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Managing aflatoxin – producing fungi using indigenous atoxigenic strains of aspergillus species in groundnut in Mtwara region, Tanzania

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MANAGING AFLATOXIN – PRODUCING FUNGI USING INDIGENOUS ATOXIGENIC STRAINS OF *Aspergillus species* IN GROUNDNUT IN MTWARA REGION, TANZANIA

Juma Mfaume

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Master`s in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

April, 2019

ABSTRACT

The present study was conducted from January to October 2018 in Mnanje, Mpeta and Naliendele villages to determine aflatoxin levels in dried groundnut kernels, to identify toxigenic and atoxigenic strains of *Aspergillus* section *Flavi* from soil samples and to test the effect of atoxigenic strains against aflatoxin-producing fungi in groundnut. Isolation of fungi was done on MRBA and AFPA media and analysis of aflatoxin levels in groundnut was conducted using HPLC. The results showed that, there was significant differences ($p < 0.05$) in total aflatoxins level between groundnut samples. Eleven (24.4%) out of 45 samples had aflatoxin levels above the Tanzania Food and Drugs Authority's standard (10 µg/kg). Total aflatoxin contamination levels ranged from higher i.e. 5.86 to 16.81 µg/kg at Mnanje village to lowest at Mpeta village (0.05 to 15.02 µg/kg). The results also indicated that 20.4% of the fungal composition in soil samples were toxigenic strains while 79.5% were atoxigenic implying that these strains can be exploited for biological control to mitigate aflatoxin contamination risks in groundnuts. This was proved through the study that also indicated that total aflatoxin levels in groundnut kernel samples were lower in atoxigenic treated groundnut plots than in controls plots. Thus, there is need to conduct further studies involving a wide geographical location on testing the efficiency of the atoxigenic strains identified in this study for further characterization and formulation of a bio-control against aflatoxin producing-fungi in Tanzania.

DECLARATION

I, Juma Mfaume do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation titled "Managing aflatoxin producing-fungi using indigenous atoxigenic strains of *Aspergillus spp* in groundnuts in Mtwara region, Tanzania" is my original work and has never been submitted for a degree award in any other university.

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Dr. Athanasia Matemu (Supervisor 2)	**Date**

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CERTIFICATION

This is to certify that, the dissertation titled, "Managing aflatoxin – producing fungi using atoxigenic strains of *Aspergillus spp* in groundnut in Mtwara region, Tanzania" submitted by Juma Mfaume (M315/ T.16) in partial fulfillment of the requirements for the award of Master`s degree in Life Sciences of the Nelson Mandela African Institution of Science and Technology**.**

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Dr. Ernest R. Mbega (Supervisor 1) **Date**

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Dr. Athanasia Matemu (Supervisor 2) **Date**

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DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving wife Zahra Said Majili whose words of encouragement and push for tenacity ring in my ears. My children Zainab, Ramadhani, Faraji, Husna, Aisha and Sharifa have never left my side and are very special. I also dedicate this dissertation to my many friends and colleagues who have supported me throughout the process. I will always appreciate all they have done. Both of you have been my best cheer leaders.

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LIST OF ABBREVIATIONS AND SYMBOLS

AF	Aflatoxin
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFPA	*Aspergillus flavus* and *Aspergillus parasiticus* agar
ANOVA	Analysis of variance
EPA	Environmental Protection Agency
CAST	Council for Agricultural Science and Technology
CI	Confidence interval
CDC	Centre for diseases control
CFU	Colony forming units
CMC	Carboxyl methyl cellulose
CO_2	Carbon dioxide
CZA	Czapeks agar
EPA	Environmental Protection Agency
FAO	Food and Agricultural Organization
Fig	Figure
FAOSTAT	Food and Agricultural Organization Statistics
g	Gram
GAP	Good agronomic practices
GDP	Gross Domestic Product
GPS	Global positioning system
HPLC	High performance liquid chromatography
IARC	International Agency Research for Cancer

ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IITA	International Institute of Tropical Agriculture
IPP	Industrial Products Promotions
KBr	Kobra Cell
Km^2	Square kilometre
Lab	Laboratory
LoQ	Limit of quantification
m	Metre
MEA	Malt extract agar
MRBA	Medium Rose Bengal agar
MT	Metric tons
mg/kg	Milligram per kilogram
mL	Mililitre
NaOH	Sodium hydroxide
nm	Nanometer
NM-AIST	Nelson Mandela African Institution of Science and Technology
No	Number
NPC	National Population Census
OA	Oatmeal agar
O_2	Oxygen gas
P	Probability
pH	Acidity or alkalinity
spp	Species
TARI	Tanzania Agricultural Research Institute
TFA	Trifluoroacetic acid
TFDA	Tanzania Food and Drugs Authority

TaF	Total aflatoxins
TL II	Tropical Legume II
TVC	Total viable count
USA	United States of America
USDA	United States Department of Agronomy
US$	United State dollars
WHO	World Health Organization
^{o}C	Degree centigrade

CHAPTER ONE

INTRODUCTION

1.1 General background

Groundnut also known as peanuts (*Arachis hypogaea* L.) is an annual plant species in the family leguminosae with its original in the Latin Americas (Hammons, 1982). The crop was introduced to the African continent from Brazil by the Portuguese in the 16th century (Abalu and Etuk, 1986; Adinya *et al.*, 2010). Groundnut is the world's 13th most important food crop, 4th most important source of edible oil (40-50%) and 3rd most important source of vegetable protein (20-50%), (Taru *et al.*, 2008) which also contains 10-20% carbohydrates (Sørensen *et al.*, 2011).

In Tanzania, groundnut is among the most important crops for smallholder farmers, providing both food and income for households (Sibuga *et al.*, 1992). Groundnut is very nutritious with a number of useful ingredients such as fat, protein, carbohydrates, vitamins and minerals all of which are important in human and livestock feed (Higgs, 2003). Due to the multiple uses of groundnut, it is both an important food and cash crop for domestic (Chirwa and Matita, 2012). However, Aflatoxin contamination is among key factors that can affect quality of the crop especially among food insecure households including Tanzania (Abass *et al.*, 2014). These resource constrained communities usually lack knowledge or when that is not the case they fail to adopt costly strategies for mitigating the aflatoxin problems.

Some recommendations for managing aflatoxin producing microbes in the field including timely harvesting, drying groundnut on raised platform and storage using jute bags during post-harvest crop stages have been recommended (Hell and Mutegi, 2011). There is a strong consensus that aflatoxin contamination on important crops such as groundnut and others poses a significant threat to public health, trade and livelihoods in Tanzania (Kimanya, 2014). Control measures show rarely immediate or visible effect. Most of control measures are mostly labour (management) or capital intensive (inputs) whereby there is no or little effect on bio control mitigation. With all these concern use of biological control to mitigate aflatoxin should be now the alternative approach for these farmers' households (Dorner *et al.*, 2003). This study aimed to reduce aflatoxin producing fungi population and their corresponding impact to an acceptable level using native strains of *Aspergillus* section Flavi.

1.2 Problem Statement and Justification

Aflatoxin contamination has been associated with groundnut throughout the world over the years. Cardwell *et al.* (2004) reported the annual loss in corn, groundnut and wheat crops in Africa due to mycotoxin contamination is estimated to be over $750 million. Report by Food (2003); 25% of the world's food crops are significantly contaminated with mycotoxins; aflatoxin being the one. The first reported case of acute aflatoxin in Tanzania occurred in the year 2016 when 65 cases were reported from two districts (Chemba and Kondoa) in Dodoma region (part of central Tanzania); where 19 people died (Mohamed, 2017). Since aflatoxins are potent toxins and carcinogenic, their quantity in food and feed must be closely monitored and regulated in most countries worldwide.

Efforts for aflatoxin prevention is complicated because it requires a series of intervention strategies such as hand sorting of kernels (with removal of damaged kernels), drying kernels on natural fibre mats, estimating the completeness of a sun-drying period and supplying wooden pallets to store the bags; to be merged with the traditional farming practices (Lewis, 2005; Bryce *et al.*, 2005; Wild *et al.,* 2015). Of all the aflatoxin control methods, use of antagonistic organisms as bio control agents has been cited as a potential approach for mitigation of fungal growth in the field (Yin *et al.,* 2008). Numerous organisms have been tested for biological control of aflatoxin contamination including bacteria, yeasts, and non-toxigenic (atoxigenic) strains of the causal organisms of which only atoxigenic strains have reached the commercial stage (Dorner, 2009).

In some countries such as the United states of America (USA) biological control of aflatoxin production in crops has been approved by the Environmental Protection Agency (EPA) and already two commercial products based on atoxigenic *Aspergillus flavus* strains are being used (e.g. afla-guard® and AF36®) for the prevention of aflatoxin in peanuts, corn and cotton seed (Dorner, 2009). In Africa, atoxigenic strains of *Aspergillus flavus* have been identified to competitively exclude toxigenic fungi in the maize and peanut fields; Atehnkeng *et al*. (2008b) reported that atoxigenic strains reduce aflatoxin concentrations in both laboratory and field trials by 70 to 99%. A mixture of four atoxigenic strains of *A. flavus* of Nigerian origin has gained provisional registration as AflaSafe® to determine efficacy in on-farm tests. However, none of the reported strains have been marketed in Tanzania. Furthermore, since they are not produced locally, ordering and buying from abroad would be

costly to farmers thus, searching for locally available fungal strains for mangling aflatoxin producing fungi is urgently needed in Tanzania.

1.3 Objectives

1.3.1 Main objective

To develop affordable bio control technology for mitigation of aflatoxin in groundnuts using native atoxigenic strains of fungi in Tanzania

1.3.2 Specific objectives

(i) To determine aflatoxin levels in dry groundnut kernels.

(ii) To identify toxigenic and atoxigenic *Aspergillus* strains in the study area of Mtwara, Masasi and Nanyumbu districts.

(iii) To formulate groundnut seed based inoculant and test it *in vivo.*

1.4 Research questions

(i) What are the levels of aflatoxins present in groundnut kernels produced and traded by growers in Southern region of Mtwara?

(ii) What is the distribution and occurrence of *Aspergillus* section *Flavi* in the study sites?

(iii) Biological control method is effective to manage aflatoxin in groundnuts?

1.5 Research hypothesis

Ho: use of biological control to mitigate aflatoxin in groundnut production is effective method.

Hi: use of biological control to mitigate aflatoxin in groundnut production is not effective method.

1.6 Significance of the research study

This study provides succinctly data on the aflatoxin occurrence and contamination levels on groundnuts as well as it brings awareness and more knowledge to farmer's households in reducing risk in aflatoxin contamination in the study area. Furthermore, it promotes the use of indigenous atoxigenic strains of *Aspergillus spp* to manage toxigenic fungi in groundnut fields. Lastly it sets a basis for further research on bio control methods for mitigation of aflatoxin in Tanzania.

CHAPTER TWO

LITERATURE REVIEW

2.1 Global groundnut production

Groundnuts are produced in the tropical and subtropical regions of the world, on sandy soils (Vilane, 2016). The worldwide groundnut is grown in 26.4 million hectares with a total production of 37.1 million metric tons and an average productivity of 1.4 metric t/ha; globally groundnut is grown over 100 countries (Fig. 1) whereby developing countries constitute 97% of the global area and 94% of the global production of this crop (USDA, 2014). The production of groundnut is concentrated in Asia and Africa with 56% and 40% of the global area and 68% and 25% of the global production, respectively.

