 2015). Early maturity exhibited by the L-opv also gave them an advantage over the C-hy since late maturity coupled with longer drying periods potentially extends the conditions ideal for fungal colonization and mycotoxins production (Rose et al., 2017). In terms of yield, C-hy produced more per unit area compared to L-opv. Therefore, in addition to high yield, there is a need for breeders to incorporate mycotoxin resistance in germplasm improvement (Mutiga et al., 2017; Okoth et al., 2017).

4.4. Fusarium mycotoxins

Among the *Fusarium*-derived mycotoxins, of primary concern in the Kenyan maize were, fumonisins (F), produced by *Fusarium verticillioides* and *F. proliferatum*. Over 52.2% of samples from Lake Victoria region and 38% of samples from Western region had FB_1 levels surpassing 1000 μg/kg. F are associated with liver and kidney toxicities, neural tube defects and esophageal carcinomas (Bakker et al., 2018; Kibe, 2015). ZEN produced by *F. graminearum*, *F. cerealis* and *F. sambucinum* linked with estrogen toxicity was also of concern in the Kenyan maize more so since it is unregulated. Of the tested samples, 18% had ZEN levels above 1000 μg/kg (Bakker et al., 2018; Cai et al., 2018; Kibe, 2015). A family of terpenoid toxins known as trichothecenes were also present in the Kenyan maize. Of concern was the high levels DON with over 25% of the samples from Lake Victoria region and 17% of the samples from Western region surpassing the 1000 μg/kg. Trichothecenes are produced by

