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### Occurrence of *Aspergillus flavus* and *Aspergillus parasiticus* on Stored Maize in Morogoro municipality and Makambako district, Tanzania

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#### Abstract

Maize is an important cash and food crop grown in Tanzania and other parts of the world. The crop is susceptible to fungal infestation and subsequent mycotoxins contamination that negatively affects human and animal health as well as the socio-economic status of the farmers and respective stakeholders. The study was conducted to determine occurrence of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) on maize stored in warehouses found in Morogoro municipality and Makambako district. A total of 226 maize samples were collected from six wards in the selected study areas then analysed for aflatoxigenic fungal infestation. Potato Dextrose Agar (PDA) was used for isolation of the fungi which were then identified to species level via observation of morphological characteristics with aid of taxonomic keys. The proportional occurrence of *A. parasiticus* and *A. flavus* with respect to areas where samples were collected was presented using descriptive statistics. The result showed that only 15% (34/226) of the collected samples were contaminated with *Aspergillus flavus* and *Aspergillus parasiticus*. In the collected samples, the incidence of *A. flavus* was higher than that of *A. parasiticus* whereby 28 samples were contaminated with *A. flavus* and 6 samples with *A. parasiticus*. The study elucidated that the methods used in the storage of this major staple food used by majority of Tanzanians makes it vulnerable to infestation and subsequent contamination by aflatoxigenic fungi. We therefore recommend that responsible sectors should implement appropriate intervention strategies designed to reduce occurrence of aflatoxigenic *Aspergillus* species on stored maize.

#### 1. Introduction

*Aspergillus* species are filamentous, cosmopolitan and ubiquitous fungi found naturally in different components of environment including soil, air and contaminated food (Palencia *et al.*, 2010; Karthikeyan *et al.*, 2013). They are diverse in terms of habitat and are versatile in terms of their ability to produce toxins as secondary metabolites. Many of them are pathogenic to plant and animals while others are useful in industrial food production (Palencia *et al.*, 2010). Under favourable conditions, various *Aspergillus* species can grow in maize grains during pre-harvesting, processing or storage stage and lead to maize spoilage by mycotoxins production (Nazir *et al.*, 2014; Egbuta *et al.*, 2015). The growth as well as toxins-producing ability of the species is favoured by important environmental conditions particularly high moisture and temperature (Karthikeyan *et al.*, 2013; Balendres *et al.*, 2019). Other studies reported that humidity due to leakage of stores and insect damage are prerequisite factors for the growth of mycotoxins-producing fungi on well-dried grains (Shitu *et al.*, 2018). Aflatoxins, fumonisins and ochratoxins are important mycotoxins produced by the *Aspergillus* species. These toxins

have gained global public health significance due to their deleterious effects on both human and animal health (Palencia *et al.*, 2010; Chilaka *et al.*, 2012). Aflatoxins have gained more attention compared to other mycotoxins produced by *Aspergillus* species due to their harmful effects in human and animal health. *Aspergillus flavus* and *Aspergillus parasiticus* are members of the *Aspergillus* genus, commonly known to produce aflatoxins on a wide range of agricultural commodities especially on cereal grains (Dhanasekaran *et al.*, 2011; Guchi, 2015). Ellis *et al.* (1991) and Odhiambo *et al.* (2013) reported that these species can produce aflatoxins on a wide variety of substrates under specific conditions of temperature, humidity and water activity. However, the presence of aflatoxins on a substrate is not determined by the presence or absence of aflatoxigenic mould on that substrate since the toxins may persist long after the mould growth has disappeared (Odhiambo *et al.*, 2013). Currently, several aflatoxins have been discovered but four major types of aflatoxins are considered more potentially dangerous to humans and domestic animals health. These are aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), aflatoxin B<sub>2</sub> (AFB<sub>2</sub>), aflatoxin G<sub>1</sub> (AFG<sub>1</sub>) and aflatoxin G<sub>2</sub> (AFG<sub>2</sub>) (Balendres *et al.*, 2019; Khan *et al.*, 2020). Apart from these, aflatoxin M<sub>1</sub>