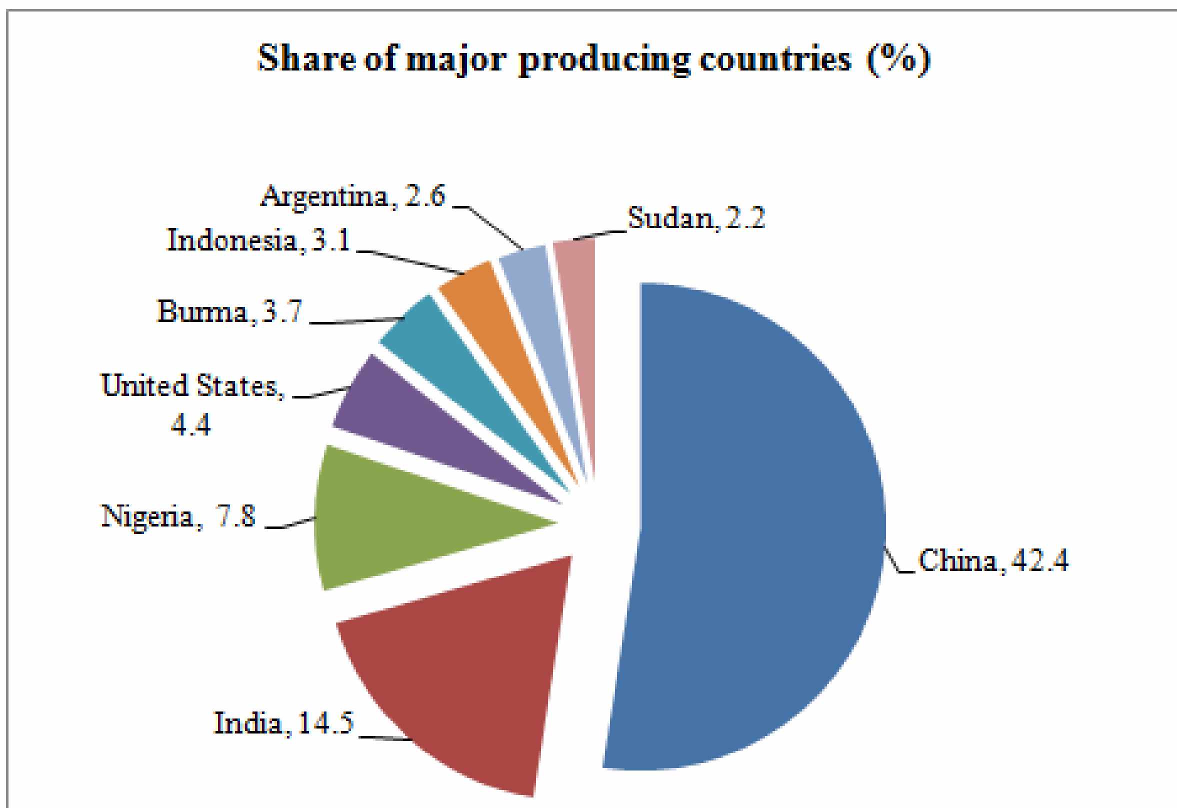

Figure 1: Groundnut production worldwide. Source USDA (2010)

2.2 Groundnut production in Tanzania

Tanzania is among the top ten groundnut producing countries in Africa with production of 810 000 metric tons (MT) and the second producing country in Africa, after Nigeria which produces 3 070 000 MT. Tanzania overall groundnut area and production has almost doubled since 2005 from 300 000 MT to 800 000 MT (FAOStat, 2015) in 2014 (Fig. 2) which accounted for 2.9% of the global area for groundnut cultivation and 1.7% of global production.

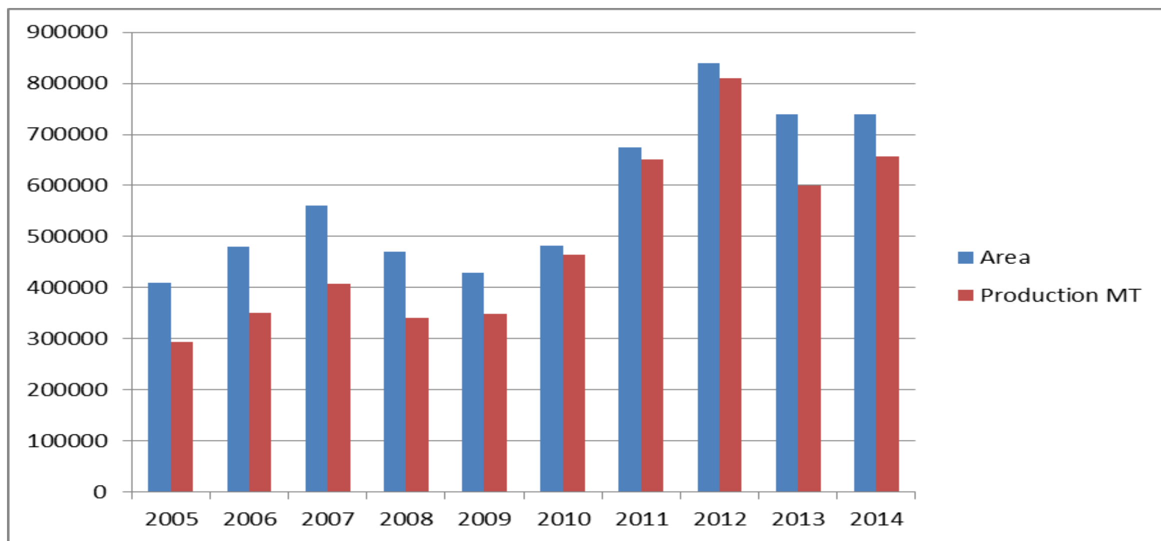

Figure 2: Tanzania Groundnuts Area (Ha) and Production (MT) 2005-2014: Source: FAOSTAT 2015.

The most important groundnut growing regions in Tanzania are Mtwara, Tabora, Shinyanga, Kigoma, Dodoma and Mwanza (Katundu *et al.*, 2014). Smallholder farmers hold the key to agriculture all over the world, and it is no different in Tanzania (Monyo and Varshney, 2016). The reported increase in annual groundnut production in Tanzania is due to the efforts of the smallholder farmers who have been involved in various projects promoting groundnut production in the country.

2.3 Importance of groundnut in Tanzania

Commercial groundnuts production in Tanzania started in 1946 at Kongwa (Dodoma), Urambo (Tabora) and Nachingwea (Mtwara) under the Groundnut Scheme. However, the scheme failed and was subsequently abandoned in 1951. Groundnut is amongst the most important crops for smallholder farmers in Tanzania, providing both food and income for households (Katundu *et al.,* 2014). According to the report by other researchers (Hermelin *et*

al., 2003), groundnut is a nutritious source of fats, protein, carbohydrates, vitamins and minerals for human consumption and parts of the crop can be used for livestock feed.

Groundnut is one of the major oilseeds produced in Tanzania. However, there is virtually no oil produced commercially from groundnut in the country, since groundnuts in nut form have higher value than converted into oil (Sibuga *et al.,* 1992). It is not economical to press groundnuts to extract edible oil as there are several cheaper sources such as sunflower seeds. A study by Osage (2010) in Nigeria reported that most of the groundnuts are consumed as snacks in the following forms: raw nuts; roasted with salt and non-salted nuts; roasted spiced peanuts; stir fried groundnuts mixed with other foodstuffs. Some groundnuts are ground into powder, which is done by a peanut powder grinding machine. "Kashata" for instance, is a peanut brittle traditionally sold by street vendors in Tanzania along with black coffee.

2.4 Impact of aflatoxin in groundnut production

Aflatoxin contamination is a serious quality problem at various stages of groundnuts production, from pre-harvest practices during crop growth up to post-harvest management practices for example during storage. Aflatoxins are a family of toxins produced particularly by certain *Aspergillus flavus* and *Aspergillus parasiticus* that are found on agricultural crops such as maize (corn), peanuts, cotton seed, and tree nuts; which are abundant in warm and humid regions of the world. Aflatoxin-producing fungi can contaminate crops in the field, at harvest, and during storage. The organism survives in spores (conidia), which are carried by wind or insects to the growing crop; and any condition that interferes with the integrity of the seed coat allows the organism to gain entry into individual kernels (Manizan *et al.*, 2018).

Aflatoxin contamination causes economic losses many crops including groundnuts and other commodities, and on economic losses of processed food and feedstuffs worldwide (Coppock *et al.*, 2018). The Food and Agriculture Organization (Bryden, 2007) has estimated that 25% of the world's crops are affected by mycotoxins each year, with annual losses of around 1 billion metric tons of foods and food products. Economic losses occur because of: -

(i) Yield loss due to diseases induced by toxigenic fungi;
(ii) Reduced crop value resulting from mycotoxin contamination;
(iii) Losses in animal productivity from mycotoxin-related health problems; and
(iv) Human health costs (Shephard, 2008).

Africa loses an estimated US$ 670 million in export trade alone due to aflatoxin contamination in food stuffs (IPP Media, 2017); with 40 percent of the foodstuffs on the continent contaminated and the fact that aflatoxins are responsible for the estimated 30 percent of liver cancer cases. Because of its natural presence in soils and the difficulty in verifying its presence, production, trade and consumption of aflatoxin-contaminated groundnuts is a significant concern in developing countries including Tanzania.

2.5 Effect of aflatoxin contamination on health

Aflatoxins occur naturally as four related chemicals, designated B1, B2, G1, and G2, that are not only acutely toxic, but are also highly carcinogenic (Eaton and Groopman, 2013). Chronic exposure to the B1 form of aflatoxins causes liver cancer (Kew, 2013), and is linked to cirrhosis of the liver (Kuniholm *et al.*, 2008) as well as to immune suppression in humans (Williams *et al.,* 2004). It has been reported that approximately 4.5 billion people living in developing countries are chronically exposed to uncontrolled amounts of aflatoxins (Klich and Pitt, 1988). According to Daniel *et al.* (2011) aflatoxins contamination of food is a significant risk factor for human health, particularly in developing countries that lack detection, monitoring, and regulating measures to safeguard the food supply. In Tanzania, the Tanzania Food and Drugs Authority (TFDA) (https://allafrica.com/stories/2016) reported high levels of aflatoxin amounting to between 5.7 to 204.5 micro grams per kilo, way above the required 5 micrograms per kilo for, which were found in 115 samples of maize cereals, maize flour, and humans tested by the Centre for Disease Control and Prevention (CDC) in Atlanta, United States of America (USA).

2.6 Factors influencing aflatoxin occurrence on groundnuts

In the semi-arid tropics, a number of crops are often contaminated by mycotoxins (Aidoo, 2016) groundnut, maize, cotton seed, sorghum, millet, rice, Brazil nuts, pecans, pistachio nuts, spices (particularly chilies), walnuts and products made from these crops. According to Klich and Pitt (1988), the production of aflatoxin is equally influenced by physical, chemical and biological factors; aflatoxins are reported to be produced between 25°C - 35°C optimum temperature, and acidic pH; high humid areas between 83% - 88% and appropriate level of CO_2 and O_2 has also been reported to influence the mold growth and aflatoxin production. A study by Report by Nautiyal *et al.* (2002) explained that, contamination can occur in any stage of production from pre-harvest to post-harvest in areas, which lies within the 40ºN and 40ºS. Pre-harvest contamination primarily occurs under heat and drought stress most

especially during the later growing stage when growth rate of the groundnut plant is in decline stage.

2.7 *Aspergillus* section *Flavi*

Aspergillus is one of the most important genera of micro fungi, with many species having great impact on various fields of interest (Gams, 2007): as human, animal and plant pathogens, as spoilage agents of food commodities or as producers of bioactive and/or toxic secondary metabolites. Additionally, some of the most important microorganisms used in food fermentations and in industrial bioprocesses are part of this genus (Pandey *et al.*, 2000). Classic systematics of *Aspergillus* section Flavi has been based primarily on differences in morphological and cultural characteristics (Raper and Fennell, 1965; Klich, 1993).

Aspergillus section Flavi includes six economically important species that are very closely related morphologically and phylogenetically: *Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius, Aspergillus oryzae, Aspergillus sojae* and *Aspergillus tamarii* (Rodrigues *et al.*, 2011). The second specific objective of this research study aimed to identify isolates from *Aspergillus* section Flavi which can be useful in developing groundnut based inoculant as stipulated in the specific objective three

CHAPTER THREE

MATERIAS AND METHODS

3.1 Study location and materials

This study was conducted in three villages (Mpeta, Mnanje and Naliendele) of Masasi, Mtwara and Nanyumbu districts in Mtwara region, located in the extreme Southern part of Tanzania. The region lies between longitudes 38° and 40° 30" East of Greenwich and latitudes; 10° 05" and 11° 25" South of the Equator and covers a total landmass of 16 710 square kilometres (km^2). Mtwara region shares boundary with Mozambique to the south, Indian Ocean to the east and borders the Ruvuma region to the west and Lindi region to the north (Brinda *et al.*, 2014). The study sites were selected based on the evidence of dominance of groundnut amongst the food and cash crops grown in these districts (Fig. 3). Mtwara climate is of tropical with an average temperature of 26.3 °C and the annual precipitation average is 1024 mm.

