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DETECTION OF ZEARALENONE MYCOTOXIN AND CHARACTERIZATION OF *FUSARIUM* SPECIES IN COMMERCIAL MAIZE GRAINS FROM NORTHERN GHANA

ABDUL RASHID HUDU

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BY

ABDUL RASHID HUDU

(B.Sc. Food Processing Technology)

(UDS/MBT/0004/19)

THESIS SUBMITTED TO THE DEPARTMENT OF BIOTECHNOLOGY, FACULTY OF BIOSCIENCES, UNIVERSITY FOR DEVELOPMENT STUDIES IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY IN BIOTECHNOLOGY

NOVEMBER, 2022

DECLARATION

Student

I hereby declare that this dissertation/thesis is the result of my original work and no part of it has been presented for another degree in this University or elsewhere:

Candidate:

Signature: …… Date: ……

Name: ……

Supervisors

We hereby declare that the preparation and presentation of the thesis was supervised following the guidelines on supervision of thesis laid down by the University for Development Studies.

Principal Supervisor's

Signature: …… Date: …….

Name: …….

Co-Supervisor

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Name: ……

GENERAL ABSTRACT

The fungal genus *Fusarium* contains many important plant pathogens as well as endophytes of wild and crop plants. Together with their derivative mycotoxins such as zearalenone, can inhibit eukaryotic protein synthesis and cause toxicosis in humans and animals as well exhibit antimicrobial and phytotoxic activities. Maize (*Zea mays* L.) is among the major cereal staples in Africa including Ghana, but it is highly susceptible to *Fusarium* infestation. The focus of this study was to measure the level of zearalenone (ZEA) in commercial maize from northern Ghana, to assess traders' knowledge and awareness of mycotoxins and isolates and to characterize *Fusarium* species (ZEA producing ones). A total of 75 maize samples were randomly purchased from 11 different market centres in northern Ghana. ZEA levels were determined using HPLC. Knowledge and awareness of mycotoxin among traders were assessed using face-to-face interviews. *Fusarium* species were isolated using selective media and PDA growth media and molecularly characterized using species-specific primers. ZEA was found in 33.3 % of the samples (25/75), although generally at low levels, 0.61 to 3.05 ng/g with a mean concentration of positive samples of 1.50 ng/g. More than half (53.7%, n=22) of the traders have no knowledge of mycotoxins contamination of maize grain. Among those who were aware of mycotoxin occurrence, younger traders (age group < 20) and traders with more years in selling (above 5 years) maize are more likely to be aware of mycotoxins occurrences in maize. *Fusarium verticillioides* were the most predominant species. Most of the isolates were from the Upper East region. Despite the low health risk of the population to ZEA, this research points to the importance of raising traders' awareness to mycotoxins risk and control strategies.

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DEDICATION

To my Family

And

Islamic Development Bank Group

TABLE OF CONTENT

LIST OF PAPERS

Paper I. Zearalenone and its Associated Producing Fungi in Food: A Review of Emerging Trends, Control Strategies, and Legislation in Africa. (Submitted to *Comprehensive Reviews in Food Science and Food Safety*).

Paper II. Zearalenone Prevalence and Concentration Levels in Maize and Traders' Awareness of Mycotoxins in Northern Ghana. (*To be submitted to Mycotoxin Research* -- Springer*).*

Paper III. Mycotoxigenic *Fusarium* species on commercial maize kernels in northern Ghana. (*To be submitted to Journal of Fungi – MDPI*).

LIST OF TABLES

LIST OF FIGURES

1. CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Maize (*Zea mays* L. commonly known as corn) is one of the principal cereal crops widely grown and consumed throughout the world. It is an important human food and industrial crop in sub-Saharan Africa, where the estimated consumption range from 52 to 328 g/person/day (Ranum & Pe, 2014). In Ghana, maize is a widely cultivated food crop (Figure 1.2) for human consumption (72.09 %) and feedstock formulations (26.99 %) (MoFA-SRID, 2021). The crop is mainly cultivated under rain fed in northern Ghana, and production has increased gradually in the past few years. The per capita consumption is higher in the northern-savannah zone (MoFA-IFPRI, 2020). Maize plays a dynamic and diverse role in Ghana's agri-food system including food/nutrition security as well as source of livelihood for many rural farmers.

1.2 Problem Statement

Fusarium species and their derivative mycotoxins are involved in numerous postharvest and grain quality losses. In maize, the estimated yield losses attributable to *Fusarium* diseases may be more than 50% (Mielniczuk & Skwaryło-bednarz, 2020; Nathawat et al., 2020) depending on the type of cultivar, severity of infestation, soil fertility, and climatic conditions (Capo & Blandino, 2021; Golinski et al., 2010). Apart from the yield and quality losses, *Fusarium* produces various mycotoxins in grains. The most harmful groups, zearalenone, fumonisin, and trichothecenes, can inhibit eukaryotic protein synthesis (Han et al., 2022), and cause toxicosis in humans and animals as well as exhibit antimicrobial and phytotoxic activities (Kalagatur et al., 2018; González et al., 2021).

Their recent occurrence has become more pronounced in food and feed samples from Africa (Figure 1.1).

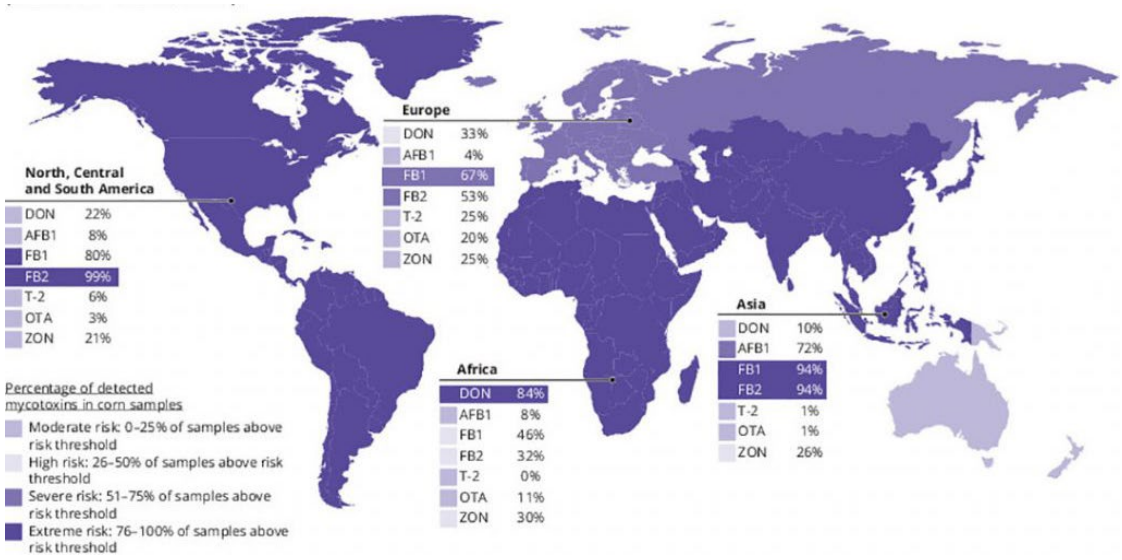

Figure 1:1: Global prevalence of mycotoxins in different regions based on percentage positive corn sample (October 2018 – March 2019)

Sources: Raj et al. (2019)