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(AFM<sub>1</sub>) and aflatoxin M<sub>2</sub> (AFM<sub>2</sub>) are other significant members of the aflatoxin family commonly found in milk, dairy products, meat and eggs (Guchi, 2015; Wu, 2015). Previously conducted studies highlighted that aflatoxin-contamination in crops is associated with ill-health effects in humans and animals including kidney and liver infections, immuno suppression, mutagenesis and teratogenesis (Bbosa et al., 2013; Seetha et al., 2017). On the broader sense, aflatoxin-contamination leads to greater economic losses by reducing the trade potential of the crops exported for international trade (Seetha et al., 2017). Maize production assures a source of income, poverty reduction and food security as it is grown and used as a staple food crop in all regions of Tanzania (Lyimo et al., 2014; Suleiman and Kurt, 2015). The contamination of this crop by aflatoxins prevents maize producers from accessing international market, suppresses economic opportunities and unfortunately affects consumer health (Bbosa et al., 2013; Wu, 2015). The present study was conducted to determine the occurrence of aflatoxigenic fungi (*A. parasiticus* and *A. flavus*) on this important food crop stored inside facilities in Morogoro municipality and Makambako district, Tanzania.

## 2. Material and methods

### 2.1 Description of the Study Area

The study was carried out in six wards selected from two regions; three out of these were Kihonda, Uwanja wa Taifa and Kingo from Morogoro municipality in Morogoro region while the other three were Mwembetogwa, Mjimwema and Utengule from Makambako district in Njombe region. The wards were selected purposefully based on the availability of maize warehouses to maximize the chances of obtaining stored maize samples. Significant variations in some climatic conditions was the major criteria considered in selection of the two study areas. Variation in temperature between the two selected regions was most crucially considered as the climatic condition greatly influences the existence and survival of the pathogen under study.

Morogoro municipality is one of the six districts of Morogoro region, it is the region's headquarters covering an area of 531 km<sup>2</sup> with a population of 396,481, according to population projection of 2019. The municipality is located at about 195 kilometres West of Dar es Salaam and situated on the lower slope of Uluguru Mountains whose peak is about 1600 feet above the sea level (URT, 2017). The municipality lies at the crossings of longitudes 37033' and 37051' East of the Greenwich Meridian and between latitudes 6037' and 6055' South of the Equator. It is characterised by an annual average temperature ranging between 16°C to 28°C in the cold dry season and 21°C and 33°C in the warm wet season. The municipality has an average annual rainfall range of between 821mm and 1505mm (URT, 2017). The average relative humidity is 63% to 83% in March through May and 46% to 82% from July to September. The major economic activities in Morogoro municipality include primary and secondary industrial activities, commercial farming, small-scale enterprises and commercial wholesale and retail (URT, 2017).

Makambako district is one among the six districts in Njombe region located at a junction between Njombe, Iringa and Mbeya region. The district lies approximately 40 miles North of the district capital of Njombe. The district is bordered Mufindi, Njombe and Wanging'ombe districts in the North and East, South and West respectively (URT, 2017). It lies at the crossings Longitudes 33°05' and 35°08' East of the Greenwich Meridian and Latitude 8°08' and 9°08' South of the Equator. The district covers an area of 884km<sup>2</sup> with a population of 93,827 according to the 2012 Tanzania Nation Census (TNC) (URT, 2017). The district displays an annual temperature range of between 15°C

and 25°C and experiences mild and sunny weather throughout the year, with maximum temperature observed in September and December while minimum temperature occurs in June and August when the temperatures fall to 15°C. The rainy season starts between October and November and ends in March and April with an annual average rainfall range of between 600mm and 1000mm (URT, 2017). The average relative humidity is 80-91% in March through June and 58-75% from July to October. The major economic activities in Makambako district are agriculture and trade (URT, 2017).

### 2.2 Study Design

A cross-sectional study design was adopted in this study. A simple random sampling technique was employed to collect maize samples from warehouses for further laboratory analysis.

### 2.3 Sample Size Calculation

The sample size for the study was calculated based on previously established prevalence by Seetha et al. (2017) using the formula;

$$n = [Z^2 P (1-P)] / d^2$$

Whereby;

n = Sample size.

Z= Statistic for level of confidence (1.96 at confidence level of 95%)

P= Expected prevalence (18% based on study by Seetha et al., 2017)

d= Precision (5%)

Therefore;  $n = 1.96 \times 1.96 \times 0.18 (1 - 0.18) / 0.05 \times 0.05 = 226$ .

Therefore, the calculated sample size was 226, and for each sample about 200g of stored maize grains was collected from warehouses in different wards.