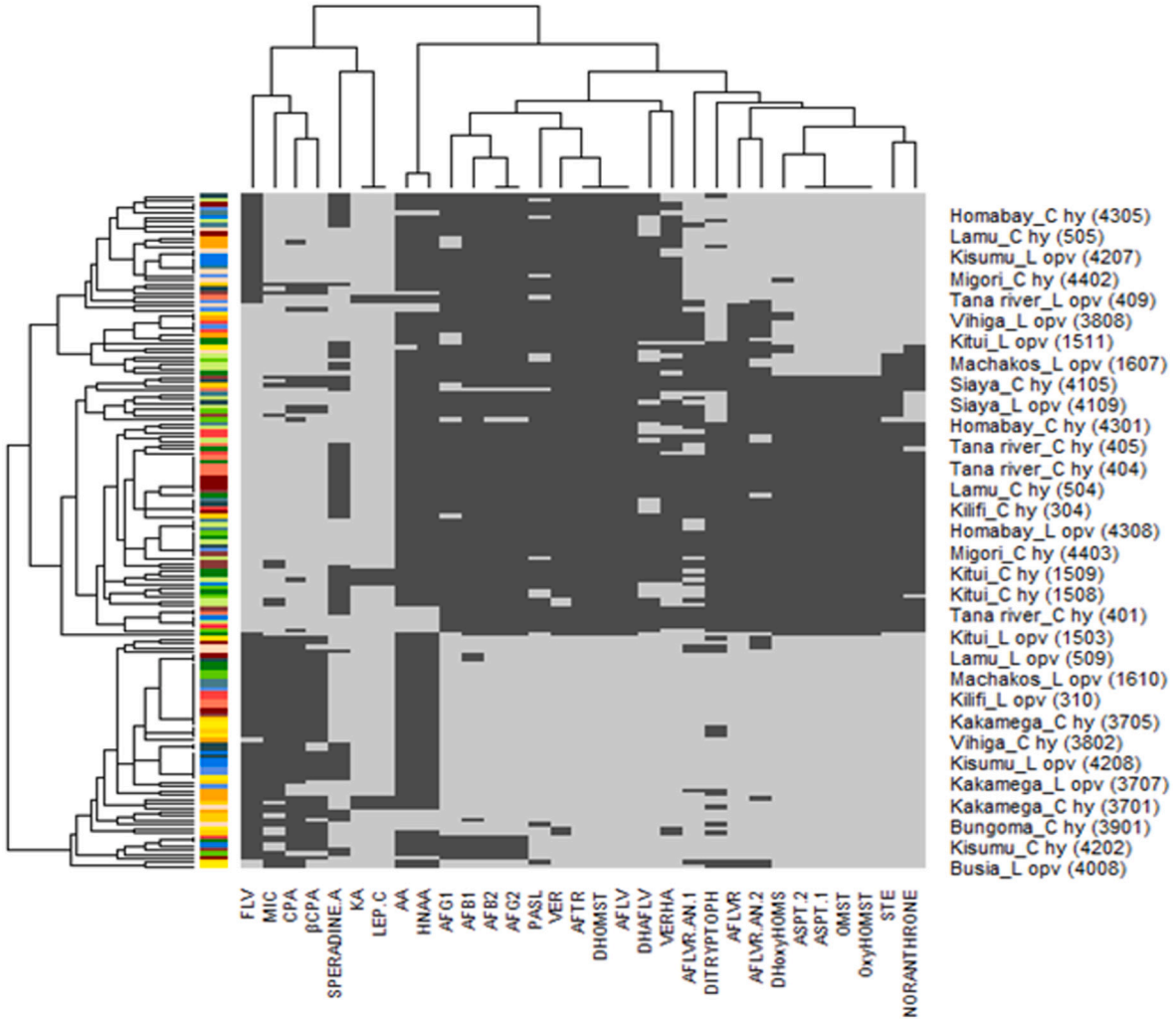

Fig. 6. Incidence of *A. flavus* metabolites in counties from Eastern, Western, Coastal and Lake Victoria regions of Kenya in 2018. On the vertical axis, to the right, counties of sample collection are shown. On the horizontal axis, at the bottom, the different *A. flavus* metabolites detected are shown. The dendrogram on top of the graph clusters the *A. flavus* metabolites and the dendrogram on the left side clusters the samples based on place of origin. Samples originating from the same county have the same color in the color bar on the left. Dark grey color indicates presence of an *A. flavus* metabolite, while light grey indicates the absence of a metabolite. *A flavus* metabolites detected are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), sterigmatocystin (STE), dihydro-*O*-methylsterigmatocystin (DHOMST), *O*-methylsterigmatocystin (OMST), dihydroxyl-*O*-methylsterigmatocystin (DHoxyHOMST), oxy-*O*-methylsterigmatocystin (OxyHOMST), aspergillic acid (AA), hydroxyneoaspergillic acid (HNAA), aflavinine (AFLV), dihydroxy-aflavinine (DHAFLV), aflavarin (AFLVR), aflavarin analogue-$_1$ (AFLVR AN $_1$), aflavarin analogue-$_2$ (AFLVR AN $_2$), aflatrem (AFTR), cyclopiazonic acid (CPA), beta-cyclopiazonic acid (β CPA), aspertoxin ASPT $_1$, aspertoxin (ASPT $_2$), paspalinine (PASL), versiconol (VER), versiconol hemiacetal acid (VERHA), kojic acid (KA), flavacol (FLV), methylcitreo-isocoumarin (MIC), leporine C (LEP C) and ditryptophenaline (DIRTYPTOPH). Western counties (yellow) are Busia, Kakamega, Bungoma and Vihiga. Lake Victoria counties (blue) are Kisumu, Migori, Homabay and Siaya. Eastern counties (green) are Kitui, Machakos and Makueni. Coastal counties (red) are Lamu, Kilifi, Taita taveta and Tana River. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

members of the *Fusarium graminearum* species complex (FGSC). FGSC comprises *F. graminearum*, *F. poae*, *F. cerealis*, *F. sambucinum*, *F. sporotrichioides*, *F. culmorum*, *F. acuminatum* and *F. tricinctum*. Trichothecenes have been reported to have the ability to inhibit eukaryotic protein synthesis (Bakker et al., 2018; Kibe, 2015; Proctor et al., 2018).