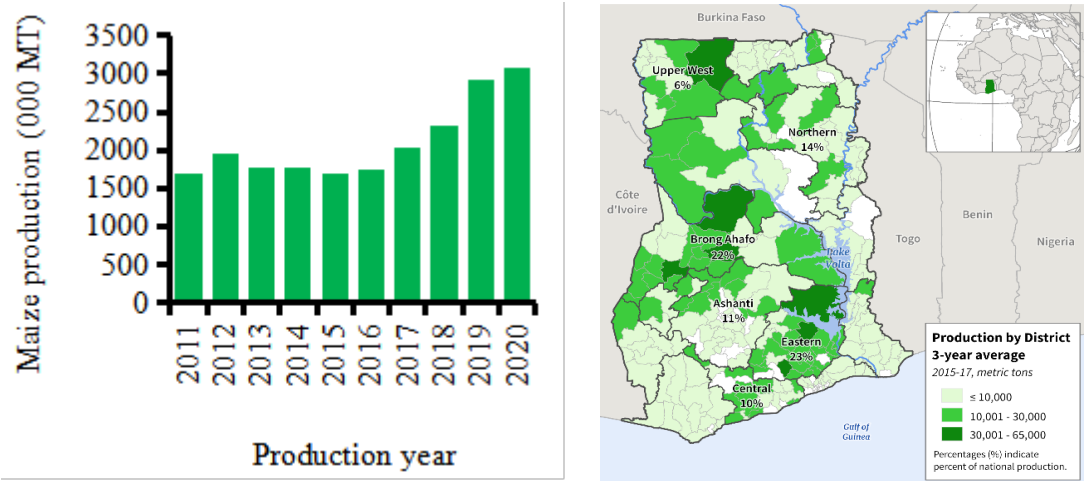

Figure 1:2: Maize production statistics (a) and the major producing regions (b) in Ghana

1.3 Justification of Research

Among the principal cereal crops grown in Africa for human and industrial usage, maize (*Zea mays* L.), is exceptionally susceptible to *Fusarium* infestations and zearalenone contamination. About 95% of feed and feed raw materials from Africa are contaminated with multiple *Fusarium* mycotoxins, more than twice the prevalence rate of *Aspergillus* mycotoxins (Gruber-Dorninger et al., 2018). The reason could be the wide and repeated misuse of mineral fertilizer coupled with preharvest and postharvest factors such as water stress, pest infestation and storage conditions. Excess nitrogen favored increased infestation of *Fusarium* fungi and subsequent production of zearalenone (ZEA) (Podolska et al., 2017); severe water stress causes production of mycotoxins in kernels (Daou et al., 2021). Consequently, animals and humans may frequently be exposed to different *Fusarium* toxins.

In Ghana, studies on *Fusarium* mycotoxins, particularly ZEA are very limited, few publications studied *Fusarium* spp. prevalence rate using morphological methods. These studies indicate growing evidence of ZEA producing species (*F. verticillioides, F. graminearum*) (Korley et al., 2022). Therefore, this thesis enters within these frameworks of evaluating food quality with respect to ZEA and its associated producing fungi in commercial maize from northern Ghana using molecular techniques.

1.4 Main Objective

The main objective was to estimate zearalenone concentration level and determine the diversity of its associated producing fungi in commercial maize from northern Ghana as well as to assess traders' knowledge and level of awareness of mycotoxins.

1.4.1 Specific Objective

1. To estimate and compare the levels of zearalenone in commercial maize kernel in the five Northern Regions of Ghana.
2. To assess maize traders' knowledge and awareness of mycotoxins.
3. To isolate and characterize naturally occurring ZEA producing fungi in commercial maize kernel in Northern Ghana.

1.5 Overview of Thesis Chapters

This thesis is designed and presented as manuscripts. The chapters are manuscript either under review or yet to be submitted in scientific journals. **Chapter one**, presents the layout of the thesis including the background, the main and specific objectives, conceptual framework and general methods of the study. The next chapter, **Chapter two,** is a literature review on zearalenone and its associated producing fungi with special focus on preharvest and postharvest factors influencing their occurrences, control strategies and legislations in Africa. Zearalenone concentration in commercial maize kernels and traders' awareness of mycotoxins in five regions in northern Ghana was carried out and presented as **Chapter three.** A survey for mycotoxigenic *Fusarium* species on the maize kernels using molecular techniques, was examined in **Chapter four**. A general summary, conclusion and recommendation based on all the findings of this thesis are outlined in **Chapter five**.

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2. CHAPTER TWO

LITERATURE REVIEW

Zearalenone and its Associated Producing Fungi in Food: A Review of Emerging Trends, Control Strategies, and Legislation in Africa

Abstract

The fungal genus *Fusarium* contains many important plant pathogens as well as endophytes of wild and crop plants. Globally, *Fusarium* toxins in food crops are considered one of the greatest food safety concerns. Their occurrence has become more pronounced in Africa in recent times. Among the major *Fusarium* mycotoxins with food and feed safety concerns, zearalenone is frequently detected in finished feeds and cereals in Africa. However, the impact of indigenous agricultural practices (pre- and postharvest factors) and food processing techniques on the prevalence rate of *Fusarium* species and zearalenone occurrence in food and feed have not been collated and documented systematically. This review studies and analysis recent reports on zearalenone contamination in agri-foods with a special focus on maize from Africa, including its fungi producers, indigenous processes impacting their occurrences, preventive measures, removal/decontamination methods, and legislations regulating their limits. Reports from relevant studies demonstrated a high prevalence of *Fusarium verticillioides* and *Fusarium graminearum* as the main producers of zearalenone in Africa. We also found that most countries do not know the status of zearalenone in their food supply chain; as such limited regulations exist to control its occurrence.

2.1 Introduction

Fusarium species are widely distributed and are well known as one of the world's most damaging fungi pathogens. The most harmful groups, zearalenone, trichothecenes, and fumonisin producers, continue to threaten sustainable agriculture in many regions. Recent reports have shown their common occurrence in African food and feed samples (Biomin, 2018, 2019; Gruber-Dorninger et al., 2019). Their postharvest occurrance, coupled with pre-and-postharvest factors such as water activity, temperature, or repeated misuse of mineral fertilizer are causing significant crop yield losses, and accumulation of mycotoxins (Mielniczuk & Skwaryło-Bednarz, 2020). For example, excess nitrogen favoured increased infestation of *Fusarium* fungi and subsequent production of zearalenone (ZEA) (Blandino et al., 2008; Podolska et al., 2017; Yi et al., 2001). Rapid infestation of grains by *Fusarium* species may cause a yield reduction of up to 19 % (Lobulu et al., 2021), and severe water stress could cause the production of mycotoxins in kernels (Daou et al., 2021). As a result, foods and animal feeds are exposed to multiple mycotoxins simultaneously (Rodrigues et al., 2011). Mycotoxins are secondary metabolites of fungi that have adverse health effects on plants, animals, and humans. Among the identified *Fusarium* mycotoxins with food and feed safety concerns, ZEA is frequently detected in feeds and cereals in Africa (Gruber-Dorninger et al., 2018). ZEA and its metabolites are the most studied mycotoxin with endocrine-disrupting activity (Eze et al., 2018). In Africa, especially South Africa, ZEA has been associated with the clinical diagnosis of gynecomastia with testicular atrophy in males (Shephard, 2008). Its prevalence rate in agri-food is becoming a prioritized area of research, particularly in Africa.