### 2.4 Sample Collection

Samples collection sites were selected based on the information provided by Ward Executive Officers and District Agriculture and Livestock Development Officers. A total of 226 samples, each containing 200g of stored maize grains were collected randomly from warehouses in different wards of Morogoro municipality and Makambako district in Tanzania between March and May 2021. A total of 113 samples were collected from Morogoro municipality and the remaining 113 samples were collected from Makambako district. The distribution of samples collected from warehouses in Kihonda, Uwanja wa Taifa and Kingo wards within Morogoro municipality was 38, 38 and 37 respectively. Also, 33, 46 and 34 samples were collected from warehouses in Mwembetogwa, Utengule and Mjimwema wards respectively in Makambako district. These samples were collected from a total 14 warehouses, eight of these from Morogoro municipality and the remaining six from Makambako district. It was observed that most of the collected samples were stored between three to six months.

In the warehouses, maize was commonly stored in polypropylene bags/sacks each, weighing an average of 100kgs. The sampling procedure was determined by the total stock of sacks in a particular warehouse. A sample was taken from each sack in a warehouse with a total stock of 10 or fewer sacks. In warehouses with more than 10 total stock sacks, only 10 sacks were randomly selected for sampling. The number of samples drawn from each sack was determined by the position and posture of the sack. The sample from the sacks were taken by using local vendor tools (Locally made probes in Kiswahili

known as “Bambo”) or hands covered by sterile gloves. The collected samples were packed in sterile zipper bags, sealed and labelled appropriately, then transported to the laboratory at Sokoine University of Agriculture. In the laboratory, the samples were stored in the cold room with a temperature below 4°C prior to analysis.

## 2.5 Determination of Moisture Content (MC)

Moisture content of each collected sample of stored maize grains was measured by using oven-drying method prior to analysis. Ten grams (10g) of each collected sample was placed in the aluminium foil, sealed and then labelled properly. The sealed samples were then kept in the hot air oven at 80°C for 24 hours after which they were removed from the oven and their weight was re-measured by the analytical balance to determine the MC. The MC of each sample was calculated based on previously formula described by **Reeb et al. (1999)** as:

$$MC (\%) = \frac{\text{Initial weight (g)} - \text{Oven dryweight(g)}}{\text{Initial weight(g)}} \times 100\%$$

Where by:

Initial weight= Weight of sample before drying

Oven dry weight= Weight of sample after drying

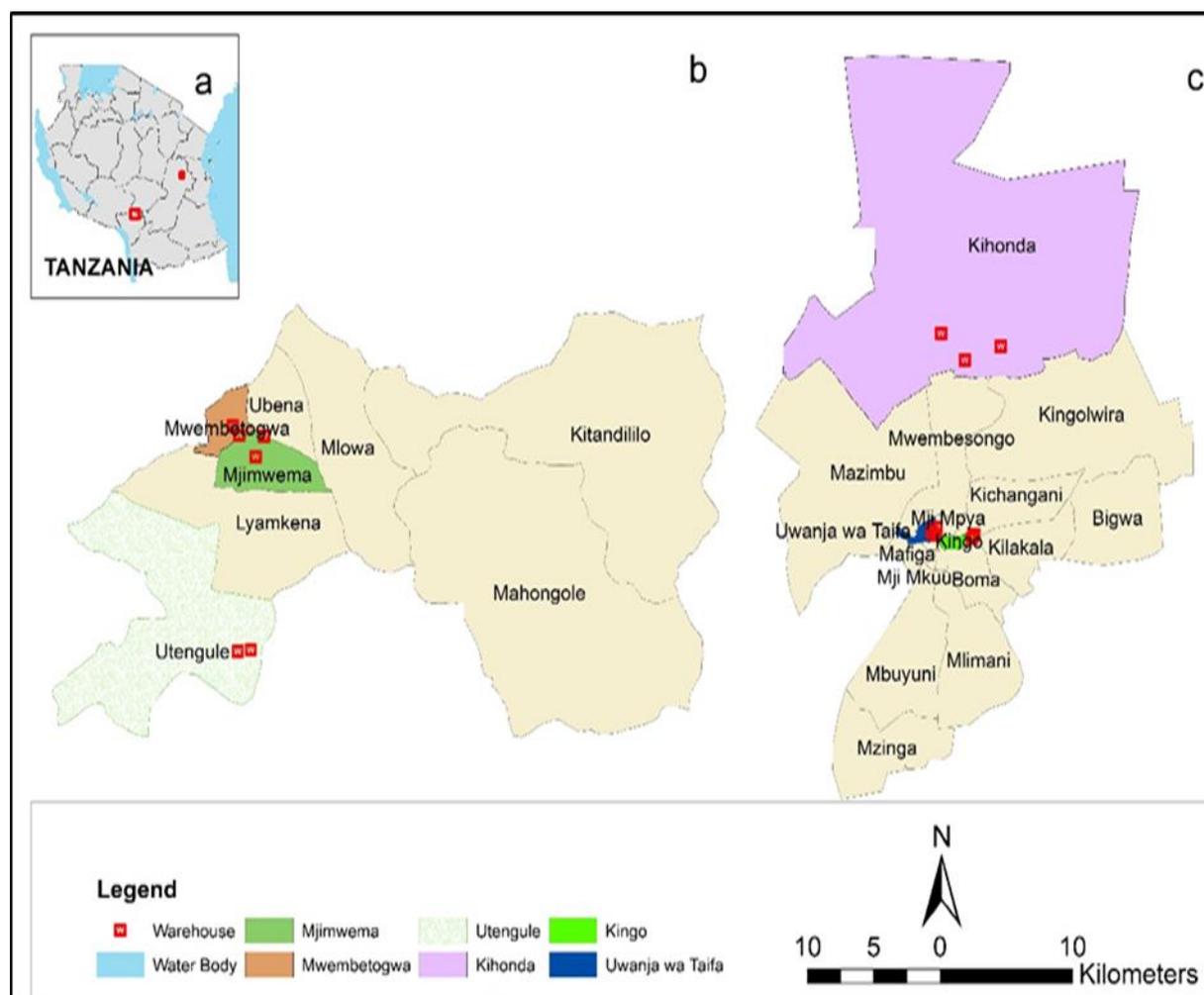
## 2.6 Preparation of Maize Samples

In the laboratory, maize grains were randomly taken from each of the 200g packages of collected maize samples and then

weighed on an analytical balance to obtain 30g. The 30g obtained from each package was ground using an electronic grain mill grinder to obtain a homogenous flour mixture. The grinder was cleaned with distilled water then sterilized with 70% of ethanol by using cotton wool after grinding each of 30g portions to overcome the problem of sample contamination. The ground samples were then packed in sterile zipper bags, labelled appropriately and stored in room temperature for further study.

## 2.7 Isolation of *A. flavus* and *A. parasiticus* from maize samples

Isolation of *A. flavus* and *A. parasiticus* from samples was conducted using a procedure as described by **Shitu et al. (2018)**. Five gram of each ground samples was weighed separately on an analytical balance and then mixed with 20ml of normal saline in the test tubes. The mixture was shaken vigorously and allowed to settle for 10 minutes. One millilitre of the mixture was pipetted from each test tube, using a micropipette then poured and spread on plates containing sterile PDA to support growth of primary fungal cultures. The plates were then incubated at 37°C for one up to three days. The incubated plates were examined daily for *Aspergillus* growth and spore formation. Colonies with morphological features of *Aspergillus* were transferred onto new PDA media plates using sterilized inoculation needles and forceps for sub-culturing to obtain pure culture. The sub-cultured plates were then incubated at 37°C for one up to seven days for further processing.



**Figure 1** Map of United republic of Tanzania (a), Makambako District (b) and (c) Morogoro Municipality showing the study sites.

### 2.8 Identification of the isolates (*A. flavus* and *A. parasiticus*)

Identification of *A. flavus* and *A. parasiticus* species from a pure culture of isolates obtained from the samples was conducted based on macroscopic and microscopic features. The macroscopic features used in identification of the isolates were; the colour of the colony, diameter of the colony, colony textures, colony reverse colour, production of sclerotia and presence of exudates in plates as explained by Khan et al. (2020) and Sukmawati et al. (2018). On the other hand, microscopic identification of the isolates was conducted utilizing a staining technique described by Egwurochi et al. (2015). The staining procedure was conducted as follows; A few drops of lactophenol cotton blue were placed on a microscopic slide. A small portion of the colony was taken from the pure culture using sterilized forceps and then placed on the smeared side of the slide. A clean cover slip was then placed over the portion of the colony on the slide that was then placed on the stage of a light microscope to be observed by switching objectives to obtain convenient resolution. Presence and parameters of microscopic features such as conidia head, conidiophore, phialides, matulae and vesicles were observed. The identification the isolates to species level by observing macro and micro morphological characteristics was conducted with aid of taxonomic keys as described by McClenny (2005).