4.5. Co-occurrence of mycotoxins

All the *Fusarium* mycotoxins co-occurred and were prevalent in samples from the Lake region. *Aspergillus* mycotoxins also co-occurred and were prevalent in samples from the Eastern region. Notably, *Aspergillus* and *Fusarium* mycotoxins had negative correlations attributable to the fact that the fungus *Aspergillus* is saprophytic, while *Fusarium* is pathogenic, suggesting that they occupy different niches in the ecosystem (Amaike and Keller, 2011; Ma et al., 2013). In the collerogram Fig. 4, positive correlation means that if one mycotoxin is present, then it is very likely that the other mycotoxin which it correlates with is also present. A negative correlation means that if one mycotoxin is present, then it is very unlikely that the other mycotoxin which it negatively correlates to will be present. Co-occurrence can prevail when multiple moulds co-infect crops at any point along the value chain, and they produce mycotoxins or when a single mould with the ability to produce several mycotoxins infect the crop. In this study, mycotoxins co-occurrence was as a result of both scenarios. For example, in cases where ZEA, OTA, NIV and ROQ-C all were present in one sample, it meant several moulds had co-infected the crop since ZEA and NIV are synthesized by *F. graminearum*, OTA by *A. ochraceus* and ROQ-C by *P. roqueforti*. While in cases where NIV, DON and ZEA were all present in one sample, it meant that possibly the crop had been infected with *F. graminearum*, which could synthesize all the 3 toxins. Mould interaction at substrate level may influence mycotoxins production synergistically or even antagonistically (Kemboi et al., 2020; Magan et al.,