Numerous studies on the natural occurrence of *Fusarium* species and ZEA in food and feed in Africa have been conducted (Tables 2.2 and 2.3). The effect of ZEA on human and animal health has prompted some countries to establish appropriate, permissible levels in foodstuff intended for human and animal use. In the European Union, the maximum level for ZEA is pecked between 0.5 and 400 µg/kg in various products intended for human and animal consumption (European Commission, 2006b, 2006a). The maximum acceptable level for ZEA in China for example is 60 µg/kg (Ward, 2018). Currently, few legislations (South Africa and Morocco) are available for regulating ZEA in food and feed in Africa (Ankwasa et al., 2021; Lahouar et al., 2018).

There are few comprehensive databases on the prevalence rate of *Fusarium* species and ZEA occurrence in Africa. To facilitate data consolidation on the prevalence of *Fusarium* species and ZEA occurrence in Africa, this article highlights detailed information on ZEA and its associated producing fungi, current exposure levels, and regulatory regime in Africa. Furthermore, it summarizes stress levels leading to the production of ZEA in food and feed in Africa.

2.2 Trends in maize consumption and nutrient supply in Africa

Although it is clear that maize is a multipurpose crop, maize use as direct human food is repeatedly high in Africa (54 %) compared to its global 56 % use in feed production and 13 % use in direct human consumption. Maize grain is the first most consumed cereal in Africa with an intake ranging from 50 to > 330 g/person/day (Palacios-Rojas et al., 2020; Ranum & Pe, 2014), with the eastern and southern countries accounting for the majority of its use. Therefore, maize and its derived products form large proportions in food

formulations, including infant formulas. Among the major cereal consumed in Africa, maize provides more than 30 % of total calorie intake (Nuss & Tanumihardjo, 2010).

The average dietary energy intake per capita in Africa is 399 kcal for maize as food compared to a total intake of 80 kcal for Asia, 301 kcal for America, 59 kcal for Europe and 38 kcal for Oceania: the energy supply per day from maize is high in eastern and southern Africa (556.0 kcal/capita/day) compared to 287 kcal/capita/day for West and Central Africa and 318.3 kcal/capita/day for northern Africa (Erenstein et al., 2022). Furthermore, the average dietary supply of protein and fat intake per capita from maize as food is respectively 14 and 5 kcal/capita/day in eastern and southern Africa, 7.6 and 4 kcal/capita/day for West and Central Africa and 8.3 and 4 kcal/capita/day for northern Africa (Erenstein et al., 2022). Despite these significant contributions of maize to human nutrition in Africa, the grain is highly susceptible to *Fusarium* infection and ZEA contamination, and their effect on health is largely undetected in developing countries including Africa.

2.3 ZEA and its Producers in Africa

Zearalenone, a metabolite produced by various species of *Fusarium,* has been observed as a natural contaminant of cereals, in particular maize, in many countries in Africa and Europe, and the USA. High levels of ZEA production are associated with *Fusarium graminearum* and *Fusarium culmorum*. However, *F. oxysporum, F. sporotrichioides, F. proliferatum and F. verticillioides* have also been linked to ZEA production (Beev et al., 2013). Although these fungi have been widely reported to colonize a variety of food crops including maize, sorghum, wheat, rice, millet, and other legumes products, recent

evidence suggests that ZEA can also contaminate water, meat, fish, and dairy commodities (Alaboudi et al., 2022; Falkauskas et al., 2022; Gonkowski et al., 2018; Jafari-Nodoushan, 2022).

The chemical structure of ZEA and its 5 known conjugate metabolites are shown in Figure 2.1. The toxin is soluble in solvents such as acetonitrile, acetone, methyl chloride, and alcohol. In terms of stability, ZEA is stable to conventional food processing temperatures (EFSA, 2011). Globally, ZEA and its metabolites are well known for their estrogenic activities, resulting in reproductive system dysfunction. Furthermore, ZEA exhibits cytotoxicity by modifying biological macromolecules such as DNA, proteins, and nucleic acid (Eze et al., 2018; Jafari-Nodoushan, 2022; Rai et al., 2020).

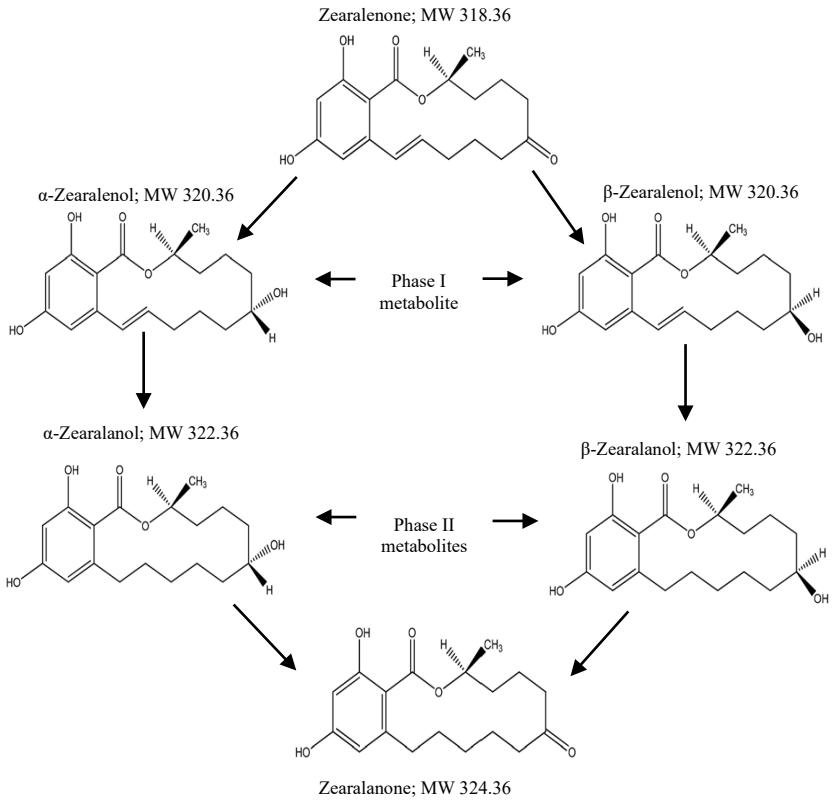

Figure 2:1: Chemical structure of zearalenone and its associated metabolites

Over the past decade, morphological and molecular analysis performed on various food crops from Africa showed the presence of several fungi genera. Among these fungi genera, *Fusarium* species are the most dominant fungi frequently isolated (Akello et al., 2021; Ekwomadu et al., 2018; John et al., 2018). The most common isolated *Fusarium* species from food crops in Africa are *F. graminearum*, *F. verticillioides, F. culmorum, F. Sporotrichioides, and F. equiseti* (Table 2.1). Their occurrence rate ranged from 3% to 100%, showing the ubiquitous presence of potential mycotoxigenic *Fusarium* species in maize, wheat, sorghum and other food produce.

2.4 Legislation on Zearalenone in Africa

As part of managing the exposure risk of animals and humans to different toxic compounds, many countries have established regulatory limits for mycotoxin concentrations in food and feedstuff. For many African countries, most of these regulations are limited to aflatoxins and fumonisin (Chilaka et al., 2022). Data on ZEA occurrence and evidence of high dietary exposure in individual countries are woefully inadequate for use in the establishment of the maximum permitted levels in food and feed raw material. As a result, many of these countries have adopted the European Commission and Codex Alimentarius Commission standards for ZEA (Ankwasa et al., 2021; Imade et al., 2021; Lahouar et al., 2018). However, only South Africa and Morocco have set regulatory limits for ZEA concentration in food and feed products. In South Africa, the maximum limits for ZEA in feeding stuff for sow/pigs, piglets, and calves/dairy cattle have been pegged at 5000 µg/kg, 3000 µg/kg, and 500 µg/kg/kg respectively (Government Notice, 2010). In Morocco, Zinedine and Mañes (2009) proposed ZEA limits of 200 µg/kg for all cereals intended for human consumption.