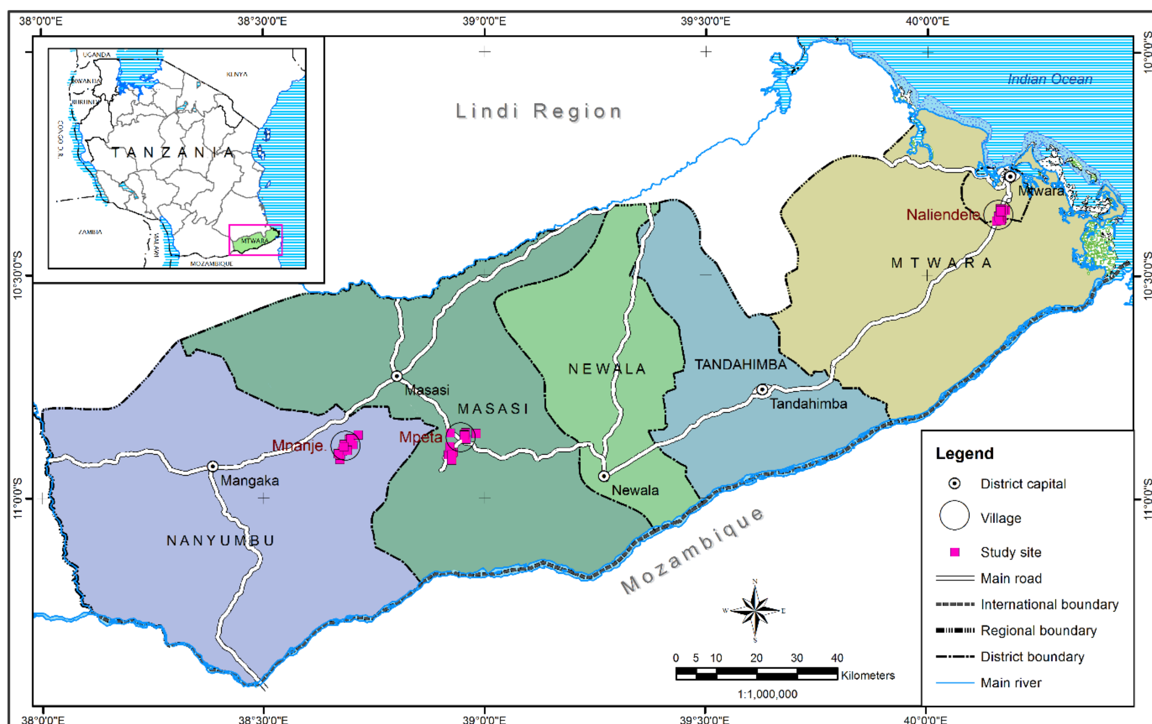

Figure 3: Map showing Aflatoxin study villages in Mtwara region Tanzania

3.2 Sampling Design

For the purpose of this study a multi-stage sampling procedure was used in the data collection. Firstly, three districts namely; Masasi, Nanyumbu and Mtwara were purposively selected. Three villages with fifteen farmer's households each were randomly selected from list prepared by the author with guidance from agriculture extension staff.

The selected villages include Mpeta for Masasi district, Naliendele for Mtwara district and Mnanje for Nanyumbu district. The selection criteria of these villages were based on the dominance of groundnut production and their contribution to the districts production and the region at large.

3.2.1 Sample collection

Each composite dried groundnut kernel and soil samples were collected from study villages of Mnanje, Mpeta and Naliendele. A total of forty-five groundnut kernel samples were collected whereby each site provided fifteen samples stored in sterile khaki envelopes and stored in cool box and transported to Nelson Mandela African Institution of Science and Technology (NM-AIST) for aflatoxin determination.

Forty-five soil samples were collected and processed as described by Dorner (2009). Three to five scoops of soil samples were randomly taken from each farmer's household field, thoroughly mixed to form a composite sample. Spoons used to scoop the soil at 4 - 10 cm depth and were surface sterilized using 70% ethanol to avoid cross contamination. The same procedure was repeated for all the randomly selected soil sample points in the same famer's household field, which were distant at least four meters apart.

A one-kilogram sub-sample was drawn from the composite soil sample and labelled with the name of the farmer, village, Global Positioning System (GPS) co-ordinates, and the date of collection (Appendix 1). The labeled samples were put in zip lock bags and placed in a cool box transported to laboratory for further analysis.

3.2.2 Reagents and consumables

Methanol (CL chem. Lab) HPLC grade, Sodium chloride and Acetic acid were obtained from Wagtech Projects Ltd, Berkshire, UK. HPLC grade water, Acetonitrile, and Trifluoroacetic acid (TFA), were purchased from ROMIL, UK. Anhydrous sodium sulphate was purchased

from Prabhat Chemicals, (Mumbai, India). AFB_1, AFB_2, AFG_1 and AFG_2 were purchased from Immunolab GmbH (Kassel, Germany).

3.3 Analysis of Aflatoxin

Determination and identification of aflatoxins B_1, B_2, G_1, and G_2 in dried groundnut kernel samples were carried out by High Performance Liquid Chromatography (HPLC) protocol (Scott, 1995); AOAC method, 2008), with some recommended modifications (Appendix 2).

3.3.1 Extraction of Aflatoxin

All dried groundnut kernel samples were ground and homogenized in a laboratory grinder [Mode DXF-20D, Rotational speed 25000 rpm, Capacity 1000g = 1 kg, Fineness of finished product (80 – 250 mm)]. Twenty-five grams of milled groundnut kernel samples was later placed in a 250 mL Erlenmeyer flask, transferred in blender jar and 100 mL of extraction solution (60:40 ethanol: water, v/v) was added and mixed on high speed for 3 min, then filtered through 24 cm Whatman No. 1. Homogenized sample mixture was filtered through a 1.5 µm microfibre filter, then 4 mL of extract solution transferred into sample container and 8 mL of phosphate buffer solution (PBS) was added while pH was adjusted to 6 using sodium hydroxide (NaOH).

An aliquot, in the quantity of 12 mL was then passed through the immunoaffinity column (VICAM, Watertown, MA, USA). The solvent flow in the columns was kept at 2-3 $mL \cdot min^{-1}$ After washing with deionized water (10 mL) the column was eluted with Methanol (1 mL) and the eluate was collected in an amber vial for further analysis step.

3.3.2 Derivatization

This was obtained by adding water: trifluoroacetic acid: acetic acid (7:2:1, v/v/v) to the eluate. The extracts were filtered through a 0.2 µm PTFE membrane (Sigma Aldrich) and were kept at 65 °C for 15 min. An aliquot of 25 µL of a sample was injected into a High Performance Liquid Chromatography (Shimadzu 10 VP liquid chromatograph, Kyoto, Japan) with a 10 AXL fluorescence detector (excitation at 365 nm and emission above 450 nm). A Phenomenex (Torrance, CA, USA) C18 column (4.6 × 250 mm, 45 µm).

3.3.3 Aflatoxin standards

Calibration curves were prepared using standard solutions of aflatoxins B_1, B_2, G_1 and G_2 (Sigma, St Louis, MO, USA) previously evaluated individually according to (Scott, 1995); AOAC method, 2008.

Standard volume of 1, 5, 10, 15 and 20 µg/L were prepared by diluting the working standard solutions in water/methanol/acetonitrile (60:30:10, v/v/v) with a flow rate of 1.0 mL min^{-1}. A limit of quantification (LoQ) for the AFB1, AFB2, AFG1, and AFG2 was 0.05 µg/kg for each toxin, as determined by the minimum amount of toxin that could generate a chromatographic peak three times over the baseline standard deviation. The mean coefficient of determination (r) values obtained for AFB_1, AFB_2, AFG_1 and AFG_2 standard curves were 0.992, 0.943, 0.995 and 0.991 respectively. The aflatoxin extracts were injected in the HPLC and quantified by the retention time and peak area respectively.

3.4 Identification of toxigenic and atoxigenic Aspergillus strains

3.4.1 Isolation and enumeration of fungal species

Scientists use a number of different methods to determine the number of microorganisms that are present in a given population. In this study serial dilution, plating and counting of live fungi to determine the number of *Aspergillus spp* in a given population were used. The total viable count (TVC) and fungal counts were determined by the dilution plate technique. The soil samples were air dried (48 - 50 °C for 48 h) and then hammered to break it into a powder. It was then passed through a 2 mm aperture laboratory test sieve (Endecott's Ltd, London, UK) to get a fine powder. Isolation and quantification of Aspergillus section Flavi were done using the dilution plate technique on Modified Rose Bengal Agar (MRBA).

Soil sample weighing 1 g each was put into a 15 mL graduated dilution tube. Nine milliliters of 2% water agar was added to make a 10 mL stock solution. The stock solution was serial diluted by transferring 1 mL of the stock to 9 mL of the diluent until a 10^{-3} dilution was attained (Fig. 4). The diluted samples were placed in a rack in a water bath at 40 ºC and plated in a semi-selective medium. Plates were incubated in the dark for 3 days at 31 °C. Colonies of *Aspergillus* section Flavi were then identified by colony morphology. About 5 - 10 isolates per soil sample were transferred to 5/2 agar (5% V-8 juice and 2% agar, pH 5.2) and grown for 5 more days, unilluminated at 31 °C. Isolates were then classified on the basis of colony characteristics and conidial morphology at X400 magnification.

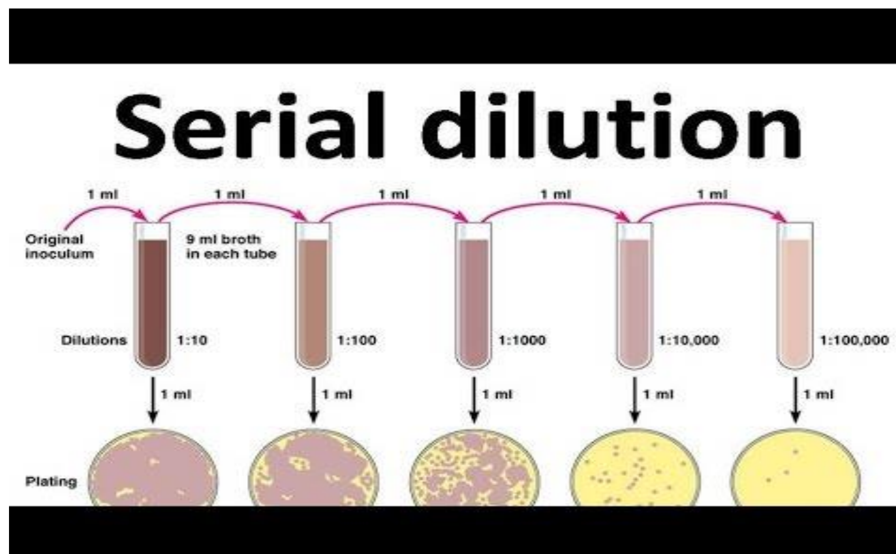

Figure 4: Serial dilution method: Source: http://www.shomusbiology.com

Fungal colonies which showed typical *Aspergillus* and their teleomorphic morphology under a stereo-microscope were transferred into malt extract agar (MEA), and incubated for 7 days, after which their genera were determined on the basis of macro morphology. Among them, strains of *Aspergillus* section Flavi (AsF) were inoculated at three points on MEA, Czapeks agar (CZA), and oatmeal agar (OA), and then incubated for 7 days. Their morphological features were evaluated in detail by stereo- and compound microscopy, and strains that evidenced characteristics identical to the other strains were removed.

3.4.2 The number of *Aspergillus* strains per mL of serially diluted soil fungi

Numbers of *Aspergillus* section *Flavi* in soils were calculated as colony forming units (CFU) per gram of soil. Numbers of 3 mm plugs of sporulating culture were transferred to 4-dram vials containing 10 mL of sterile distilled water. These conidial suspensions were maintained at 4 °C. The following equation was used to calculate the number of *Aspergillus* section *Flavi* per mL of diluted soil sample.

$$\text{Number of } Aspergillus \text{ section } Flavi \text{ per mL} = \frac{\text{Number of colony forming unit (CFU)}}{\text{volume plated (mL)} \times \text{Total dilution used}}$$

3.5 Formulation of groundnut seed based inoculant

The term 'competitive exclusion' involving physical blockage of growth or access of the toxigenic strain to the seed target has been used to describe the mechanism of biological control of aflatoxin contamination (Damnn, 2014). This direct touch-based inhibition of aflatoxin synthesis is posited to be the mechanistic basis of biological control in this system which involves the use of atoxigenic strains of *Aspergillus* section *Flavi*.

3.5.1 Field experiment

To evaluate field performance of formulated inoculants, a field experiment was carried out at Tropical Pesticide Research Institute (TPRI), Miwaleni sub station located at Uchira - Moshi District from June to October in 2018. Field trial was implemented at controlled irrigation and other good agronomic practices (GAP) which includes timely weeding and diseases control.