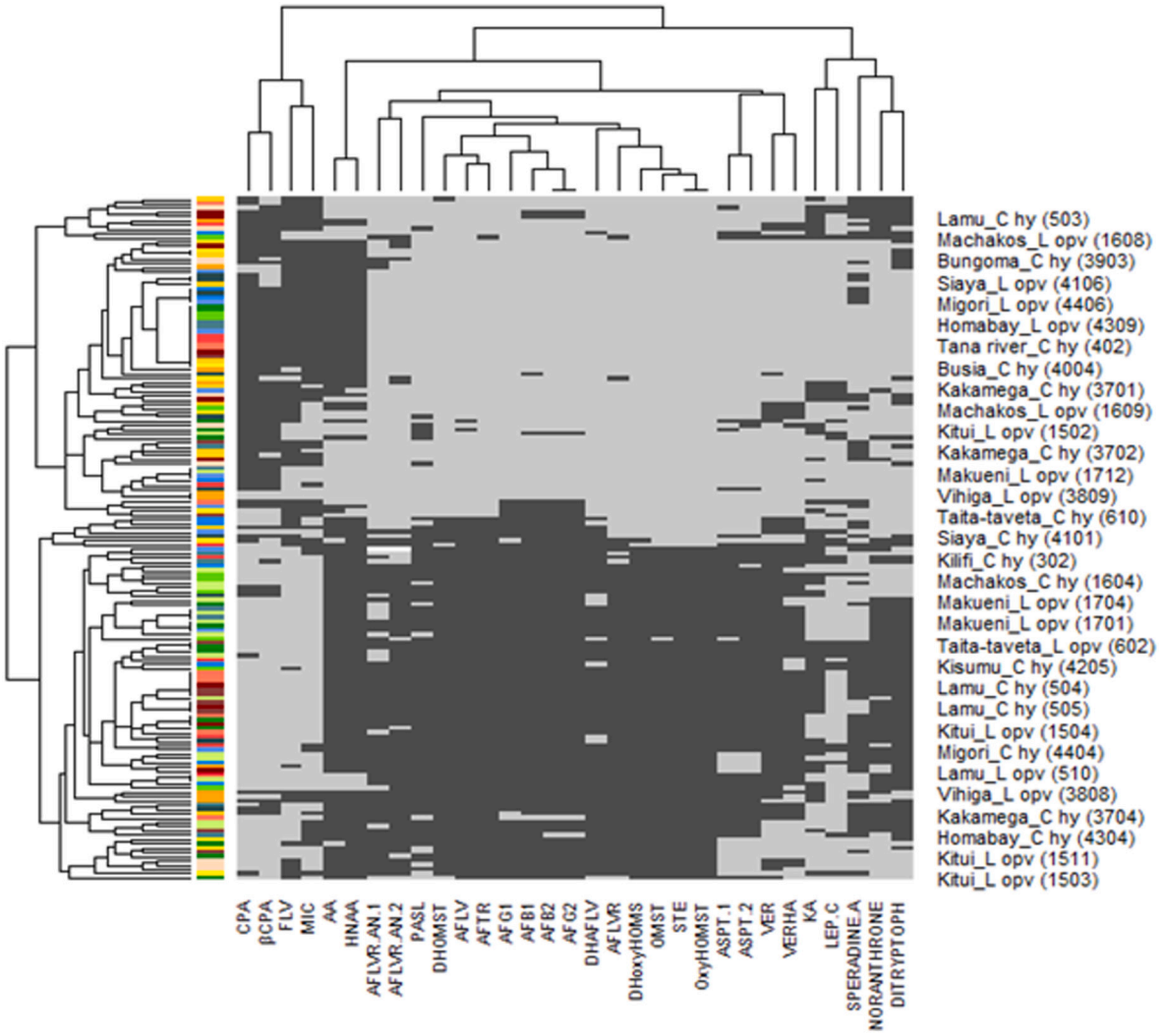

Fig. 7. Incidence of *A. flavus* metabolites in counties from Eastern, Western, Coastal and Lake Victoria regions of Kenya in 2019. On the vertical axis, to the right, counties of sample collection are shown. On the horizontal axis, at the bottom, the different *A. flavus* metabolites detected are shown. The dendrogram on top of the graph clusters the *A. flavus* metabolites and the dendrogram on the left side clusters the samples based on place of origin. Samples originating from the same county have the same color in the color bar on the left. Dark grey color indicates presence of an *A. flavus* metabolite, while light grey indicates the absence of a metabolite. *A flavus* metabolites detected are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), sterigmatocystin (STE), dihydro-*O*-methylsterigmatocystin (DHOMST), *O*-methylsterigmatocystin (OMST), dihydroxyl-*O*-methylsterigmatocystin (DHoxyHOMST), oxy-*O*-methylsterigmatocystin (OxyHOMST), aspergillic acid (AA), hydroxyneoaspergillic acid (HNAA), aflavinine (AFLV), dihydroxy-aflavinine (DHAFLV), aflavarin (AFLVR), aflavarin analogue-$_1$ (AFLVR AN $_1$), aflavarin analogue-$_2$ (AFLVR AN $_2$), aflatrem (AFTR), cyclopiazonic acid (CPA), beta-cyclopiazonic acid (β CPA), aspertoxin ASPT $_1$, aspertoxin (ASPT $_2$), paspalinine (PASL), versiconol (VER), versiconol hemiacetal acid (VERHA), kojic acid (KA), flavacol (FLV), methylcitreo-isocoumarin (MIC), leporine C (LEP C) and ditryptophenaline (DIRTYPTOPH). Western counties (yellow) are Busia, Kakamega, Bungoma and Vihiga. Lake Victoria counties (blue) are Kisumu, Migori, Homabay and Siaya. Eastern counties (green) are Kitui, Machakos and Makueni. Coastal counties (red) are Lamu, Kilifi, Taita taveta and Tana River. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2003). When food is contaminated with multiple mycotoxins, even in relatively low concentrations, their potential additive effects becomes a great public health concern (Kemboi et al., 2020; Mwihia et al., 2020; Njobeh et al., 2010). Notable negative correlations were observed between the *A. flavus* mycotoxins and the other mycotoxins, suggesting that the presence of *A. flavus* potentially displace the other mycotoxin-producing fungi. The ability of one fungus to outcompete another has been exploited in development of the biological control agent Aflasafe KE01® registered in Kenya as a biocontrol formulation against AF (Kagot et al., 2019; Migwi et al., 2020). Since aflatoxins did not co-occur with other mycotoxin, then production of aflatoxins likely confers an ecological advantage to *A. flavus* in its niche.

4.6. *Aspergillus flavus metabolites and emerging mycotoxins*

The *A. flavus* genome has 56 secondary metabolites gene clusters therefore, *A. flavus* can potentially synthesize at least 56 distinct secondary metabolites. However, these gene clusters are normally not expressed on the standard laboratory media thus presenting a hurdle in attempts to unravel these metabolites (Cary et al., 2018). This study documented the presence of 31 *A. flavus* metabolites in Kenyan households maize some of which have been shown to be toxic (Uka et al., 2019). Fortunately most of these metabolites had a positive correlation with AF and with the efforts put in place to mitigate aflatoxins, then potentially these metabolites are mitigated as well. Of concern are CPA, β CPA, FLV and MIC which negatively correlated with all the other metabolites and could be present in food even when AF is effectively mitigated. Other emerging mycotoxins which were present in the