Table 2.1: Summary of recent *Fusarium* species occurrence in some African countries

Country/Region	Food crop	AT; Time	Target DNA	Primers, and probes (5' - 3') or mode of identification	Identified species	OR	Reference
Eastern Africa							
Ethiopia	Maize	53 ºC; 50 s	EF-1α	**EF1** ATGGGTAAGGAGGACAAGAC	*F. verticillioides*	42	Tsehaye et al. (2016)
				EF2 GGAGGTACCAGTCATCATGTT	*F. graminearum*	22.6	
					F. equiseti	0.5	
Kenya	Wheat	57 ºC; 60 s	EF-1α	**EF1** ATGGGTAAGGAAGGACAAGAC	*F. verticillioides*	30.0	Kheseli et al. (2021)
				EF2 GGAGGTACCAGTCATCATGTT	*F. equiseti*	25.3	
	Maize	53 ºC; 30s	TEF 1-α	**EF1** GTGGGGCATTTACCCCGCC **EF2** ACGAACCCTTACCCACCTC	*F. verticillioides*	-	Alaro (2021)
Tanzania	Maize	62.5 ºC; 60s		NS	*F. culmorum*	3.8	Degraeve et al. (2016)
					F. graminearum	75	
					F. culmorum	5	
					F. sporotrichioides	15	
					F. verticillioides	95	
Uganda	Maize, rice, millet, Sorghum	52 ºC; 30 s	EF-1α	**rp32** ACAAGTGTCCTTGGGGTCCAGG **rp33** GATGCTCTTGGAAGTGGCCTACG,	*F. verticillioides*	20	Wokorach et al. (2021)
			TEF 1-α	**EF1** GTGGGGCATTTACCCCGCC **EF2** ACGAACCCTTACCCACCTC	*F. equiseti*	6.9	
					F. proliferatum	1.3	
Zambia	Maize	-	-	Morphological	*F. verticillioides*	37.1	Kankolongo et al. (2009)
Southern							

Country/Region	Food crop	AT; Time	Target DNA	Primers, and probes (5' - 3') or mode of identification	Identified species	OR	Reference
Africa							
South Africa	Commercial maize	58 ºC; 45s	ITS	**ITS1** TCCGTAGGTGAACCTGCGG **ITS4** TCCTCCGCTTATTGATATGC	*F. oxysporum* *F. verticillioides* *F. proliferatum* *F. oxysporum* *F. graminearum*	20 88 73 65 48	Chilaka et al. (2012)
South Africa	Maize	58 ºC; 45s	ITS	**FF2** GGTTCTATTTTGTTGGTTTCTA **FR1** CTCTCAATCTGTCAATCCTTATT	*F. verticillioides* *F. oxysporum* *F. graminearum* *F. equiseti* *F. solani*	76 60 18 18 1	Ekwomadu et al. (2018)
Angola	Maize	-	-	Morphological	*F. verticillioides* *F. graminearum* *F. graminearum* *F. pseudoanthophilium*	83 68 22.5 13.4	Panzo (2011)
Botswana	Maize meal	-	-	Morphological	*F. verticillioides* *F. proliferatum*	20.9 2.5	Mokgatlhe et al. (2011)
	Sorghum meal			Morphological	*F. verticillioides* *F. proliferatum*	20 3.4	
Western Africa							
Burkina Faso	Onion	52 ºC; 30s		**EF1** ATGGGTAAGGARGARGACAAGAC **EF2**	*F. oxysporum* *F. proliferatum*	44.44 41.66	Kintega et al. (2020)

Country/Region	Food crop	AT; Time	Target DNA	Primers, and probes (5' - 3') or mode of identification	Identified species	OR	Reference
				GGARGTACCAGTSATCATCATGTT			
Nigeria	Oil palm	65 ºC; 30s	ITS	**ITS1** TCTGTAGGTGAACCTGCGG **ITS4** TCCTCCGCTTATTGATATGC	*F. oxysporum* *F. equiseti* *F. verticillioides* *F. proliferatum*	41.37 20.68 5.17 3.44	Chidi et al. (2020)
Ivory Coast	Multiple samples	55 ºC; 45s	ITS	**ITS1** TCCGTAGGTGAACCTGCGG **ITS4** TCCTCCGCTTATTGATATGC	*F. culmorum* *F. graminearum* *F. proliferatum*	7.4 18.5 25.9	Aasa et al. (2022)
Ghana	Groundnut	-	-	Morphological	*F. verticillioides*	20.7	Korley et al. (2022)
Nigeria	Maize	-	-	Morphological	*F. verticillioides* *F. Sporotrichioides* *F. verticillioides* *F. graminearum* *F. proliferatum* *F. Sporotrichioides*	31 96 82 50 40 76	Ezekiel et al. (2008)
Northern Africa							
Morocco	Wheat	58 ºC; 1 min	TEF 1-α	**Fu3f** GGTATCGACAAGCGAACCAT **Fu3f** TAGTAGCGGGGAGTCTCGAA	*F. equiesti*	8.82	Ezrari et al. (2020)
		55 ºC; 30s		**FOF 1** ACATACCACTTGTTG CCTCG **FOR1** CGCCAATCAATTTGAGGAACG	*F. oxysporum*	17.65	
Egypt	Multiple products	55 ºC; 1 min	ITS	**ITS1:** TCTGTAGGTGAACCTGCGG **ITS4:** TCCTCCGCTTATTGATATGC	*F. equieseti* *F. verticillioides*		El-Rabbat et al. (2018)
	Maize	58 ºC; 1 min	TEF	**F1T-F** ATGGGTAAGGAGGACAAGAC **EF1T-R** GGAAGTACCAGTGATCATGTT	*F. proliferatum* *F. culmorum* *F. oxysporum*	19.0 52.3 14.3	Khalil et al. (2013)
Algeria	Wheat	60 ºC; 30s		**Fco1F** ATGGTGAACTCGTCGTGGC **Fco1R** CCCTTCTTACGCCAATCTCG	*F. culmorum*	68	Abdallah-Nekache et

Country/Region	Food crop	AT; Time	Target DNA	Primers, and probes (5' - 3') or mode of identification	Identified species	OR	Reference
							al. (2019)
		55 °C; 30s		**Fp1 1**CGGGGTAGTTTCACATTTCYG **Fp1 2** GAGAATGTGATGASGACAATA	*F. pseudograminearum*	10	
		57 °C; 30s		**Fum 1–654** CGGTTGTTCATCATCTCTGA **Fum 1–654** GCTCCCGATGTAGAGCTTGTT	*F. verticillioides*	3	
Algeria	Wheat	62 °C; 5s		**Fcu-F** GACTATCATTATGCTTGCGAGA**G** **Fcu-R** CTCTCATATAC	*F. culmorum*	40	Hadjout et al. (2022)
Tunisia/ Egypt	Sorghum	60 °C;	β-tubulin, and TEF-1a	**BT2A** GGTAACCAAATCGGTGCTGCTTTC	*F. equiseta complex*	62.7	Lahouar et al. (2015)
		53 °C;		**BT2B** ACCCTCAGTGTAGTGACCCTTGGC	*F. verticillioides*	6.7	
				ET1: ATGGGTAAGGARGACAAGAC **EF2:** GGARGTACCAGTSATCATGTT	*F. proliferatum*	3.4	
					F. graminearum	1.7	
Central Africa							
Cameroon	Maize, peanut, poultry feed	55 °C; 20s	IGS region	**IGSF** AAGGAATTCAGGAATTCTCAATTG **IGSR** GTCCACCGGCAAATCGCCGTGCG	*F. verticillioides*	90	Kana et al. (2013)