### 2.9 Statistical Analysis

Descriptive analysis was conducted using Microsoft Excel 2013. Tables and graph were presented to describe moisture content of the samples and proportional occurrence of *A. flavus* and *A. parasiticus* with respect to areas of collection.

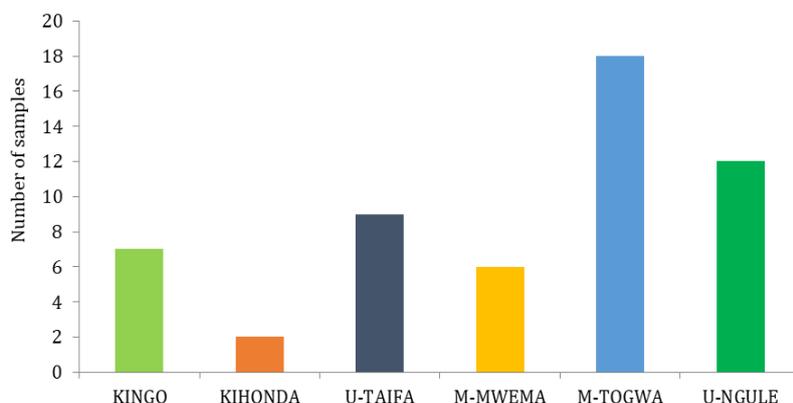
## 3. Results

### 3.1 Moisture Contents of the Samples with Respect to Site of Collection

There was a statistical significance in variation of moisture content of the samples taken from warehouses in all wards. This study found that the MC of the samples ranged from 10% to 19%. Out of the 226 samples collected, 54 (23.9%) samples had a MC above 14% while 172 (76.1%) had a MC below 14%. The proportion of samples observed with MC level above 14% were Mwembetogwa (55%), Utengule (26%), Uwanja wa Taifa (23.7%), Kingo (18.9%), Mjimwema (17.6%) and Kihonda (5.3%) wards (Table1).

**Table 1** Moisture content levels of the samples with respect to site of collection

Study sites	Moisture Level N (% of the samples)		
	Below 14%	Above 14%	Total
Kingo	30(81.1)	7(18.9)	37
Kihonda	36(94.7)	2(5.3)	38
Uwanja wa Taifa	29(76.3)	9(23.7)	38
Mjimwema	28(82.4)	6(17.6)	34
Mwembetogwa	15(45.5)	18(54.5)	33
Utengule	34(73.9)	12(26.1)	46
<b>Total</b>	<b>172(76.1)</b>	<b>54(23.9)</b>	<b>226</b>



**Figure 2** Sample with moisture contents above 14% with respect to site of collection

### 3.2 Screening for Fungal Contamination from Collected Samples

Among the total 226 samples collected from the six wards for fungal screening, only 48(21.2%) samples revealed fungal contamination in their primary cultures. The 48 contaminated samples were then subjected to further screening of aflatoxigenic fungi of interest (*A. flavus* and *A. parasiticus*) on pure cultures where only 34 (15%) samples were positive. The results show that there was at least one sample that was contaminated with *A. flavus* and *A. parasiticus* from each of the six wards. The prevalence of samples that were contaminated with *A. flavus* and *A. parasiticus* from each ward are summarized on Table 2.

**Table 2** *A. flavus* and *A. parasiticus* contamination among the collected samples

Sites	Screened Samples	Contaminated samples	Prevalence (%)
Uwanja wa Taifa	38	5	13.2
Kihonda	38	1	2.6
Kingo	37	10	27.0
Mjimwema	34	5	14.7
Mwembetogwa	33	6	18.2
Utengule	46	7	15.2
<b>Total</b>	<b>226</b>	<b>34</b>	<b>15.2</b>