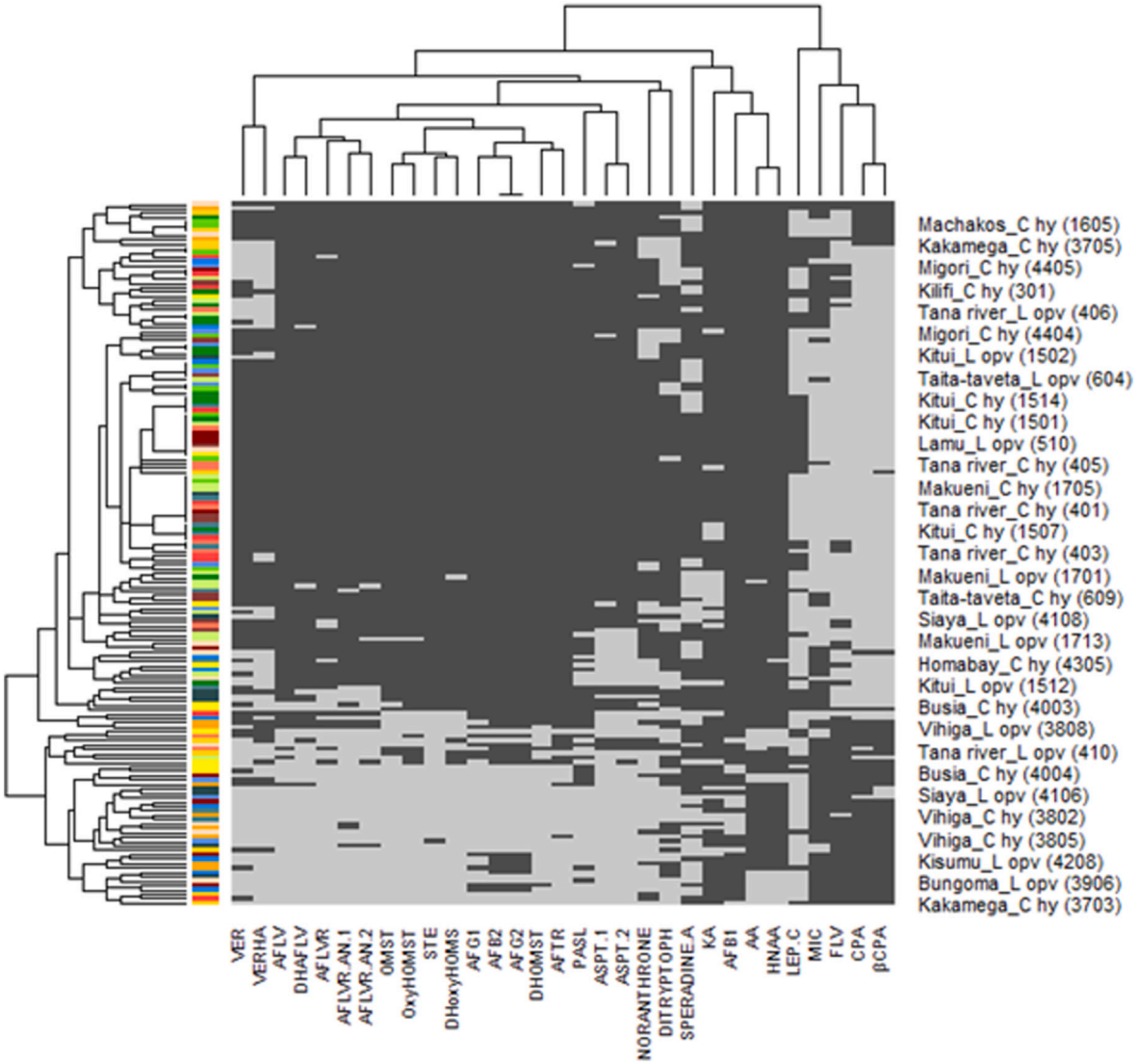

Fig. 8. Incidence of *A. flavus* metabolites in counties from Eastern, Western, Coastal and Lake Victoria regions of Kenya in 2020. On the vertical axis, to the right, counties of sample collection are shown. On the horizontal axis, at the bottom, the different *A. flavus* metabolites detected are shown. The dendrogram on top of the graph clusters the *A. flavus* metabolites and the dendrogram on the left side clusters the samples based on place of origin. Samples originating from the same county have the same color in the color bar on the left. Dark grey color indicates presence of an *A. flavus* metabolite, while light grey indicates the absence of a metabolite. *A flavus* metabolites detected are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), sterigmatocystin (STE), dihydro-*O*-methylsterigmatocystin (DHOMST), *O*-methylsterigmatocystin (OMST), dihydroxyl-*O*-methylsterigmatocystin (DHoxyHOMST), oxy-*O*-methylsterigmatocystin (OxyHOMST), aspergillic acid (AA), hydroxyneoaspergillic acid (HNAA), aflavinine (AFLV), dihydroxy-aflavinine (DHAFLV), aflavarin (AFLVR), aflavarin analogue-$_1$ (AFLVR AN $_1$), aflavarin analogue-$_2$ (AFLVR AN $_2$), aflatrem (AFTR), cyclopiazonic acid (CPA), beta-cyclopiazonic acid (β CPA), aspertoxin ASPT $_1$, aspertoxin (ASPT $_2$), paspalinine (PASL), versiconol (VER), versiconol hemiacetal acid (VERHA), kojic acid (KA), flavacol (FLV), methylcitreo-isocoumarin (MIC), leporine C (LEP C) and ditryptophenaline (DIRTYPTOPH). Western counties (yellow) are Busia, Kakamega, Bungoma and Vihiga. Lake Victoria counties (blue) are Kisumu, Migori, Homabay and Siaya. Eastern counties (green) are Kitui, Machakos and Makueni. Coastal counties (red) are Lamu, Kilifi, Taita taveta and Tana River. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

samples and are not synthesized by *A. flavus* were ROQ C, AME and AOH. More attention should be given to emerging mycotoxins and studies on exposure and toxicological effects should be done so as to determine the risk posed by these mycotoxins (Milićević et al., 2010).

4.7. Mycotoxins regulatory limits

Over 40% of the samples had mycotoxins above the regulatory limit for AF and fumonisins in Kenya- the only two mycotoxins currently being regulated in Kenya. The presence of unregulated (OTA, DON and ZEA) mycotoxins in concentrations above the regulation for European countries should be of concern to all stakeholders (Mutiga et al., 2021). Mycotoxin limit regulation is a last resort measure to protect consumers from the adverse health effects posed by these contaminants. While suggestions to borrow or harmonize regulatory standards have been made and have been welcomed by the international food and feed business community, this approach remains impractical. A robust regulatory framework must factor in food preferences and feeding patterns. For instance, in Kenya, maize-based foods are consumed at least twice daily in many homes, therefore Kenyan regulation for maize should be low compared to a place where maize is consumed once a week (Matumba et al., 2017). While acknowledging the current scientific discourse on the actual toxicity of some mycotoxins, regulatory limits for established toxicants like OTA, ZEN and DON (Battacone et al., 2010; Liang et al., 2015) should be considered without further debate among stakeholders.