OR; Occurrence rate

2.5 Agricultural practices that influence zearalenone accumulation in food

Being an emerging area of research in Africa, the impact of African indigenous farming methods on ZEA concentrations is limited. Preharvest and postharvest practices high in repeated misuse of chemical fertilizer, poor harvesting conditions, poor drying conditions, and poor storage structures result in fungi growth and mycotoxin production (Lehmane et al., 2022; Tang et al., 2019). Hence, ZEA concentrations in food are underpinned by three powerful factors – Preharvest factors identified as fertilization levels, fungicides use and harvest practices; postharvest factors such as drying techniques, storage conditions and transboundary trade; and food processing factors.

2.5.1 Pre-harvest factors

2.5.1.1 Nitrogen Fertilization

The rising per capita consumption of maize and population growth in Africa have stretched the use of chemical fertilizers in maize production. However, information on the use of mineral fertilizers, particularly nitrogen has come under intense criticism for their influence on mycotoxin (ZEA) levels in maize grain (Borràs-Vallverdú et al., 2022). It has become something of a truism that nutrient deficiencies or high nitrogen doses (> 200 kg N/ha) increased ZEA accumulation in maize grains (Podolska et al., 2017; Scarpino et al., 2022). Conventionally, in sub-Saharan Africa, the production processes of maize mostly employed suboptimal methods often resulting in farmers not being able to find a balance between nitrogen fertilizer, and maize uptake (Obour et al., 2022). As a result, under or over-application of nitrogen-based fertilizer is a common practice.

2.5.1.2 Use of fungicides

The use of fungicides has been extensively reported in Africa; however, often of over or under (sub-lethal) applications, a phenomenon that may trigger the production of various mycotoxins. This is so because fungicides have the potential to induce the production of hydrogen peroxide (H_2O_2), which is a key precursor that influences the biosynthesis pathway of secondary metabolites (Audenaert et al., 2010; Ferrochio et al., 2013). Studies on the impact of fungicides on the biosynthesis of mycotoxins of *Fusarium* spp. fungi have been reported. Cendoya et al. (2021) studied the impact of commercial fungicides: epoxiconazole + metconazole, tebuconazole, pyra-clostrobin + epoxiconazole, and prothioconazole on fumonisin accumulation by *F. proliferatum* and observed that all the studied fungicides used in sub-lethal doses enhanced fumonisin production except prothioconazole. The impact of H_2O_2 on deoxynivalenol, fumonisin, and nivalenol has been summarized in Table 2.2. However, the influence of oxidative stress on ZEA accumulation and ZEA gene expression has not been reported globally.

Table 2.2: Impact of oxidative stress factors on *Fusarium* species activities

Stress compound	Effect on *Fusarium* growth, and mycotoxin production	*Fusarium* strain used	Reference
H_2O_2 (0.5 mM, 2.0 mM)	Increase fumonisin production by up to 300% Enhance FUM gene expression	*F. verticillioides*	Ferrigo et al. (2015)
H_2O_2	Increase DON, and acetyl-DON production Enhance TRI gene expression	*F. graminearum*	Ponts et al. (2007, 2009)

Stress compound	Effect on *Fusarium* growth, and mycotoxin production	*Fusarium* strain used	Reference
H_2O_2 (0.05%)	Reduce fungi growth rate	*F. graminearum*	Zheng et al. (2012)
SDS (0.05%)	Enhance the growth of colony diameter	*F. graminearum*	Zheng et al. (2012)
H_2O_2 (0.5 mM)	Increase DON, and NIV production	*F. culmorum*	Ponts et al. (2009)
NaCl	Increased mycelial growth, mycelial biomass, sporulation, and microconidia	*F. oxysporum*	Maharshi et al. (2021)
EC (2-4 dS m^{-1})	Significantly enhanced fungal growth Increased biomass of fungal up to 90 %	*F. oxysporum*	Shoaib et al. (2018)
Sodium dodecyl sulphate (0.02%)	Decrease FUM gene expression	*F. verticillioides*	Nagygyörgy et al. (2014)
Sublethal dose Prothioconazole	Increase DON production	*F. graminearum*	

2.5.1.3 Harvesting practices

In Ethiopia and most countries in Africa, farmers use bent-down kernels that are completely dry and show a black layer at the base to determine harvest time (Mohammed et al., 2022). Other indigenous methods used by farmers to estimate maize moisture content for harvesting and storage include puncturing kernels with their thumbnails or biting kernels with their teeth or using the sounds made by kernels when agitated by hand (Joseph et al., 2015; Kagot et al., 2022; Liu et al., 2016). These practices coupled with maize cobs being heaped on farms before threshing and transportation or being dehusked and dried on cemented/bare floors have been accepted practice in most of Africa. These practices may influence *Fusarium* infestation and ZEA production. A study conducted to

evaluate the impact of grain maturation, harvesting time and late-season rainfall, showed that grains harvested late and exposed to pre-harvest rainfall had higher ZEA than those harvested early and therefore are not exposed to rainfall, at maturity (Moraes et al., 2022). Similarly, Edwards & Jennings (2018) investigated the impact of agronomic factors on *Fusarium* mycotoxins and reported that a 1-month delay in harvest increased ZEA concentration in wheat by 25-fold when compared to crops harvested at the average regional harvest date. It was previously reported that hay dried on the field had more ZEA than those dried under the shed (Taffarel et al., 2013). *Fusarium* fungi are more susceptible to mycotoxin production when subjected to heat shock, mainly with alternating temperatures, especially the daytime and night.

2.5.2 Post-harvest Factors

2.5.2.1 Storage conditions

In Africa, storage methods and structures differ by ethnic groups and agroecological area. Most storage structures in Africa are characterized by high relative humidity and temperatures. These structures and environmental conditions result in high relative humidity (> 60 %), temperature build-up, and insect propagation may lead to ZEA accumulation (Song et al., 2019). A study conducted in three climatic locations in Africa on the influence of storage practices on ZEA contamination in rice showed that storage location (N'diaye in Senegal, Cotonous in Benin and Yaoundé in Cameroon), processing type (plastic woven bags and pallets) and duration of storage (0, 90 and 180 days) affect ZEA concentration. The authors observed that storage structures with high relative humidity and low temperatures recorded significantly increased levels of ZEA (80 % RH;

24.4 ºC; 400.3 ppm ZEA) for grains stored for six months compared to storage structures with low relative humidity (62.8 % RH; 27.8 ºC; 100.2 ppm ZEA).