### 3.3 Incidences and Distribution of *A. flavus* and *A. parasiticus* Isolated from Contaminated Samples

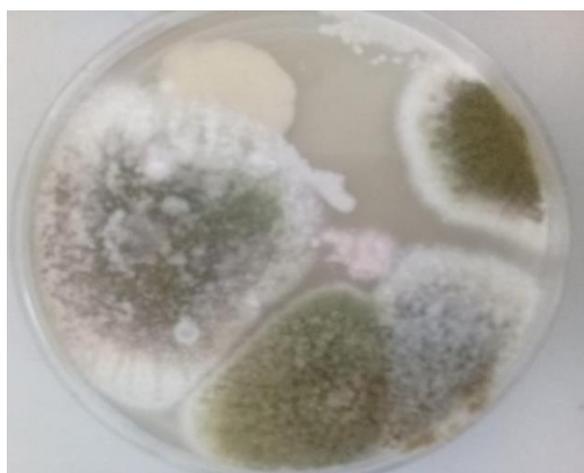
The results shows that *A. flavus* and *A. parasiticus* were isolated from 34 out of 226 maize samples collected from all wards. Out of 34 samples that were contaminated, 28 samples were due to *Aspergillus flavus* while *Aspergillus parasiticus* were found in 6 samples. Co-occurrence between *A. flavus* and *A. parasiticus* was also observed in two samples, one came from Makambako district and the other came from Morogoro municipality. Variation in the number of contaminated samples was also observed where Makambako district had the highest number of contaminated samples compared to Morogoro Municipality (Table 3).

**Table 3** Incidences and distribution of *A. flavus* and *A. parasiticus* isolated from all study sites

Study sites	Isolated aflatoxigenic fungi N (% of the isolates)		
	<i>A. flavus</i>	<i>A. parasiticus</i>	Total
Uwanja wa Taifa	5(100)	—	5
Kihonda	—	1(100)	1
Kingo	8(80)	2(20)	10
Mjimwema	4(80)	1(20)	5
Mwembetogwa	6(100)	—	6
Utengule	5(71.4)	2(28.6)	7
<b>Total</b>	<b>28(82.4)</b>	<b>6(17.6)</b>	<b>34</b>

### 3.4 Macroscopic Identification of *Aspergillus flavus* on PDA media

Macroscopic features of *Aspergillus flavus* on PDA media was almost similar in all 28 pure culture plates. Similarly, the colonies on the plates had powdered texture with slight variations in their diameters ranging between 45mm and 70mm after four days of incubation. In most cases, the colonies were flat at their borders and raised in the middle. All the plates were dominated by olive green surface color of the colony with whitish margin while their reverse sides were creamish. However, some of the plates had colonies lacking the whitish margin. Some of the *A. flavus* isolates produced colourless exudates while others did not. In addition, some of the isolates produced a compact mass of the mycelia (sclerotia) that appeared dark brown in colour.



(a)



(b)

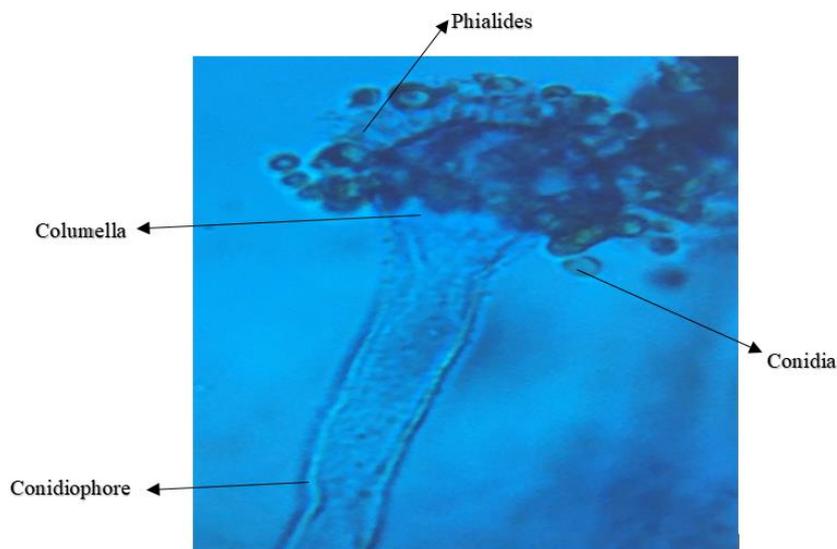


(c)

**Figure 3** Macroscopic features of *Aspergillus flavus* on PDA media: (a) Fungal growth on PDA plates after two days of incubation at 37°C, (b) Appearance of the colony surface observed after six days of incubation at 37°C and (c) Reverse side of the growth in (b).