3.5.2 Inoculum preparation and soil application of toxigenic strains

Inoculum of native *Aspergillus* section Flavi S strains (toxigenic) were multiplied on autoclaved pearl millet grain by incubating for 7 days at 28 °C in dark. This inoculum (10 g infested grain row') applied at flowering stage (40 days after seedling emergence) in the furrows adjacent to the plants, covered with a thin layer of soil, and irrigated using furrows.

3.5.3 Atoxigenic inoculum and seed treatment

Autoclaved pearl millet grains were used as a substrate to multiply atoxigenic isolates for 10 days at 28 °C in dark. The infested millet grains with atoxigenic isolates were mixed with sterile distilled water, stirred for 4-5 minutes to detach the spores, and filtered through double-layered muslin cloth to obtain spore suspension. Two hundred grams of sorghum seeds were coated with 100 mL aqueous spore suspension by adding 1 ml of 0.5% carboxyl methyl cellulose (CMC) as sticker and 20 g of bentonite powder as filler for seed dressing.

3.5.4 Testing of formulated groundnut seed based inoculant

Two plots replicated three times were prepared, one for formulated inoculant (treated) and second for infested/ control (not treated); whereby each plot had size of 4 m X 4 m and groundnut planted at a spacing of 50 cm X 10 cm. The toxigenic inoculum (10 g) was applied at 40 days after seedling emergence followed by formulated inoculant (16 g) at 50 days after seedling emergence. Formulated inoculant (atoxigenic inoculum) only applied once in a

cropping season and to be totally effective speeded by hand broadcasting onto the field. Then after maturity 10 groundnut plants were randomly harvested from each treatment in all three replicates, dried, shelled and analyzed for their aflatoxin levels using HPLC protocol.

3.6 Data analysis

R Studio version 3.3.2 (2016-10-31) and GENSTAT 11th edition was used for data analysis for variables of interest. Means for the distribution of concentrations of aflatoxins in groundnut kernels were calculated and tested for significance at 95% Confidence Interval (CI). The Dunn`s multiple comparison was further used to separate the means.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Determination of aflatoxin levels in dried groundnut kernels

Results showed that there was a significant difference in total levels of aflatoxins in the three study villages (H=12.059, p=0.0024). Post hoc analysis using Dunn's Test of Multiple Comparisons with Rank Sums revealed the difference existed between all pairs tested except Mnanje and Naliendele sites (Fig. 5).

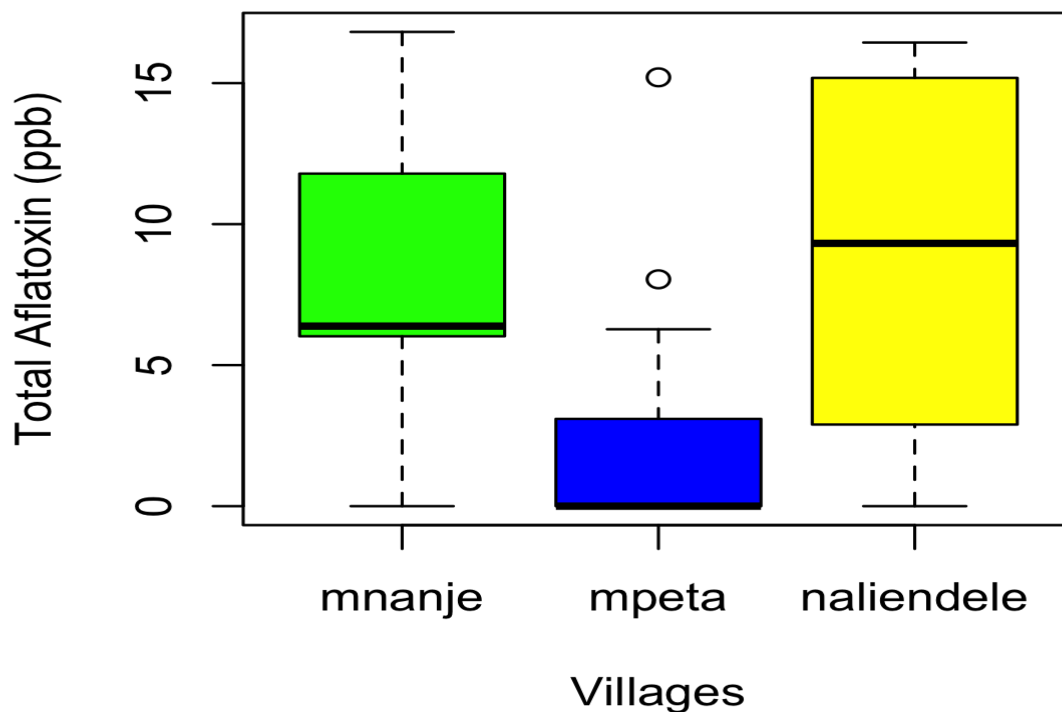

Figure 5: Total aflatoxin levels among the three study villages. (Means ± SD, n=3), error bars with different colours are significant different at $p < 0.05$

Typical levels of aflatoxins determined in dried groundnut kernel samples of Naliendele, Mnanje and Mpeta villages containing all the aflatoxins (AFB1, AFB2, AFG1 and AFG2) are summarized in Fig. 6.

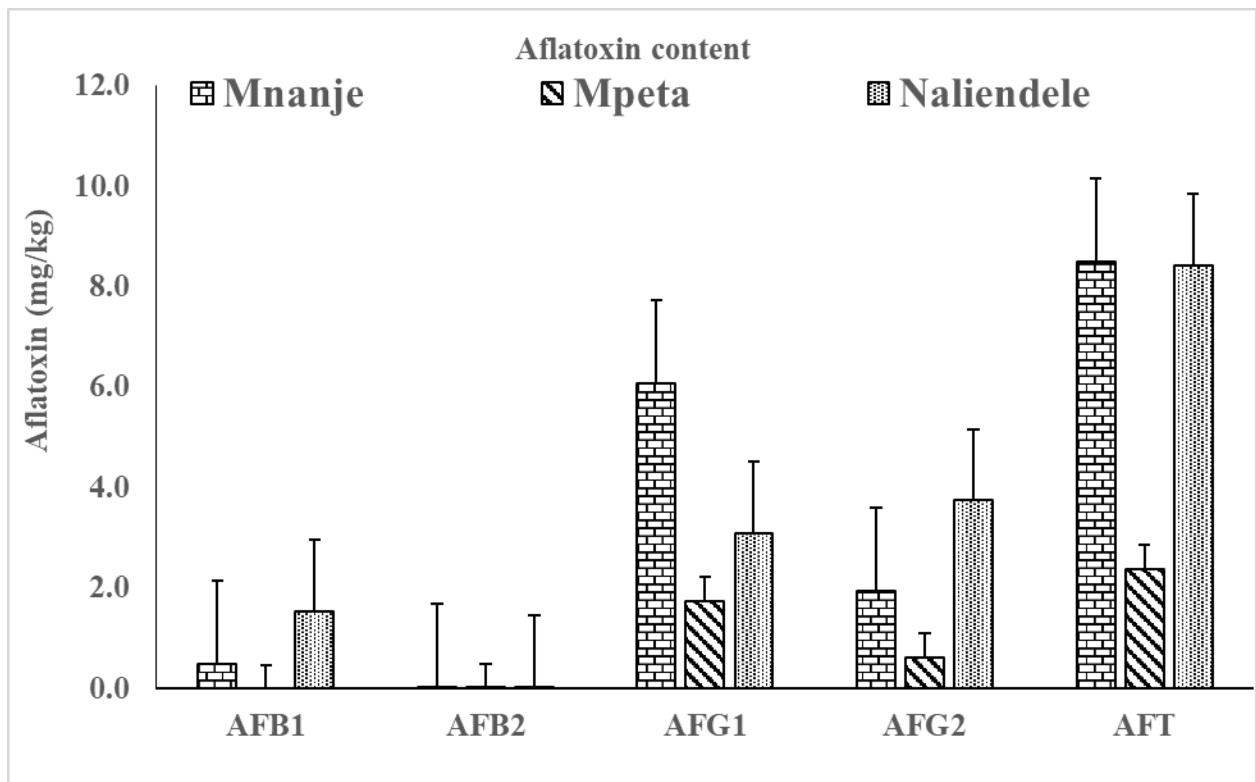

Figure 6: Aflatoxin types contamination levels in each study village

Frequency and occurrence of contamination and mean recovery percentage of aflatoxins in the validation assay for Naliendele, Mnanje and Mpeta dried groundnut kernels spiked with 2 and 20 µg/kg are summarized in Tables 1.

Table 1: Occurrence of aflatoxins in dried groundnut kernels in three villages of southern Mtwara region

Aflatoxin type	Village name	Frequency of Occurrence (n/N)	Range of concentration (μg/kg)	Mean ± SD
	Mnanje	1/15	0-7.25	7.30
AFB_1	Mpeta	0/15	0	0
	Naliendele	4/15	5.78-5.85	5.80±0.03
	Mnanje	1/15	0-0.24	0.24
AFB_2	Mpeta	2/15	0.05-0.27	0.16±0.15
	Naliendele	2/15	0.10-0.45	0.28±0.25
	Mnanje	14/15	5.86-8.75	6.50±0.77
AFG_1	Mpeta	4/15	5.87-7.78	6.50±0.86
	Naliendele	7/15	5.86-9.50	6.60±1.30
	Mnanje	3/15	9.44-10.11	9.40±0.03
AFG_2	Mpeta	1/15	0-9.33	9.30
	Naliendele	6/15	9.31-9.41	9.40±0.03
	Mnanje	14/15	5.86-16.81	9.11±4.40
Total Aflatoxin	Mpeta	5/15	0.05-15.02	7.11±5.40
	Naliendele	11/15	5.78-16.44	11.5±0.03

n=15; N=45

The number of groundnut kernel samples from three villages of Naliendele, Mnanje and Mpeta that contained at least one of the AFB1, AFB2, AFG1 or AFG2) at levels equal to or higher than the limit of quantification (0.05 μg/kg) shown in Table 2.

Table 2: Acceptable and non-acceptable aflatoxin contamination levels from the study villages.

	Aflatoxin levels µg/kg					
Farm Code	**AFB1**	**AFB2**	**AFG1**	**AFG2**	**Total Aflatoxin**	**Acceptable (+) / Not Acceptable (-)**
MN01	0.00	0.00	0.00	0.00	0.00	+++
MN02	0.00	0.00	6.70	0.00	6.70	++
MN03	0.00	0.00	5.90	0.00	5.90	++
MN04	0.00	0.00	6.40	0.00	6.40	++
MN05	0.00	0.00	8.80	0.00	8.80	++
MN06	0.00	0.00	6.70	10.1	16.8	-
MN07	0.00	0.00	6.00	0.00	6.00	++
MN08	0.00	0.00	6.30	0.00	6.30	++
MN09	0.00	0.00	6.80	0.00	6.80	++
MN10	0.00	0.00	5.90	0.00	5.90	++
MN11	0.00	0.00	6.30	0.00	6.30	++
MN12	0.00	0.00	6.00	0.00	6.00	++
MN13	7.30	0.20	7.30	0.00	18.8	-
MN14	0.00	0.00	6.20	0.00	15.6	-
MN15	0.00	0.00	5.90	9.40	15.3	-
MP01	0.00	0.00	0.00	0.00	0.00	+++
MP02	0.00	0.00	6.10	0.00	6.10	++
MP03	0.00	0.00	6.30	0.00	6.30	++
MP04	0.00	0.30	7.80	0.00	8.10	+
MP05	0.00	0.00	5.90	9.30	15.2	-
MP06	0.00	0.00	0.00	0.00	0.00	+++
MP07	0.00	0.00	0.00	0.00	0.00	+++
MP08	0.00	0.00	0.00	0.00	0.00	+++
MP09	0.00	0.00	0.00	0.00	0.00	+++
MP10	0.00	0.00	0.00	0.00	0.00	+++
MP11	0.00	0.00	0.00	0.00	0.00	+++
MP12	0.00	0.00	0.00	0.00	0.00	+++
MP13	0.00	0.10	0.00	0.00	0.00	+++
MP14	0.00	0.00	0.00	0.00	0.00	+++
MP15	0.00	0.00	0.00	0.00	0.00	+++
NL01	0.00	0.00	0.00	0.00	0.00	+++
NL02	0.00	0.00	0.00	0.00	0.00	+++
NL03	5.90	0.50	9.50	0.00	15.8	-
NL04	0.00	0.00	0.00	9.30	9.30	+
NL05	0.00	0.00	0.00	9.40	9.40	+
NL06	0.00	0.00	5.90	0.00	5.90	++
NL07	0.00	0.00	0.00	0.00	0.00	+++
NL08	5.80	0.00	0.00	9.30	15.1	-
NL09	5.80	0.10	6.30	0.00	12.3	-
NL10	0.00	0.00	5.90	9.40	15.3	-
NL11	0.00	0.00	0.00	0.00	0.00	+++
NL12	5.80	0.00	0.00	0.00	5.80	++
NL13	0.00	0.00	5.90	9.40	15.3	-
NL14	0.00	0.00	5.90	0.00	5.90	++
NL15	0.00	0.00	7.10	9.40	16.4	-

Scale of aflatoxin levels: **1**: 0 – 3.9 µg/kg (+++) =highly acceptable, **2**: 4 – 6.9 µg/kg (++) = Acceptable, **3**: 7 – 9.9 µg/kg (or +) = minimum acceptable and **4**: > 10 µg/kg (or - = highly not acceptable).