Similarly, extremely high levels of ZEA (51.8 to 468.6 ppm) were observed in grain from spikes exposed to 100 % relative humidity at all tested temperatures. At relative humidity ≤ 90 %, ZEA concentrations were very low (0.1 to 3.6 ppm) at all tested temperatures. At 100% relative humidity, mean ZEA contamination was significantly higher at 20 and 25 ºC (235.1 and 278.2 ppm) than at 30 ºC (104.7 ppm) (Moraes et al., 2022). Additionally, management practices such as improper cleaning of grains may result in *Fusarium*-contaminated plant debris being carried into storage. High proportions of plant debris such as cobs and husks may increase ZEA levels in the stored grains as this debris contain high levels of ZEA (Bamba et al., 2020; Tang et al., 2019).

2.5.2.2 Transboundary trade and ZEA occurrence

Transboundary trade is another major factor contributing to the spread and distribution of mycotoxigenic fungi and their mycotoxins, such as zearalenone, in agricultural produce in Africa. Africans' net import of 16.2 metric tonnes of maize makes it the second-highest continent, in terms of maize grain importation (Erenstein et al., 2022). A survey carried out in Nigeria has reported higher concentration levels of deoxynivalenol, and ZEA in imported wheat grains (mean 858.7 µg/kg for deoxynivalenol; 50.1 µg/kg for ZEA), whereas relatively lower levels (mean 517.8 µg/kg for deoxynivalenol; 14.5 µg/kg for ZEA) were detected in samples obtained from local farmers' stores or markets (Egbontan et al., 2017). In a similar study, Manizan et al. (2018) evaluated the occurrence of ZEA in local and imported grains in Cote d'Ivoire. They recorded no ZEA in the 41

imported rice samples, with 44.4 % of the local samples being contaminated with ZEA at varying levels. Although ZEA in the import wheat which was relatively high, its levels were low in rice samples in Cote d'Ivoire. This levels are however expected since ZEA is not a major issue in rice in the EU, USA and Asian countries, and may count for the low detection in rice samples.

2.6. Incidences and levels of Zearalenone Contamination in Africa

2.6.1 Zearalenone in raw maize

Zearalenone is a field contaminant of crops with toxin production occurring before harvest and continuing to accumulate in storage under favourable conditions. The toxin is classified as a stable mycotoxin and is not degraded under storage (Krska et al., 2003). ZEA is frequently detected in stored grains from Africa, with varying incidence rates and levels among grains and countries. In eastern Africa, ZEA concentrations were investigated in a total of 100 maize samples collected from smallholder farmers' stores in Ethiopia (Getachew et al., 2018). ZEA was detected in 96 % of the samples, with mean and maximum concentrations of 92 and 1656 µg/kg, respectively. Of the 96 % positive samples, 13.5 % contained levels above the European Union (EU) recommended value for unprocessed cereals (100 µg/kg). In another study in the south and south-western Ethiopia, Mesfin et al. (2021) randomly sampled 176 stored maize from various households and analysed them for *Fusarium* mycotoxins: ZEA concentration was up to 2447 µg/kg. In a similar study, Kamala et al. (2015) investigated multiple mycotoxin levels in 300 maize samples collected from various rural households in Tanzania. For the ZEA-positive samples (10 %), 66 % contained levels exceeding the EU maximum permitted limits. In a later study by Suleiman et al. (2017), 30 samples of maize

purchased from farmers and traders in Tanzania, were analysed for ZEA. All 30 samples tested positive for ZEA with levels ranging from 50-189.9 µg/kg. More recently in Kenya, maize samples collected between 2018 -2020 for multiple mycotoxin profiling revealed that 18 % of the 480 maize samples collected from Kenyan households had ZEA levels above 1000 µg/kg (Kagot et al., 2022).

Several studies in southern Africa have also reported maize contamination with ZEA. In a survey conducted between 2001 and 2021 in Eswatini, 892 maize and maize-based products were sampled and screened for ZEA contamination. In these samples, 7 % of maize grain and 17 % of maize meal were contaminated with ZEA (Dlamini et al., 2022). A total of 100 maize samples were randomly selected from small-scale and commercial farmers in South Africa and investigated for ZEA contamination using HPLC. The results showed that more than half of the small-scale farmer and commercial maize samples were contaminated with ZEA at mean levels below the South African regulatory limits (Ekwomadu et al., 2021). In Zimbabwe, a study conducted to assess the impact of storage duration (0, 90 and 180 days) on ZEA contamination in maize showed an increase in ZEA concentration by 89.6 % and 173.6 % in 90 and 180 days storage periods respectively (Hove et al., 2016).

Within the western Africa sub-region, higher concentration levels of ZEA have been observed in recent studies. For instance, in Côte d'Ivoire, 125 samples of maize were analysed for ZEA, and all the samples were contaminated with ZEA with 40 % of the samples exceeding the EU regulatory limits (Bamba et al., 2020). In 2019 and 2021, Oyeka et al. (2019) and Olopade et al. (2021) respectively investigated ZEA concentrations in maize from different agroecological zones in Nigeria. While none of

the samples analysed by Olopade et al. (2021) has levels exceeding the maximum permitted levels, 16.7 % of the 36 maize samples analysed by Oyeka et al. (2019) had ZEA concentrations above the EU maximum level for maize intended for direct human consumption. Additionally, all 20 samples were positive for ZEA phase I metabolites; alpha and beta zearalenol (Olopade et al., 2021). In another study in Togo, ZEA was detected in only 1 of 52 maize samples in lower concentrations (Hanvi et al., 2019).

Generally, the incidence rate of ZEA is fairly low for maize samples from northern Africa. Mahdjoubi et al. (2020) evaluated Algerian maize kernels from different local markets and reported that only one of seven ZEA-positive samples exceeded the EU maximum permitted levels. In Egypt, Sebaei et al. (2020) evaluated multiple cereals including 55 maize samples for ZEA contamination and observed that 2 samples had ZEA at levels 10 and 108 µg/kg. Previous research by Abdallah et al. (2017) also revealed that 10 out of 79 maize samples collected from farms and market centress in Egypt were contaminated with ZEA.

Data on ZEA occurrence in maize grains from Central Africa are quite real. A survey conducted on multiple products including 37 maize samples in Cameroon revealed that about 89 % of the maize samples were contaminated with ZEA in levels ranging from 0.2 to 309 µg/kg (Abia et al., 2013). In the Democratic Republic of Congo Mulunda et al. (2013) 40 maize samples collected from main markets were analysed for various *Fusarium* mycotoxins and 92.5 % of the samples were positive for ZEA with concentrations ranging from 24 to 811.2 µg/kg.

2.6.2 Zearalenone in Processed food and beverages

A survey evaluated ZEA levels in 50 traditionally maize-fufu food collected from households in Bamunka in Cameroon. Forty per cent and 90 % of the samples contained detectable levels of alpha and beta zearalenol. All the 50 traditional maize-fufu samples were ZEA-positive with levels ranging from 5 to 150 µg/kg (Abia et al., 2017). In another survey, 101 maize porridge sampled from households across three rural villages in Tanzania were screed for mycotoxins and the results showed that ZEA levels ranged from 10.20 to 269.9 µg/kg in 31 % of the samples (Geary et al., 2016). In another study in South Africa, Shephard et al. (2013) analyzed maize-based evening meals and porridge donated by 54 females in Transkei, a region with high oesophageal cancer for multiple mycotoxin occurrence. The author observed that all the samples were contaminated with ZEA with values ranging from 0.2 to 239 µg/kg for maize-based food and 0.44 to 239 µg/kg for porridge samples. Also, in Limpopo (South Africa), analyses of 20 maize porridge showed no contamination of ZEA and beta zearalenol. However, alpha zearalenol was detected in 19 of the 20 samples at levels between 10.25 and 61.5 µg/kg (Tebele et al., 2020). A study on the occurrence of ZEA in cooked maize porridge and dietary exposure of Tanzanian households to ZEA was performed in 2016. It was reported that 23 % of the processed maize-based porridge had ZEA concentration above the limit for infant food (ZEA > 20 µg/kg) (Geary et al., 2016).