### 3.5 Microscopic identification of *Aspergillus flavus*

Microscopic identification of *Aspergillus flavus* under the microscope showed that the conidiophores bearing the vesicles were colorless and thick walled. The conidial heads were biseriate or uniseriate with vesicles, which varied in size and appeared with globose or sub-globose shape. In the biseriate conidial heads, the phialides grew from the matulae while in the uniseriate the phialides grew on the vesicle. In addition, the conidial head of the species had a globose shape with variable size and aseptate hyphae.



**Figure 4** Microscopic observation of *Aspergillus flavus* under the light microscope

### 3.6 Macroscopic identification of *Aspergillus parasiticus* on PDA media

Macroscopic features of *Aspergillus parasiticus* on PDA media was almost similar in all six pure culture plates. Similarly, the colonies on the plates had powdered texture with slight variations in their diameters ranging between 35mm and 65mm after four days of incubation. All the plates were dominated by

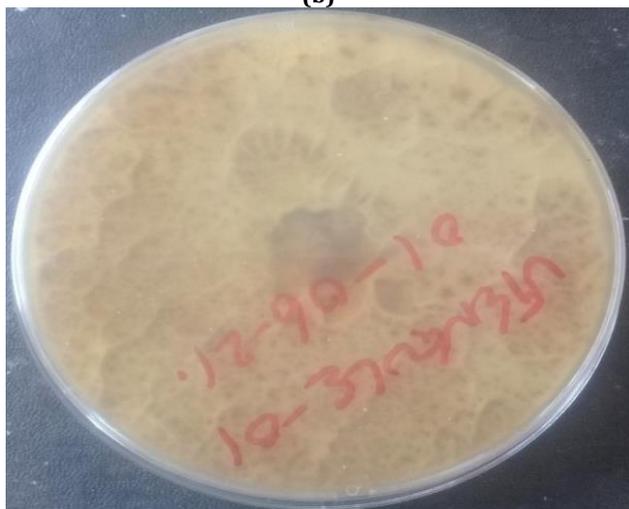
dark green surface color of the colony while their reverse sides were yellowish-cream. *A. parasiticus* isolates lacked exudates and some produced pigments with brown colour on the reverse side of the growth. In addition, some of the isolates produced a compacted mass of mycelia (sclerotia) with a dark colour.



(a)



(b)

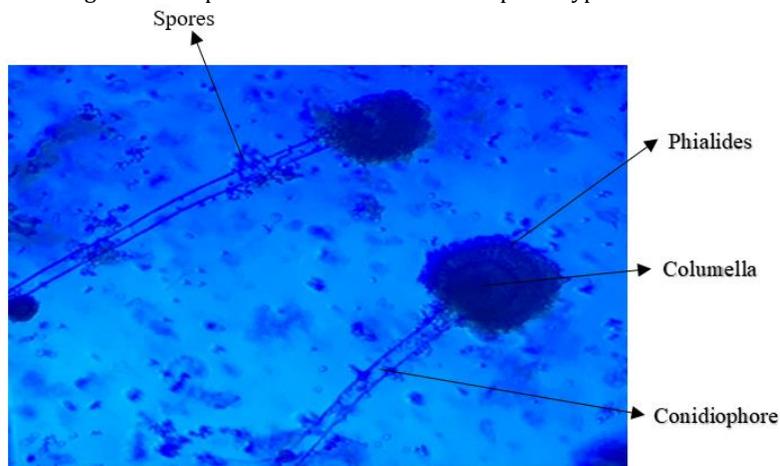


(c)

**Figure 5** Macroscopic observation of *Aspergillus parasiticus* on PDA media: (a) Fungal growth on PDA plates after two days of incubation at 37°C, (b) Appearance of the colony observed after six day of incubation at 37°C and (c) Reverse side of the growth in (b).

### 3.7 Microscopic observation of *A. parasiticus*

Microscopic identification of *Aspergillus parasiticus* under the microscope showed that the conidiophores bearing the vesicles were colorless, rough, aseptate and thin walled. At the tip of the conidiophores, there were columella with various sizes and globose shape. The conidial heads were biserial and appeared with globose shape. In addition, the conidial head of the species had a globose shape with variable size and aseptate hyphae.