From the 45 groundnut kernel samples analyzed, 25 samples (55.6%) had aflatoxin contamination above the limit of quantification (> 0.05 µg/kg) for AFG1, followed by 10 samples (22.2%) for AFG2. Finally, 5 samples (11.1%) each for AFB1 and AFB2, respectively.

Mnanje village site had the greatest number of positive samples for AFG1 which was 14 and a total aflatoxin occurrence of 19, followed by Naliendele village (6) with a total aflatoxin occurrence of 19. Mpeta village had lowest number of positive samples for AFB2 (2) with a total aflatoxin occurrence of 7. Eleven groundnut kernel samples (24.4%) of the total analyzed samples had levels above 10 µg/kg, with a mean total aflatoxin concentration equal to 9.25 µg/kg. These total aflatoxin (TAF) concentration included 5 groundnut kernel samples with AFB1, 5 groundnut kernel samples with AFB2, 25 groundnut kernel samples with AFG1 and 10 groundnut kernel samples with AFG2 that had mean concentrations of total aflatoxins of 6.1 µg/kg, 0.2 µg/kg and 9.4 µg/kg.

4.1.2 Identification of toxigenic and atoxigenic *Aspergillus* strains

Morphological identification of *Aspergillus* section *Flavi* was done as described by Domsch *et al*. (1980). The fungal species were identified on the basis of morphology which comprises both macroscopic and microscopic characteristics. Plates were observed for colony colour using colour chart. All colonies that showed yellowish orange or pale yellow colour on the reverse side of colonies or black heads on the top of colonies were counted.

The colour of the colonies (Fig. 7a) was used for enumeration and identification of the sections. *Aspergillus* section *Flavi* had the highest frequency of (40/45) from soils of the total *Aspergillus* species. Two species were identified namely *Aspergillus flavus* and *Aspergillus tamarii* with their respective colonies observed.

(i) *Aspergillus flavus* (MN3)

Colony observed after 7 days of incubation at 31°C; the colonies were yellow green with white mycelia at the edges; formed sporulation rings; the conidia were rough; did not produce exudates and soluble pigments; Reverse colour was cinnamon brown as shown in Fig. 7b.

(ii) *Aspergillus tamarii* (N5 and MP10)

Colony observed after 7 days of incubation at 31°C. On PDA the colonies were cinnamon in colour with white mycelia and rough conidia. They produced exudates but no soluble

pigments. Reverse was cream yellow with deep yellow colour at the edges as shown in Fig. 7c1 and 7c2.

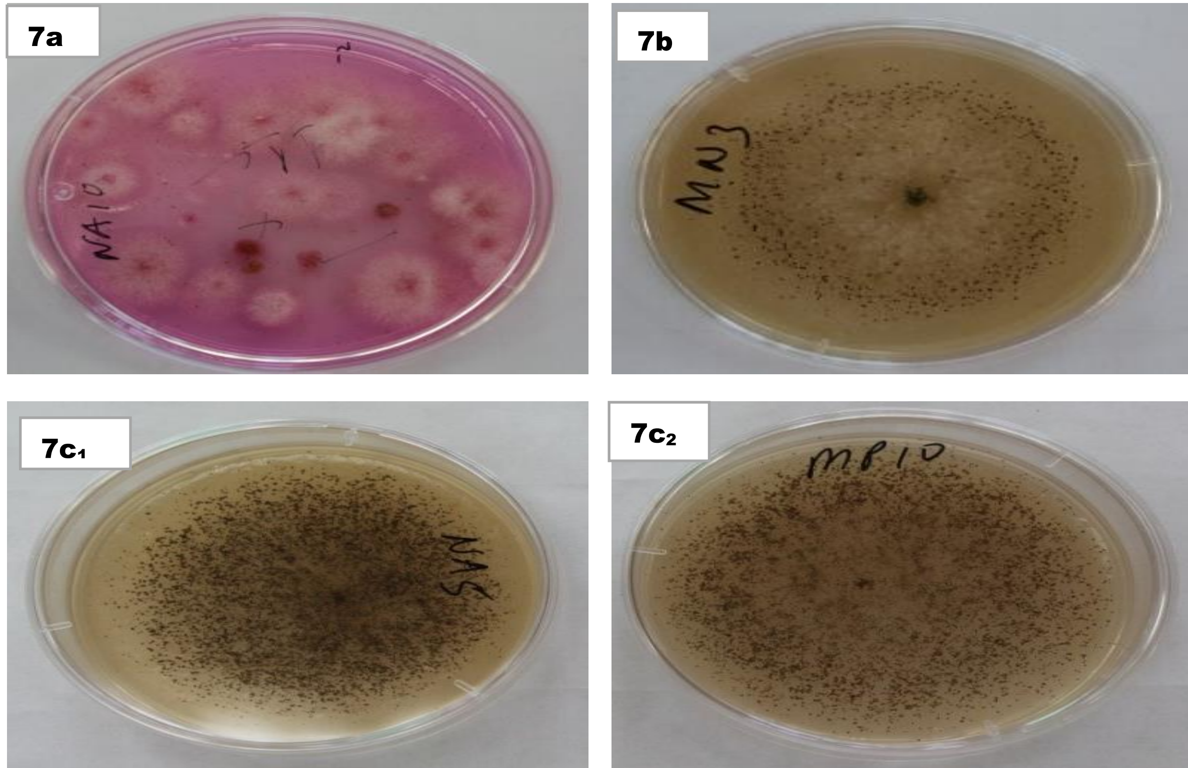

Figure 7a: Colonies of soilborne fungi growing on Modified Dichloran Rose Bengal medium (MDRB) after dilution and ready for counting, 7**b)** *Aspergillus flavus* colonies on selective media and 7c_1 and c_2: *Aspergillus tamarii* colonies on selective media from Naliendele and Mpeta village respectively.

The results of this study indicated a high incidence of *Aspergillus* species from the soils of groundnut farmer's household fields in Southern Mtwara. *Aspergillus* section *Flavi* population average ranged between 8.479 x 10^2 in colony forming unit (CFU)/g and 8.2136 x 10^3 CFU/g in all the three study villages summarized in Table 3. In this study I documented the population densities of *Aspergillus flavus* across three villages in Southern Tanzania. Population densities varied among study villages.

Table 3: Average quantity of *Aspergillus* section *Flavi* population in soil from 45 groundnut fields after harvest season

District[a]	Village name[b]	CFU/g[c]
Masasi	Mpeta	8.479×10^2
Mtwara	Naliendele	4.1158×10^3
Nanyumbu	Mnanje	8.2136 x 103

[a]Adminstrative districts; [b]Experiment localization; [c]Colony forming unit (CFU) of *Aspergillus* section *Flavi* in dry soil after harvest.

Aspergillus section *Flavi* was detected in 36 soil samples from 45 fields situated within the studied sites. A total of 402 section Flavi colonies were successfully transferred from MRBA to 5/2 agar and subsequently identified by macroscopic, microscopic and growth characteristics in AFPA medium.

Distribution of *Aspergillus* section *Flavi* (Table 4) indicated *Aspergillus* species was the most predominant fungal genera identified. Among *Aspergillus*, *Aspergillus flavus* was the most predominant where L-strains constituted 79.5% of the species identified, followed by S-strains constituted 18.4% while the frequency of the *Aspergillus tamarii* constituted 2%.

Table 4: Average percentage of *Aspergillus* section *Flavi* strains identified across study villages

Site name	Average L strains (%)	Average S strains (%)	Average *A. tamarii* (%)
Mnanje	68.0	32.0	0
Mpeta	85.3	13.3	1.3
Naliendele	85.3	10.0	4.7
Mean	79.5	18.4	2.0

[a]Adminstrative districts; [b]Experiment localization; [c]Colony forming unit (CFU) of *Aspergillus* section *Flavi* in dry soil after harvest.

Results from Table 4 shown average high incidence of L strains observed (85.3%) at Mpeta and Naliendele villages respectively; which followed by Mnanje village (68%); while average low incidence of S strains were observed at Naliendele village (10%), followed by Mpeta village (13.3%), and finally Mnanje village (32%). The presence of both L and S-strains indicated the possibility using them to develop sustainable bio control method by using atoxigenic ones.

Results indicated that there was significant ($p < 0.05$) differences between infested (control) and treated groundnut plots (Table 5). These results supports that the atoxigenic formulated inoculum was effective in significantly reducing aflatoxin contamination.

The total aflatoxin levels in groundnuts were lower in treated groundnut plots than in controls (infected) groundnut plots.

4.1.3 Effect of formulated groundnut seed based application on groundnut plants

Results indicated that there were significant ($p < 0.05$) differences between infested (control) and treated groundnut plots (Table 5). These results supported that the atoxigenic formulated inoculum was effective in significantly reducing aflatoxin contamination. The total aflatoxin level in groundnut was lower in treated groundnut plots than in controls (infected) groundnut plots.

Table 5: Paired Samples t-test results with the null (Ho) hypothesis that the mean of Infected – Treated is equal to 0 for all replications

	Paired Differences							
	Mean	Std Deviation	Std Error Mean	95% Confidence Interval of the Difference: Lower	95% Confidence Interval of the Difference: Upper	t	df	Sig (2 - tailed)
Pair 1 INFESTED - TREATED	6.896	6.457	1.179	4.487	9.309	5.85	29	.001

Results of total aflatoxin levels of ten randomly selected groundnut plants analyzed from each treatment and replication are shown in Table 6. Highest frequency of occurrence of total aflatoxin levels (> 10 μg/kg) for infested groundnut plants observed in replication one (5/10) representing 50% followed by replication three (4/10) representing 40% and finally replication two (1/10) representing 10%.

Table 6: Total aflatoxin levels of 10 selected groundnut plants for each replication

Replication	Infested (μg/kg)	Treated (μg/kg)
1	0.00	0.00
1	0.00	6.14
1	15.80	6.27
1	9.32	7.78
1	15.20	9.37
1	5.88	0.00
1	0.00	0.00
1	15.13	0.00
1	12.26	0.00
1	15.24	0.00
2	0.00	0.00
2	5.79	0.00
2	5.27	0.05
2	5.89	0.00
2	16.44	0.00
2	0.00	0.00
2	6.67	0.00
2	5.87	5.85
2	6.38	0.00
2	8.76	0.00
3	16.82	0.00
3	6.01	0.00
3	6.31	5.78
3	6.76	5.82
3	5.91	0.00
3	6.25	0.00
3	6.04	5.79
3	14.83	0.00
3	15.65	0.00
3	15.31	0.00

Total aflatoxin levels of 10 selected groundnuts for infested (toxigenic inoculum) and treated (atoxigenic inoculum) from each replication.

4.2 Discussion

The observed significance different of Aflatoxin levels amongst the study villages were attributed by differences in environmental conditions including temperature and moisture. Naliendele village is characterized by higher temperatures compared to other two study sites (Mnanje and Mpeta), which explains the higher concentration of aflatoxins observed. The temperature affects the physiological, biological and chemical processes of groundnuts and supports the survival of the fungus. The findings of this study are in line with a study by (Kamika *et al.*, 2014) in Congo and (Waliyar *et al.*, 2015) in Mali. This study indicated eleven dried groundnut kernel samples, (24.4%) of the total analyzed samples to contain aflatoxin levels above the maximum tolerable limit of 10 μg/kg, set for total aflatoxin (TAF) and five groundnut kernel samples (11.1%) were above the maximum tolerable limit of 5 μg/kg, set for AFB1 respectively. In the 2012 assessment report by Abt Associates (Leader, 2016) found that aflatoxin (AFB1) present in 20% of groundnut samples from Manyara (Northern zone) and Mtwara (Southern zone) and in 8% of samples from Shinyanga (Western zone) were above the maximum tolerable limit of 5 μg/kg, set for AFB1 in groundnut for human consumption in Tanzania.