In Cameroon, a selection of 14 traditional maize beers and 8 dagwa – a dry fried snack made from milled maize and groundnuts – samples produced from maize were screened for the presence of ZEA and other mycotoxins. Of these samples, 86 % of the traditional beer and 100 % of the dagwa samples were confirmed to contain ZEA in levels ranging

from 1.6 to 35 µg/kg and 6 to 57 µg/kg respectively (Abia et al., 2013). The authors observed that mycotoxin-related knowledge was low among fermented food sellers. Contrary to this observation, Adekoya et al. (2017) evaluated 32 maize-based beers from South Africa for the co-occurrence of mycotoxins and reported that none of the samples contained detectable levels of ZEA despite the detection of fumonisin in 53 % of the samples. Tables 2.3 and 2.4 provide a summary of published accounts on ZEA levels in other food and beverage samples in Africa.

2.6.3 Occurrence of ZEA in animals and animal products

Apart from grains, a few surveys also investigated the occurrence of ZEA in animals and animal products in Africa, where it was reported not to be as frequent as in grains. For instance, in South Africa, the mycotoxin survey in red meat from rural subsistence farmers and registered abattoirs by van Deventer et al. (2021) reported no ZEA contamination in tested samples (LOD; 20 µg/kg). Meanwhile, in Zambia (another Southern African country), Gonkowski et al. (2018) investigated the presence of ZEA and its analogue products in 27 sun-dried Kapenta fish collected from three cities where they recorded the presence of ZEA and α-ZEA in all samples with concentration levels ranging from 27.2 to 53.9 µg/kg, and <3.0 to 71.1 µg/kg respectively, 66.7 % tested positive for β-zearalenone with levels ranging from <12 to 59.8 µg/kg (Gonkowski et al., 2018).

In the West African sub-region, where 33 % of 108 dried beef samples in Nigeria tested positive for α-zearalenone with concentration levels ranging from 47.6 to 167.34 µg/kg (Dada et al., 2020), ZEA, and β-zearalenone were absent.

Table 2.3: Summary of zearalenone occurrence in raw food, and feed from Africa

Country/Region	Food crop	Sample-size (positive sample)	Method	Mean (range) (µg/kg)	Reference
Eastern Africa					
Kenya	Fish feed	78 (40)	HPLC	136 (<38-757.9)	Mwihia et al. (2020)
Ethiopia	Sorghum	80 (19)	HPLC	3.62 (<LOD-121)	Mohammed et al. (2022)
Kenya	Animal feed	25 (19)	HPLC	67 (61-167)	Rodrigues et al. (2011)
Kenya	Feed	10 (6)	LC-MS/MS	NS (11.2-28.2)	Warth et al. (2012)
Tanzania	Cassava	405 (154)	LC-MS/MS	NS (21.4-8493)	Sulyok et al. (2015)
Rwanda	Cassava	222 (104)	LC-MS/MS	NS (100-2826)	Sulyok et al. (2015)
Southern Africa					
South Africa	Chicken feed	62 (62)	LC-MS/MS	100 (NS-610)	Njobeh et al. (2012)
South Africa	Cattle feed	25 (24)	LC-MS/MS	72 (NS-123)	Njobeh et al. (2012)
South Africa	Horse feed	3 (3)	LC-MS/MS	43 (NS-46)	Njobeh et al. (2012)
South Africa	Swine feed	2 (2)	LC-MS/MS	148 (NS-170)	Njobeh et al. (2012)
Madagascar	Cassava	126 (41)	LC-MS/MS	83.8 (16.3-286.7)	Abass et al. (2019)
Namibia	*Kalahariturber pfeilii*	8 (8)	ELISA	NS (45 – 9680)	Hainghumbi et al. (2022)
Western Africa					
Côte d'Ivoire	Cobs	125 (125)	HPLC	NS (38.61-234.87)	Bamba et al. (2020)
Côte d'Ivoire	Spathes	125 (125)	HPLC	NS (94.54-341.84)	Bamba et al. (2020)
Nigeria	Sorghum	20 (18)	LC–MS/MS	18 (<LOQ-20)	Olopade et al. (2021)
Nigeria	millet	20 (17)	LC-MS/MS	64 (<LOQ-396)	Olopade et al. (2021)
Nigeria	Granola	18 (61.1)	LC-MS/MS	1.73 (0.81-5.99)	Ezekiel et al. (2020)
Nigeria	Popcorn	19 (5.3)	LC-MS/MS	6.40	Ezekiel et al. (2020)
Nigeria	Millet	87 (14)	LC/MS	419 (0-1399)	Chilaka et al. (2016)
Nigeria	Rice	41 (33)	HPLC	203.6 (0.7-570.6)	Egbuta et al. (2015)
Togo	Sorghum	12 (2)	LC-MS/MS	22 (19-24.6)	Hanvi et al. (2019)
Northern Africa					
Tunisia	Wheat	155 (123)	HPLC	110 (0-560)	Zaied et al. (2012)
Algeria	Wheat	30 (19)	UHPLC-MS/MS	102 (9.6-295)	Mahdjoubi et al. (2020)

Country/Region	Food crop	Sample-size (positive sample)	Method	Mean (range) (µg/kg)	Reference
Algeria	Rice	30 (6)	UHPLC-MS/MS	9.9 (8.6-15.5)	Mahdjoubi et al. (2020)
Egypt	Wheat	15 (6)	HPLC	1.55 (0.53-2.5)	El-Desouky & Naguib (2013)
Egypt	Barley	15 (4)		1.5 (0.70-1.77)	El-Desouky & Naguib (2013)
Egypt	Animal feed	77 (71)	LC-MS/MS	NS (NS-791)	Abdallah et al. (2017)
Central Africa					
Cameroon	Edible non-timber products	210 (194)	ELISA	62.7 (<15-500)	Djeugap et al., (2019)
Cameroon	Peanut	35 (15)	LC-MS/MS	4 (<LOQ-45)	Abia et al. (2013)
Cameroon	Soybean	10 (10)	LC-MS/MS	15 (12-18)	Abia et al. (2013)
Congo	Bean	30 (27)	TLC	185.2 (12.5-273.2)	Mulunda et al. (2013)

NS: not stated; nd: not detected

Table 2.4: Summary of zearalenone occurrence in beverages

Country	Product	No. of samples	Detection Method	Detection range (µg/L)	Reference
Nigeria	Fermented melon	25 (8)	HPLC	33 (21-45)	Adekoya et al. (2017)
	Fermented locust bean	8 (5)	HPLC	18 (11-33)	Adekoya et al. (2017)
	Fermented African oil bean	13 4)	HPLC	72 (39-117)	Adekoya et al. (2017)
Nigeria	Kumu	-	LC-MS/MS	0.2	Ezekiel et al. (2015)
	Pito	-	LC-MS/MS	0.2	
Cameroon	Maize beer	14 (12)	LC-MS/MS	17 (1.6-35)	Abia et al. (2013)
	Dagwa	8 (8)	LC-MS/MS	32 (6-57)	

2.6.4 Zearalenone in Water Sample

Analysis of the water samples from Zambia showed the presence of ZEA in three out of four water samples from lakes, with values ranging from <2.0 to 18.0 ng/L (Gonkowski et al., 2018).