**Figure 6** Microscopic observation of *A. parasiticus* under the light microscope

### 4. Discussion

The present study found that about 23.9% of all samples collected from both study areas had their moisture content (MC) above 14%. MC below 14% inhibits the growth of aflatoxigenic fungi while that above 14% favours their growth on stored crops at various temperatures according to **Shekhar et al. (2018)** and **Shitu et al. (2018)**. The two study areas have varying degrees of temperature and humidity where Makambako is relatively more humid and cool compared to Morogoro municipality. Therefore, most of study samples (with MC above 14%) were from Makambako district (36) while the remaining (18) were from Morogoro municipality. With the fact that temperature and humidity are climatic conditions known to influence the behaviour and survival of fungi that infest stored crops (**Achaglinkame et al., 2017; Weinberg et al., 2008**), this indicates that the significant variation of these conditions between the two study areas could be the reason why a higher number of samples that had a MC above 14% were from Makambako district. This study corresponds to the study conducted by **Mpuchane et al. (1997)** in Botswana who found that some of the maize samples collected from warehouses had a MC above 14%. Similarly, the study conducted by **Danso et al. (2019)** in Ghana found that some of the maize samples collected from warehouses had a MC above 14%. This implies that the measures taken to reduce the moisture content in the maize prior to storage in many African countries are inadequate. The MC of the stored maize could also be affected by other factors including variations of temperatures and humidity within the warehouses during storage.

This study also revealed that about 21.2% of maize samples collected from warehouses were contaminated with fungal species. This percentage of contaminated samples is significantly low compared to that revealed by other studies such as that conducted by **Olagunju et al. (2018)** in Durban, South Africa where 70% of maize samples collected from stores were contaminated with fungal species. Similarly, another study conducted in South Africa by **Chilaka et al. (2012)** found that about 87% of commercial maize samples collected from stores

were contaminated with fungal species. This variation in percentage of contaminated samples could reflect a parallel variation in the practices employed prior to storing maize. Furthermore, the variations of the percentage of contaminated samples is also a consequence of the great variations in the climatic conditions of the two areas (Tanzania and South Africa). Furthermore, the study also revealed that *Aspergillus* species were the most dominant fungal species contaminating the maize in storage facilities. This finding is in line with the findings from another study conducted by **Saleemi et al. (2012)** in Pakistan who also found that *Aspergillus* species were the most dominant fungal species compared to others capable of contaminating stored maize. Similarly, the study conducted by **Balendres et al. (2019)** in Philippines also found that *Aspergillus* species were most dominant fungal species contaminating agricultural commodities. This predominance of *Aspergillus* species in the contaminated maize samples is a result of the significantly greater ability of the species to survive in a wide range of environmental conditions as well as their capacity to infest a wide range of agricultural commodities as reported by **Mousavi et al. (2016)** and **Thathana et al. (2017)**. The study also found that there was unequal distribution *A. flavus* and *A. parasiticus* species among the isolates from the maize samples. Furthermore, the study found that the frequency of isolation of *A. flavus* (82.4%) was high compared to *A. parasiticus* (17.6%). This finding is comparable to that from a study conducted by **Iheanacho et al. (2014)** in South Africa who similarly found that *A. flavus* was more prevalent than *A. parasiticus* in agricultural crops. A study conducted in Zaria, Nigeria by **Shitu et al. (2018)** also found that there was High prevalence of *A. flavus* compared to *A. parasiticus* in millet and maize samples collected from markets. This might be influenced by resilience and ability of *A. flavus* to grow in substrates under a diverse range of environmental conditions as reported by **Nazir et al. (2014)**.

## 5. Conclusion

The study revealed that *A. flavus* was the major aflatoxigenic fungi contaminating stored maize collected from warehouses in both Morogoro municipality and Makambako district. The study also indicated that humidity and temperature conditions of Makambako district favour the growth of aflatoxigenic fungi on stored maize compared to Morogoro Municipality. In view of the fact that it is not easy to control climatic conditions of an area, we recommend that stakeholders should adopt good storage practices to minimize fungal infestation and subsequent aflatoxin contamination on stored maize. Moreover, further studies should be conducted to determine the status of other mycotoxins contaminating stored crops in different regions of Tanzania. However, education on the impacts of fungal and mycotoxins contamination on stored crops should be provided to storage facility owners and other stakeholders.

## Conflict of Interest

The authors declare that they had no competing interests.

## Acknowledgments

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