Out of 45 of samples of dried groundnut kernel samples analyzed for total aflatoxins contamination in all three villages, twenty-four samples equivalent to 53.3% were found to be accepted for human consumption as they were below permissible level (10 μg/kg). AFG1 was predominant in all positive samples followed by AFG2, AFB1 and AFB2 respectively; as compared to findings reported by Seetha *et al.* (2017) which had mean AFB1 contamination levels in oilseeds from sunflower and groundnuts of 95.9 μg/kg compared to 1.4 μg/kg in starchy cereals of maize and sorghum in Central Tanzania.

The observed differences in the frequency of occurrence of AFG1 type as compared to other aflatoxin types between the study villages were attributed by the different strains of *Aspergillus* section Flavi found in the soils of the study sites. In addition to the environmental factors, storage conditions contributed to the observed differences, since dried groundnut kernel samples were collected from farmer's household stores that had different storage conditions and different storage structures but stored in the same period of time or season.

The incidence of atoxigenic strains of *A. flavus* L-strains were higher in all the villages except in the Mnanje village; where frequency of atoxigenic strains was significantly ($p < 0.05$) higher than that of toxigenic S- strains. *Aspergillus flavus* exists in two morphotypes the large

(L) and small (S) sclerotia producing strains. The S- strains have consistent high aflatoxin-producing ability while the L-strains vary greatly in toxin production with atoxigenic strains commonly found in this group. In this study, I documented the population densities of *Aspergillus* section *Flavi* across three studied sites in southern Tanzania.

Population densities of *Aspergillus flavus* varied among sites. Mnanje and Naliendele villages which fall in warm areas had high populations of *Aspergillus flavus* as compared to Mpeta village, located in cooler weather. Recently study in Zambia documented the population densities of *Aspergillus flavus* across two agroecologies in eastern Zambia; population densities of *Aspergillus flavus* varied among districts (Njoroge *et al.*, 2016). The mean population density of *A. flavus* was 2.6, 1.8, 2.0 and 2.4 log CFU/g of dry soil in Chipata, Mambwe, Nyimba, and Petauke districts, respectively.

Presence of *Aspergillus* section *Flavi* L- strains in higher percentages in all study villages of Mnanje, Mpeta, and Naliendele (68%, 85.3% and 85.3%), respectively; led to the identification of atoxigenic strains (MN03) which was employed to formulate groundnut seed based inoculant to manage aflatoxin-producing fungi in groundnut. Similar study conducted in Kenya reported identification of atoxigenic isolates of *Aspergillus flavus* with potential value for biological control within highly toxic *Aspergillus* communities associated with maize production (Probst *et al.*, 2011).

Fungi used for competitive exclusion involved both atoxigenic and competitive strains. According to Dorner (2010), for competitive exclusion to be effective, the atoxigenic strains must be present at highly competitive levels when conditions make the crop susceptible to infection. This was supported by results in this study where atoxigenic strains were 79.5% in average population while toxigenic strains were 21.4%. After application to the field and uptake of moisture, the fungus completely colonizes the grain, and abundant sporulation provides inoculum levels sufficient to achieve a competitive advantage for the nontoxigenic strains. Similar study conducted by Kachapulula *et al.* (2017) indicated the potential of atoxigenic members of the *Aspergillus flavus* L- morphotypes for management of aflatoxin in Zambia.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

This study has demonstrated that some dried groundnut kernel samples produced and traded in the Southern region of Mtwara were contaminated with aflatoxins, although only 24.4% indicated concentrations above the tolerance limit established by Tanzania Food and Drugs Authority regulations. The highest aflatoxin levels were observed in samples of groundnut kernel from Mnanje and Naliendele sites, respectively; and this fact has created awareness on a high health risk associated with the use groundnuts, taking into consideration children consume mostly porridge and other food stuffs prepared from grounded flour of groundnut kernels. The identification of both *Aspergillus* section *Flavi* namely *Aspergillus flavus* which contained large proportion of L strains (79.5%) as compared to S- strains (18.4%) and *Aspergillus tamarii* (2%); led to the identification of atoxigenic isolates which was employed to formulate groundnut seed based inoculant to manage aflatoxin-producing fungi in groundnut. The total aflatoxin levels in groundnut was lower in treated groundnut plots than in controls (infected) groundnut plots and this supports that the atoxigenic formulated inoculum was effective in significantly reducing aflatoxin contamination.

5.2 Recommendations

(i) This study recommends further investigations to study contamination of groundnut and groundnuts product by different types of aflatoxins in all major groundnut producing regions in Tanzania, before one develops interventions to mitigate it.

(ii) Further studies at Molecular level are recommended for studying diversity and efficacy of *Aspergillus* section Flavi namely, *Aspergillus flavus* L-strains, *Aspergillus* S- strains and *Aspergillus tamarii* from a wider geographical location in Tanzania.

(iii) Developed seed based inoculum needs further investigations by conducting series of on-farm trials and evaluation before recommended to farmers and other stakeholders for adoption.

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LIST OF APPENDICES

Appendix 1: Simple questionnaire for farmer`s households in research study villages

MANAGING AFLATOXIN-PRODUCING FUNGI USING INDIGENOUS ATOXIGENIC STRAINS OF ASPERGILLUS SPP. IN GROUNDNUT IN MTWARA REGION, TANZANIA

Background information

1. Location: ……………………… Coordinates……………………………. 2. Sex: 1. Male……. / 2. Female ………. / Age……………….

Household size?1. Males (above 18yrs) … 2. Females…/ 3. Children (below 18yrs) …

Aflatoxin knowledge and awareness

Answer the question by ticking the most appropriate answer.

1=strongly not aware 2=not aware, 3=somewhat aware, 4= aware, 5=strongly aware

Question: Are you aware of aflatoxin contamination of groundnuts? **1 2 3 4 5**

Appendix 2: Method for Determination of Aflatoxin B1, B2, G1, G2, In Food and Feeds by Pre Column Derivatization Using HPLC-FLD or HPLC/MS

1.0 Scope and application

This procedure describes the method for analysis of aflatoxin B1,B2,G1 and G2 in food and feeds by HPLC-FLD or HPLC-MS

2.0 Responsibilities

All analysts in Food laboratory shall be responsible for the application of this SOP.

3.0 Personnel qualifications

Personnel must be trained on the basic principles of the HPLC and must read the material safety data sheet of the chemicals before starting analysis.

4.0 Precaution

Aflatoxins are highly toxic, use protective measures such as gloves and mask

Decontaminate any used glassware with Sodium hypochlorite 4%

Aflacolumn are designed for single use only

Aflatoxin are light sensitive,handle it in a dark environment

The consumables used must be disposed in incinarator

5.0 Procedure

5.1 Requirements

5.1.1 Equipments and glass ware

High perfomance liquid chromatograph

10ml syringes

FLD detector/MS

Erlymeyer flask-250 mL

Vortex

Measuring cylinder

Vaccum filter with adapter

Filter paper

Brander

shaker

Funnel

5.1.2 Chemicals/ consumables

Aflatoxin standards (B1,B2,G1,G2)

Aflacolumn (immunoaffinity column)

Water (HPLC grade)

Methanol (HPLC Grade)

Acetonitrile (HPLC Grade)

Phosphate buffer solution (PBS) pH6-8

Gracial acetic acid

Sodium hypochlorite 4%

Trifluoroacetic acid (TFA)

5.2 Sample preparation

Four stages are involved during sample preparation

5.2.1 Extraction stage

Weigh out 25g of sample into 250 mLerlymeyer flask

Transfer the sample in blander jar and add 100ml ml of extraction solution (60:40 methanol;water or 60:40 acetonitrile :water)

Cover blender jar and mix on high speed for 3minutes or shake using gyratory shaker for 1hr

Using a funnel filter extract into a sample container using filter paper (whatman no1)

5.2.2 Dilution stage

Take 4 mL of extract and add 8ml of phosphate buffer solution (PBS)

Adjust the PH to 6-8 using sodium hydroxide

5.2.3 Clean up stage

Place the aflacolumn into the adapter

Load the diluted extract using a syringe and allow it to pass through column, the flow rate should not exceed 3 mL/min

Rinse the column twice with 10ml of distilled water,

Use the first rinse solution to wash the container and apply the second rinse direct to the column.In case of any remaining liquid apply slight pressure on top of column

5.2.4 Elution stage

Place the vial under the column for collection of eluent

Elute the bounded aflatoxin without the use of vaccum with 1ml of Acetonitrile HPLC grade by passing it through the column. The Acetonitrile should be left on the column for a few second before elution to allow intensive contact with the gel.

Apply slight pressure on top of column or apply vacum in the bottom to remove any remaining liquid

Take 400 µL from the eluent mix with 600 µL of derivatizing reagent (70:20:10 H_20: TFA:acetic acid)

(V) Condition the mixture at 65^0C for 15 minutes,allow it to cool and inject to HPLC

5.3 Standards preparation

Prepare a mixture of aflatoxin standard solution (B1,B2,G1,G2) of the following concentration; 1ng/mL, 5ng/ml, 10ng/mL and 15ng/mL for calibration curve.

Use derivatizing reagents as a diluent ((70:20:10 H_20: TFA:acetic acid)

5.4 Determination by HPLC

5.4.1 HPLC condition

Mobile phase : 50% :40%;10% Water :Methanol :Acetonitrile

Column; C_{18}

Column temperature: 40^0C

Flow rate: 0.8 mL/min

Injection volume:20µL

5.4.2 Detector- FLD

Emission 450nm

Excitation 365nm

5.4.3 MS condition

Ion source –ESI

Drying gas temperature-350

Drying gas flow-10L/min

Capillary voltage-4000

Nebulizer - 50psig

Scan m/z 199-350

6.0 Calculaton

Concentration of the sample, ppb = $\frac{\text{conc found}\left(\frac{\text{ng}}{\text{ml}}\right)\times 1\text{ml}\times 100\,(\text{ml})\times 2.5\,(\text{dilution factor})}{4\text{ml}\times \text{weight of the sample taken (g)}}$

The results of test sample shall be reported in one decimal place

7.0 References

Council for agricultural science and technology-report,mycotoxin risk in plant ,animal and human systems, Jan 20013

Romer labs

Appendix 3: Media – Recipes and Preparation for Isolation of *Aspergillus* strains from the soil.

3.1 Media for growing and identifying *Aspergillus* section Flavi

3.1.1 Clean up (CU) – For isolation of *Aspergillus* section Flavi from soil and crop

The medium used in sample isolations, called CU ("Clean up"), restricts the growth of *Aspergillus niger* and other fast growing fungi with Dichloran and restricts bacterial growth with Rose Bengal, Chloramphenicol, and Streptomycin. The antimicrobial compounds used in this medium do not guarantee an absence of bacteria or fungi that are not Aspergillus section Flavi, but contaminating organisms are greatly reduced. Heating streptomycin makes the compound too toxic to be useful in isolation media. Only add streptomycin to CU once the media is cool enough to handle. The inclusion of NaCl in CU medium promotes sporulation in *Aspergillus* section Flavi and makes young colonies easier to spot on the medium surface.