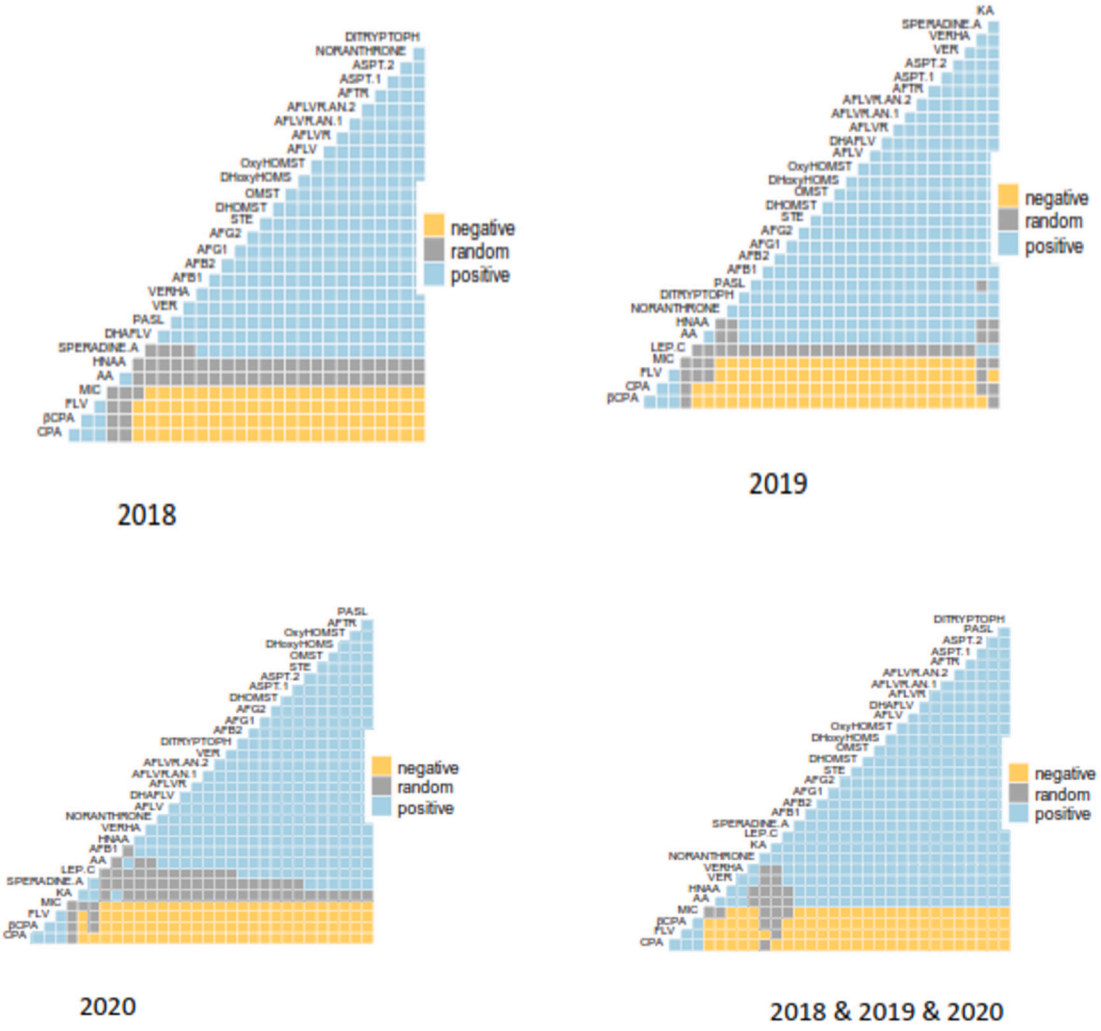

Fig. 9. Co-occurrence of detected *A. flavus* metabolites. Positive means that if one metabolite is present then it is very likely that the other metabolite with which they have a positive correlation is also present. Negative means that if one metabolite is present, then it is highly unlikely that the other metabolite with which they have negative correlation is also present. Random means no conclusion can be drawn about presence the metabolite in relation to others. *A flavus* metabolites detected are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), sterigmatocystin (STE), dihydro-*O*-methylsterigmatocystin (DHOMST), *O*-methylsterigmatocystin (OMST), dihydroxyl-*O*-methylsterigmatocystin (DHoxyHOMST), oxy-*O*-methylsterigmatocystin (OxyHOMST), aspergillic acid (AA), hydroxyneoaspergillic acid (HNAA), aflavinine (AFLV), dihydroxy-aflavinine (DHAFLV), aflavarin (AFLVR), aflavarin analogue-$_1$ (AFLVR AN $_1$), aflavarin analogue-$_2$ (AFLVR AN $_2$), aflatrem (AFTR), cyclopiazonic acid (CPA), beta-cyclopiazonic acid (β CPA), aspertoxin $_1$ (ASPT $_1$), aspertoxin $_2$ (ASPT $_2$), paspalinine (PASL), versiconol (VER), versiconol hemiacetal acid (VERHA), kojic acid (KA), flavacol (FLV), methylcitreo-isocoumarin (MIC), leporine C (LEP C) and ditryptophenaline (DIRTYPTOPH).

5. Conclusion

This study unravelled 22 mycotoxins and 31 *A. flavus* metabolites present in Kenyan maize. This should be of public health concern factoring in the potential synergistic effects that these mycotoxins could have. In addition to AF and fumonisins, regulatory authorities should also do regular surveillance and testing for OTA, DON and ZEN as comprehensive legislation is put in place to protect the public against chronic exposure to these mycotoxins regulated in other parts of the world. Further, studies on the toxicological effects of emerging mycotoxins -now confirmed present-in Kenya should be done in tandem with risk assessment and exposure tests. This will aid in filling of gaps currently existing in toxicity and exposure data that is required to support legislative decisions for mycotoxins control.