3.2 Chemical list

Bacto TM agar, Sucrose, $NaNO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4$*$7H_2O$, KCL, NaCl, Rose Bengal stock solution, Streptomycin stock solution, Chloramphenicol stock solution, Dichloran stock solution, A and M micronutrients

Protocol

1. Prepare one media for every 500 ml of medium, add a stir bar and the following in each bottle

   10 g (2%) BactoTM agar (may substitute with 13 g purified agar BMTM agar)

2. Place beaker on stir plate, add a stir bar and the following for each litre of purified water (enough for 2 media bottles)

3 g Sucrose

3 g $NaNO_3$

0.75 g KH_2PO_4

0.25 K_2HPO_4

0.5 g $MgSO4*7H_2O$

0.5 g KCL

10 g NaCl

1 ml A and M micronutrients

5 ml Rose Bengal stock solution

3. After all ingredients have dissolved, bring to final volume, and adjust pH to 6.50 while solution is stirring

4. Measure 500 mL of medium and add to individual media bottles

5. Loosely cap bottles and place to disperse agar (it will not dissolve)

6. Place bottles in microwave and heat on HIGH for 15 minutes or until agar melts. Watch after 10 minutes as it may boil before 15 minutes. DO NOT BOIL

7. Add 5 ml Chloramphenicol stock (2.5 mL/bottle)

8. Remove, place on stir plate to mix for a few minutes and then place it in autoclave basket

9. Autoclave for 20 minutes at 121 ºC. Remove from autoclave and let it cool (55-60 ºC).

10. Add in Biosafety cabinet

    10 ml Dichloran stock solution (5 mL/bottle)

    5 ml streptomycin stock solution (2.5 ml/bottle)

11. Place on heated stir plate (70 ºC) until medium is ready to pour

RESEARCH OUTPUTS

Output 1: Accepted Paper

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Distribution and occurrence of indigenous strains of atoxigenic and toxigenic *Aspergillus* section *Flavi* in groundnut producing areas of Southern Tanzania

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Key words: Distribution, *Aspergillus*, Occurrence, Atoxigenic, Toxigenic, Tanzania.

Abstract

The objective of this study was to isolate and identify atoxigenic and toxigenic strains of *Aspergillus* section *Flavi* in southern Tanzania, and investigate possible application of atoxigenic strains in control of aflatoxin levels in groundnuts. Fungal communities in soils from groundnut fields were examined to see the distributions of aflatoxin-producing *Aspergillus* species and to spot endemic atoxigenic strains. Forty-five isolates belonging to *Aspergillus* section Flavi were collected randomly from soils of groundnut fields in three districts and characterized using morphological and physiological examination. *Aspergillus* section *Flavi* was detected in 40/45 (88.89%) of the soil samples collected in Mtwara, Tanzania. Members of *Aspergillus* section *Flavi* L-strain was the most common (79.5%), followed by S-strains (18.4%) and finally *Aspergillus tamarii* (1.8%). The mean colony forming unit (CFU) of the *Aspergillus* colonies per gram of soil was highly variable ($p < 0.05$) among the districts, ranging from 8.5×10^{2} to 8.2×10^{3}. The mean pH across the gathering sites additionally varied (pH five 0.5–6.8) which is within the optimal pH requirement for the members of *Aspergillus* section *Flavi*. Non-significant ($p > 0.05$) variation in temperature across the sampling sites was observed. The results also showed that *Aspergillus* flavus was detected in all the three districts. Atoxigenic strains have a potential value to be employed as biological control agents to mitigate aflatoxin in groundnuts.

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Introduction

The soil serves as a reservoir for many microbial communities of plants and herbs which can be producing, carbon dioxide and nitrogen cycle (Fontaine *et al.*, 2003). The microorganisms play major role in soil ecosystem. Soil is associate in nursing oligotrophic medium for the expansion of fungi as a result of the plant life growth (Parkinson *et al.*, 1989). *Aspergillus* and its teleomorphs have been investigated with polyphasic methods to examine variability among species. Currently, according to the polyphasic taxonomy, (Samson *et al.*, 2014); (Yilmaz *et al.*, 2014) proposed that the genus *Aspergillus* is classified into four subgenera (*Aspergillus*, *Circumdati*, *Fumigati* and *Nidulantes*) and 20 sections and each includes a number of related species.

According to (Sugui *et al.*, 2014) *Aspergillus* section *Fumigati* is one of the most species-rich sections in the genus *Aspergillus* and includes species with overall significance for medicine, pharmacology, biotechnology, food and soil mycology. At present, the section consists of 51 taxa: 21 strictly anamorphic *Aspergillus* species and 30 *Neosartorya* species (Samson *et al.*, 2007). The most known economically important species are *Aspergillus flavus* and *Aspergillus parasiticus*, which are saprophytic (living on dead or decaying material) during most of their life-cycle (Klich, 1993). They are also plant pathogens and are found on a wide variety of crops produced in Africa including cereals, legumes, oilseeds, roots and tubers, spices, and tree nuts (Logrieco *et al.*, 2003). Furthermore, *Aspergillus flavus* and *Aspergillus parasiticus* are very useful in biological control of aflatoxin in legumes and cereals (Reddy *et al.*, 2009).

In United Republic of Tanzania, groundnut is among the foremost necessary crop for rural household's farmers, providing each food and financial gain for households (Katundu *et al.*, 2014). Groundnut is extremely alimentary with variety of useful ingredients as fats, protein, carbohydrates, vitamins and minerals all of which are important in human and livestock feed (Sibuga *et al.*, 1992). Due to the multiple uses of groundnut crop, this makes it an important food and cash crop for domestic consumption and export in many developing and developed countries (Kassie *et al.*, 2011). The consumption of groundnut and its products has received special attention in recent years due to food safety and food quality issues (Bourn & Prescott, 2002). The potential hazards associated with food include naturally occurring fungi which periodically cause severe contamination in invaded crops (Pitt, 2000). Aflatoxins are toxic metabolites produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*.

Aflatoxin B1 (AFB1) is a potent carcinogen, teratogen and mutagen (Ahsan *et al.*, 2010). Aflatoxin contamination reduces utilization options for the affected produce through complete rejection or reduced market value (Kumar *et al.*, 2017). According to (Dorner, 2009) biological control of aflatoxin production in crops in the United states of America (USA) has been approved by the Environmental Protection Agency (EPA) and two commercial products based on atoxigenic *Aspergillus flavus* strains are being used (Alfa-guard® and AF36®), for the prevention of aflatoxin in groundnuts, corn and cotton seed.

In Africa, atoxigenic strains of *Aspergillus flavus* have been identified to competitively exclude toxigenic fungi in the maize and groundnut fields. (Atehnkeng *et al.*, 2008) reported that atoxigenic strains reduce aflatoxin concentrations in both laboratory and field trials by 70 to 99% in Nigeria; a mixture of four atoxigenic strains of *Aspergillus flavus* of Nigerian origin has gained provisional registration as AflaSafe® to determine efficacy in on-farm tests. However, none known strains have been marketed in Tanzania and even if they will, there will a cost to be incurred by farmers higher than if the strains could be identified and produced in Tanzania. Therefore, objective of this research study was to identify natural occurrence of different toxigenic and atoxigenic *Aspergillus* strains in Mtwara, Masasi and Nanyumbu districts which will be useful in biological control approach.

Materials and methods

Field survey of study locations

Farmer's household fields' survey was conducted in 3 prominent groundnut producing districts of Masasi, Nanyumbu and Mtwara in Mtwara region, Southern zone of Tanzania. Three villages of Mnanje, Mpeta and Naliendele were purposively selected due to their potential in groundnut production in Mtwara region (Fig. 1). To determine fungal populations, soil samples were collected randomly from 45 groundnuts from 3 villages selected.

Fig. 1. A map of Mtwara region showing *Aspergillus* section Flavi study sites in Mtwara, Tanzania.

Sample collection and preparation

Forty-five soil samples were collected and processed as described by (Dorner, 2009). Three to five scoops of soil samples were randomly taken from each farmer's household field, thoroughly mixed to form a composite sample. Spoons used to scoop the soil at 4-10cm depth and were surface sterilized using 70% ethanol to avoid cross contamination. The same procedure was repeated for all the randomly selected soil sample points in the same famer's household field which were distant at least four meters apart.

A one-kilogram sub-sample was drawn from the composite soil sample and labelled with the name of the farmer, village, Global Positioning Systems (GPS) co-ordinates, and the date of collection. The labeled samples were put in zip lock bags and placed in a cool box transported to laboratory further analysis.

Isolation and enumeration of fungal species

The soil samples were air dried (48 - 50°C for 48 hours) and then hammered to break it into a powder. It was then passed through a 2mm aperture laboratory test sieve (Endecott's Ltd, London, UK) to get a fine powder. Isolation and quantification of *Aspergillus* section *Flavi* were done using the dilution plate technique on Modified Rose Bengal Agar (MRBA). Soil sample weighing 1g each were put into a 15mL graduated dilution tube. Nine milliliters of 2% water agar was added to make a 10mL stock solution.

The stock solution was serial diluted by transferring 1 mL of the stock to 9mL of the diluent until a 10-3 dilution was attained. The diluted samples were placed in a rack in a water bath at 40°C and plated in a semi-selective medium. Plates were incubated within the dark for three days at 31°C. Colonies of *Aspergillus* section *Flavi* were then identified by colony morphology. About five - ten isolates per soil sample were transferred to 5/2 agar (5% V-8 juice and a couple of agar, pH 5.2) and grown for 5 more days, unilluminated at 31°C. Isolates were then classified on the premise of colony characteristics and conidial morphology at X400 magnification. *Aspergillus* section *Flavi* colonies were known by their characteristic growth pattern, retention of MRBA within mycelia, and production of characteristics conidiophores after 3 days on MRBA.

Isolates with abundant small sclerotia (average diameter < 400mm) were preliminary classified as strains S while isolates with smooth conidia and large sclerotia (average diameter > 400mm) were classified as L strains of Aspergillus flavus. *Aspergillus tamarii* were initially identified by colony and spore morphology. All preliminary identification was confirmed by color reaction on *Aspergillus flavus* and *Aspergillus parasiticus* (AFPA) agar. Numbers of *Aspergillus* section *Flavi* in soils were calculated as colony forming units (CFU) per gram of soil. The following equation was used to calculate the number of *Aspergillus* section *Flavi* per mL of diluted soil sample

$$\text{Number of } Aspergillus \text{ section } Flavi \text{ per mL} = \frac{\text{Number of colony forming unit (CFU)}}{\text{Volume plated (mL) x Total dilution used}}$$

Number of 3mm plugs of sporulating culture were transferred to 4-dram vials containing 10 mL of sterile distilled water. These conidial suspensions were maintained at 4°C for additional analysis.

Results

Morphological identification of *Aspergillus* section *Flavi* was done as described by (Afzal *et al.*, 2013). The fungal species were identified on the basis of morphology which comprises both macroscopic and microscopic characteristics. Plates were observed for colony colour using colour chart. All colonies that showed yellowish orange or pale yellow colour on the reverse side of colonies or black heads on the top of colonies were counted.

The colour of the colonies (Fig. 2A) was used for enumeration and identification of the sections. *Aspergillus* section *Flavi* had the highest frequency of (40/45) which is 88.8% from soils of the total *Aspergillus* species. Three species were identified namely *Aspergillus flavus* L-strains, *Aspergillus flavus* S-strains and *Aspergillus tamarii* with their respective colonies observed (Fig 2B, $2C_1$ & & $2C_2$) respectively.

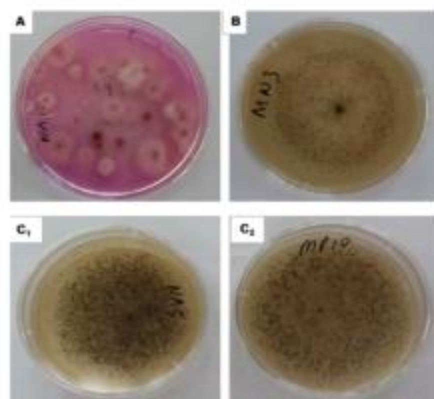

Fig. 2A. Colonies of soilborne fungi growing on Modified Dichloran Rose Bengal medium (MDRB) after dilution and ready for counting.

Fig. 2B. *Aspergillus flavus* colonies on selective media.

Fig. 2C_1 & C_2. *Aspergillus tamarii* colonies on selective media from Naliendele and Mpeta villages respectively.

Aspergillus flavus (MN3)

Colony observed after 7 days of incubation at 31°C; the colonies were yellow green with white mycelia at the edges; formed sporulation rings; the conidia were rough; did not produce exudates and soluble pigments; Reverse colour was cinnamon brown as shown in Fig. 2B.

Aspergillus tamarii (N5 & MP10)

Colony observed after 7 days of incubation at 31°C. On organizer the colonies were cinnamon in color with white mycelia and rough conidia. They produced exudates but no soluble pigments. Reverse was cream yellow with deep yellow colour at the edges as shown in Figs. 2C1 & C2.

The results of this study indicated a high incidence of *Aspergillus* species from the soils of groundnut farmer's household fields in Southern Mtwara. *Aspergillus* section *Flavi* population average ranged between 8.479×10^2 in colony forming unit (CFU)/g and 8.2136×10^3CFU/g in all the three study villages summarized in Table 1. In this study, I documented the population densities of *Aspergillus flavus* across three villages in Southern Tanzania. Population densities varied among study villages.