CRediT author contribution statement

Victor Kagot: conceptualization, investigation, data curation, writing, review and editing. **Sofie Landschoot:** data analysis, data visualization, review and editing. **George Obiero:** conceptualization, supervision, review and editing. **Marthe De Boevre:** conceptualization, resource mobilization, supervision, review and editing. **Sheila Okoth:** conceptualization, supervision, review and editing. **Sarah De Saeger:** conceptualization, resource mobilization, project administration, supervision, review and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

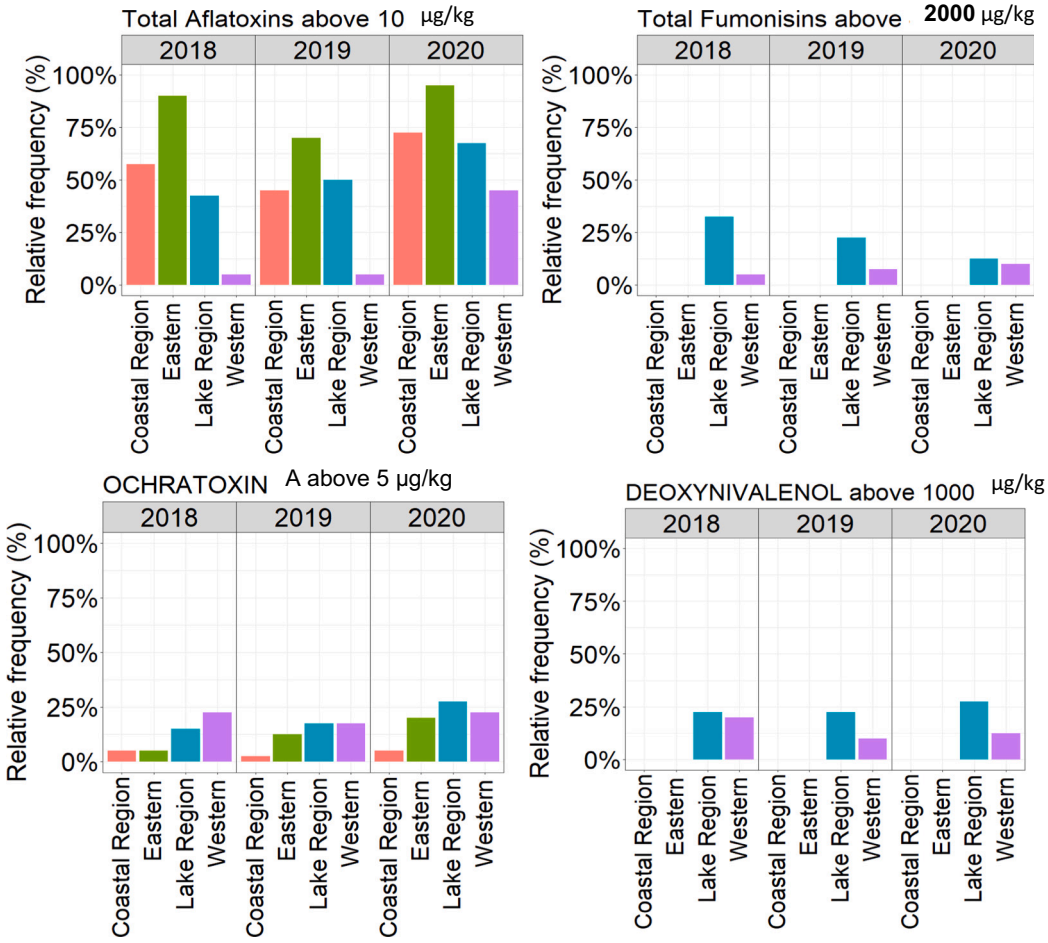

Fig. 10. Incidence of total aflatoxins and total fumonisins, above Kenyan regulation of 10 µg/kg and 2000 µg/kg respectively. OTA > 5 µg/kg and DON > 1000 µg/kg.

the work reported in this paper.

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