Table 1. Average quantity of *Aspergillus* section *Flavi* population in soil from 45 groundnut fields after harvest season.

District[a]	Village name[b]	CFU/g[c]
Masasi	Mpeta	8.479×10^2
Mtwara	Naliendele	4.1158×10^3
Nanyumbu	Mnanje	8.2136 x 103

[a]Adminstrative districts; [b]Experiment localization; [c]Colony forming unit (CFU) of *Aspergillus* section *Flavi* in dry soil after harvest.

Aspergillus section *Flavi* was detected in 36 soil samples from 45 fields situated within the studied sites. A total of 402 section *Flavi* colonies were successfully transferred from MRBA to 5/2 agar and subsequently identified by macroscopic, microscopic and growth characteristics in AFPA medium. Distribution of *Aspergillus* section *Flavi* (Table 2) indicated *Aspergillus* species was the most predominant fungal genera identified. Among *Aspergillus*, *Aspergillus*

flavus was the most predominant where L-strains constituted 79.5% of the species identified, followed by S-strains constituted 18.4% while the frequency of the *Aspergillus tamarii* constituted 2%.

Table 2. Average percentage of *Aspergillus* section Flavi strains identified across study villages.

Village name	Average L strains (%)	Average S strains (%)	Average *A. tamarii* (%)
Mnanje	68.0	32.0	0
Mpeta	85.3	13.3	1.3
Naliendele	85.3	10.0	4.7
Mean	79.5	18.4	2.0

Results from Table 2 shown average high incidence of L-strains observed (85.3%) at Mpeta and Naliendele villages respectively; which followed by Mnanje village (68%); while average low incidence of S-strains were observed at Naliendele village (10%), followed by Mpeta village (13.3%), and finally Mnanje village (32%). The presence of both L and S-strains indicated the possibility using them to develop sustainable bio control method by using atoxigenic ones.

Discussion

Morphological characterization of Aspergillus section Flavi from soil isolates

Aspergillus section *Flavi* were detected in 40 soil samples out of total 45 (88.8%) soil samples collected from different farmer's household fields of groundnut producing areas in Mtwara, Tanzania. This current study complies to previous similar research study findings in Benin (Cardwell & Cotty, 2002), Nigeria (Donner *et al.*, 2009) and Kenya (Muluvi *et al.*, 2015). Members of the *Aspergillus* section *Flavi* identified in this study includes *Aspergillus* section *Flavi* strains of L-morphotypes, S-morphotypes, and *Aspergillus tamarii* which showed to be major aflatoxin-producing contaminants in the soils of groundnut producing areas. This study supports a high incidence of *Aspergillus* section *Flavi* with *Aspergillus flavus* (L-morphotypes) being the most predominant (79.5%) as indicated in Table 2.

Distribution of Aspergillus section Flavi in Southern Tanzania

The incidence of atoxigenic strains of *Aspergillus flavus* L-strains was higher in all the villages except in the Mnanje village; where frequency of atoxigenic strains were significantly ($p < 0.05$) higher than that of toxigenic S-strains. According to (Gonçalves *et al.*, 2012) *Aspergillus flavus* exists in two morphotypes the large (L) and small (S) sclerotia producing strains; the S-strains have consistent high aflatoxin-producing ability while the L-strains vary greatly in toxin production with atoxigenic strains commonly found in this group. In this study, I documented the population densities of *Aspergillus* section *Flavi* across three studied sites in southern Tanzania.

Population densities of *Aspergillus flavus* varied among sites. Mnanje and Naliendele villages which fall in warm areas had high populations of *A. flavus* as compared to Mpeta village, located in cooler weather. Recently study in Zambia (Njoroge *et al.*, 2016) documented the population densities of *A. flavus* across two agroecologies in eastern Zambia. Population densities of *A. flavus* varied among districts. The mean population density of *A. flavus* was 2.6, 1.8, 2.0, and 2.4log CFU/g of dry soil in Chipata, Mambwe, Nyimba, and Petauke districts, respectively.

Presence of *Aspergillus* section *Flavi* L-strains in higher percentages in all study villages of Mnanje, Mpeta, and Naliendele (68%, 85.3% and 85.3%) respectively; led to the identification of atoxigenic strains (MN03) which was employed to formulate groundnut seed based inoculant to manage aflatoxin-producing fungi in groundnut. Similar study conducted by (Probst *et al.*, 2014) in Kenya, reported the majority (75%) of isolates belonged to the L-strain morphotypes of *Aspergillus flavus*; minor percentages were *Aspergillus tamarii* (6%), *Aspergillus parasiticus* (1%), and isolates with S-strain morphotypes (3%)

Aflatoxin-producing fungi vary most widely in their characteristics which includes, virulence for crops and aflatoxin-producing ability (Cotty *et al.*, 2008). In many reported cases groundnut and maize crops are very vulnerable for aflatoxin infestation. *Aspergillus flavus* identified has both L and S strains which are commonly implicated as causal agents of aflatoxin contamination (Probst *et al.*, 2014).

Currently, biological control to mitigate aflatoxin is managed by use of atoxigenic *Aspergillus flavus* L-strain isolates which are very useful to competitively exclude aflatoxin producers during crop infection and thereby limit contamination in USA (Cardwell & Cotty, 2002). In Africa particularly Nigeria these strains have been reported to reduce aflatoxin in both laboratory and field trials by 70 to 99% (Atehnkeng *et al.*, 2008). Fungi used for competitive exclusion involved both atoxigenic and competitive strains. According to (Dorner, 2010), for competitive exclusion to be effective, the atoxigenic strains must be present at highly competitive levels when conditions make the crop susceptible to infection. This was supported by results in this study where atoxigenic strains were 79.5% in average population while toxigenic strains were 21.4%. Similar study conducted by (Kachapulula *et al.*, 2017) indicated the potential of atoxigenic members of the *Aspergillus flavus* L-morphotypes for management of aflatoxin in Zambia.

Conclusion

Evidence in distribution and occurrence of both toxigenic and atoxigenic species of *Aspergillus* section Flavi, in Mtwara region confirmed the possibility of using these fungal species as biological agents to mitigate aflatoxin contamination risks in both soils and groundnuts produced and traded in southern Tanzania.

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DETERMINATION OF AFLATOXIN IN GROUNDNUT KERNELS IN SOUTHERN REGION OF MTWARA, TANZANIA

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Introduction

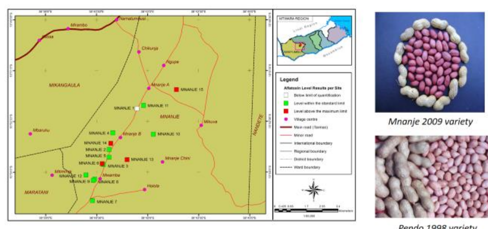
- Aflatoxins contamination is a growing threat to health, trade and food security in Sub Saharan Africa (SSA); where smallholder farmers are challenged by food production and now climate change.
- Subsequent severe outbreak of Turkey X disease in the United Kingdom (UK) lead to identification of aflatoxin, the causative toxin as a fluorogenic polyketide compound with dicoumarin structure from *Aspergillus flavus*.
- Aflatoxins occur naturally as four related chemicals, designated B1, B2,G1, and G2, that are not only acutely toxic, but are also highly carcinogenic (Eaton and Groopman, 1993). Tanzania is among SSA countries located between 40ºN and 40ºS latitude, has hot, humid, and drought-prone climates which offer suitable growing conditions for the fungi.
- The first reported case of acute aflatoxin in Tanzania occurred in the year 2016 when 65 cases reported from two districts (Chemba and Kondoa) in Dodoma region (central part of Tanzania); where 19 people have died. Since aflatoxins are potential carcinogens, their quantity in food and feed is closely monitored and regulated in most countries worldwide.
- Limited study performed for contamination of all types of aflatoxin from groundnut kernel produced and traded in Southern Tanzania.

Objectives

- ➢ To investigate the occurrence of aflatoxins from groundnut kernel produced and traded in Southern Tanzania
- ➢ To determine aflatoxin levels in dry groundnut kernel

Methodology

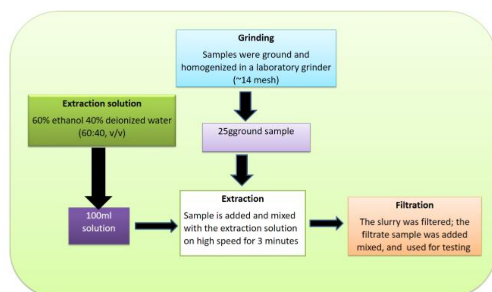
1. **Sampling**: A total of 45 groundnut kernel samples from 3 districts (Masasi, Mtwara, and Nanyumbu) of Mtwara region, Tanzania were collected between December 2017 and January 2018.



Mnanje 2009 variety

Pendo 1998 variety

2.Sample extraction



3.Aflatoxin (B1, B2, G1, G2) analysis

Analysis of aflatoxins was performed using High performance liquid (HPLC) protocol

Results and Discussion

Typical levels of aflatoxins determined in dried groundnut kernel samples of Naliendele, Mnanje and Mpeta sites containing all the aflatoxins (B1, B2, G1, and G2) summarized in Table1. Limits of quantification (LoQ) for the aflatoxins (B1, B2, G1, G2) was 0.05 μg·kg-1 for each toxin, as determined by the minimum amount of toxin that could generate a chromatographic peak three times over the baseline standard deviation. The mean coefficient of determination (r) values obtained for AFB1, AFB2, AFG1 and AFG2 standard curves were 0.992, 0.943, 0.995 and 0.991, respectively. Mean recovery percentage of aflatoxins in the validation assay for Naliendele, Mnanje and Mpeta dried groundnut kernels spiked with 2 and 20 μg·kg-1.

From the 45 groundnut kernel samples analysed, 29 (64.4%) had aflatoxin concentrations above the limit of quantification (> 0.05 μg/kg). Mnanje village was the site that had the greatest number of positive samples for aflatoxin 14 (93.3%) with a mean aflatoxin level (B1+B2+G1+G2) of 16.8 ± 5.9 μg/kg. Naliendele village followed as the site with number of positive samples 11 (73.3%) with a mean aflatoxin level (B1+b2+G1+G2) of 16.4 ± 5.7 μg/kg. Finally, Mpeta village was the site that had lowest number of positive samples 4 (26.7%) with a mean aflatoxin level (B1+B2+G1+G2) of 15.2 ± 6.1 μg/kg.

Table 1. Aflatoxin levels in groundnut kernels produced and traded in the Southern region of Mtwara, Tanzania.

Groundnut production site	Samples with aflatoxin[a]		Aflatoxin levels[b] (μg/kg)				
	>0.05 μg/kg[c] N (%)	>10 μg/kg[d] N (%)	AFB1	AFB2	AFG1	AFG2	Total aflatoxins
Naliendele	11/15 (73.3%)	6/15 (40%)	5.78-5.85	<LoQ[c]	5.85-9.50	9.31- 9.41	11.49±4.36
Mnanje	14/15 (93.3%)	4/15 (26.7%)	7.25	<LoQ	5.86-8.75	9.44-10.11	9.11±4.37
Mpeta	4/15 (26.7%)	1/15 (6.7%)	<LoQ	<LoQ	5.87-7.77	----	7.14±5.43
Total	29/45 (64.4%)	11/25 (24.4%)	5.78-7.25	0.05	5.85-9.50	9.31-10.11	9.25±4.72

[a] Number of samples analysed: 15 for each site (Total: 45 samples), [b] Results expressed as mean of samples analysed in duplicate, [c] Limit of quantification (LoQ) of the analytical method (0.05 μg·kg-1 for each aflatoxin), [d] Tolerance limit adopted in Tanzania (sum of aflatoxins AFB1, AFB2, AFG1 and AFG2), [e] Sum of aflatoxins AFB1, AFB2, AFG1 and AFG2.

Conclusion

It could be concluded that some dried groundnut kernel samples produced and traded in Mtwara region were contaminated with aflatoxins, although only 24.4% indicated concentrations above the tolerance limit established by Tanzania Food and Drugs Authority (TFDA) regulations; and this fact contribute to high health risk when considering that children are high consumers of porridge and other food stuffs prepared from grounded flour of groundnut kernels. The hazard of aflatoxin contamination in groundnuts originates in farmer fields, but can then be controlled or get exacerbated at the post-harvest and storage stages.

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