2.7 Reported exposure assessment and risk characterization in Africa

Generally, there are two pathways for measuring human dietary exposure to ZEA. The First and most widely used method is the direct exposure pathway, where dietary exposure evaluation of ZEA combines household consumption data and the concentration of ZEA in food products and expressed as nanogram/kilogram body weight/day. The Food and Agriculture Organization of the United Nations (FAO) and the Joint Expert Committee on Food Additives (JECFA) under the World Health Organization (WHO) as well as the European Food Safety Authority (EFSA) have developed various guidelines for dietary data collection including living standards monitoring survey, household income and expenditure surveys and National household budget survey.

Using this method, high exposure risks have been reported by some countries in Africa. Consumers in the derived savannah zone of Nigeria were reported to have a high risk of exposure to ZEA with % tolerable daily intake (TDI) of 395.6, 158.3, and 66 which were 1582, 633, and 264 times higher than the tolerable daily intake of 0.25 $\mu g \cdot kg^{-1}$ $bw \cdot day^{-1}$ (Adetunji et al., 2017). A study on the occurrence of ZEA in food and dietary exposure of the population to ZEA was assessed in Algeria by Mahdjoubi et al. (2020). Mean ZEA exposure in the adult population through maize was lower (0.08 kg body weight per day; TDI 31.97 %) compared to wheat (0.85 kg b.w. per day; TDI 341.4 5).

The second pathway to assessing ZEA exposure is the indirect method. This approach uses biomarkers such as urine, breast milk and serum. Across the African continent, few studies on the lactational or maternal transfer of ZEA to an infant and birth outcome are available. Recently in Nigeria, Braun et al. (2022) evaluated the association between the transfer of ZEA from food to breastmilk and infant. ZEA was detected in lactating mothers' meals (0.1-33 µg/kg) and urine (11-1142 ng/L) but was not detected in breast milk. However, urine samples from exclusively breastfed infants contained ZEA ranging from 8.6-983 ng/L (Braun et al., 2022). Similarly, in Nigeria, Ezekiel et al. (2022) demonstrated that urine samples of exclusively breastfed infants (N=23) contain ZEA ranging from 17-784 ng/L, while ZEA could not be detected in breast milk consumed by exclusively breastfed infants (N=22). Another study conducted in Ethiopia to determine the association between ZEA in pregnant women and birth outcomes showed that 50.9 % (295) out of 579 serum samples of a pregnant woman contained ZEA with a concentration of up to 9 ng/ml (Tesfamariam et al., 2022). Also, 33.9 % (196), 42 % (243), 65.3 % (378), and 64.6 % (374) were positive for zearalenone, alpha zearalenol, beta zearalenol, beta zearalanol at concentrations up to 15.6 ng/ml, 10.5 ng/ml, 13.2 ng/ml, and 12.9 ng/ml, respectively. , The study, however, found no significant association between ZEA, and its derivative compounds exposures, and birth outcomes (Tesfamariam et al., 2022). Shephard et al. (2013) used a multi-biomarker method with β-glucuronidase and immunoaffinity clean-up to evaluate ZEA exposure levels of 54 adult females from South Africa using urine sample: ZEA was detected in 100 % of the urine samples with a mean level of 0.529 ng/mg creatinine. Also, 92% and 72% of the samples contained alpha and beta zearalenol with mean concentrations of 0.614 ng/mg creatinine

and 0.702 ng/mg creatinine respectively. In Yaoundé, Cameroon, Abia et al. (2020) collected and analysed 89 Cameroonian adults' urine for various *Fusarium* mycotoxins; the authors observed that ZEA and its phase I metabolites were the most frequently detected mycotoxins (82 % of the sample analysed contained detectable quantities of ZEA, alpha zearalenol and beta zearalenol) with 2 samples exceeding the EU TDI.

Being the major staples of many African communities, cereal and legumes particularly maize and sorghum, are frequently used as complementary foods for infants and young children. However, the dietary exposure assessment for ZEA reported from different countries in Africa is based on average intake and body weight for adults. This assessment may not represent the true exposure levels for infants and young children.

2.8. Strategies to reduce risk levels of ZEA in Africa

There is an urgent need to transform African indigenous food systems to respond to the present increased occurrence of mycotoxins in food grains. It has been established that the first step in mycotoxin management is to prevent fungi infestation, that is, production systems should incorporate elements and activities that will prevent fungi infestation (reference?). Providing agricultural education and technologies aimed at changing farmers' pre-and post-harvest practices, by using demonstrations (e.g. proper fertilization and fungicide use, with an explanation of their impact on agriculture) may help reduce ZEA exposure and improve mycotoxin levels in the African food supply chain (Njeru et al., 2019; Visser et al., 2020). The scale-up of education on the use of resistant cultivars, appropriate soil amendment methods, proper weed management and harvesting techniques should be facilitated. Unfortunately, commercially available maize cultivars in

Africa do not have a specific resistance to *Fusarium species* (Tembo et al., 2022). Previous studies in South Africa, and Nigeria reported potential resistance of some maize-inbred lines to *F. verticillioides* and fumonisin accumulation (Olowe et al., 2015; Small et al., 2012). No studies have so far evaluated maize cultivars from Africa for *Fusarium* species and ZEA resistance.

2.8.1 Impact of soil amendments

Being a typical soil-bornes pathogen, *Fusarium* species (ZEA producers) survive on or within infected soil and crop residues, which remains the leading cause of grain contamination before harvest. Consequently, using soil amendments to change the micro-environment of soil should be preferred to direct unamended soil. Biochar though more expensive than common fertilizers (Latawiec et al., 2019), can reduce *Fusarium* infestation and expression in crops through its potential to fix carbon and increase soil pH and elementary composition (Akhter et al., 2015; Marra et al., 2018). Recently in Nigeria, Akanmu et al. (2020), in their study, found biochar as an effective tool in managing resident pathogens (*Fusarium verticillioides*) and have successfully used biochar from poultry faecal waste and sawdust to reduce disease severity (ear rot) caused by *Fusarium verticillioides* in maize. Previous in vitro study has shown that activated carbon would serve as an adsorbent to mycotoxins, capable of binding 100 % ZEA at 0.1, 0.25, 0.5, and 1 % dose levels (Bueno et al., 2005). This adsorption capacity and increased pH explained that biochar not only delayed conidial germination but also carried a high adsorption capacity for mycotoxin (Li et al., 2022).

Recently in Africa, the utilization of natural extract is emerging as a possible method to suppress mycotoxigenic strains' growth and their production of mycotoxins in grain under storage. Although a higher water activity (a_w) level favours *Fusarium* growth, antifungal activities of some natural extracts such as essential oils are reported to be effective at higher a_w levels (Velluti et al., 2004). Olopade et al. (2019) explored the possibility of using montmorillonite clay and or *Cymbopogon citratus* to decontaminate ZEA in stored millet. The authors observed that millet treated with 12 % *Cymbopogon citratus* reduced ZEA contamination by 98.3 % while 12 % montmorillonite-*Cymbopogon citratus* mixed showed a 66 % reduction of ZEA in millet stored for 4 weeks. A study by Ali et al. (2021) suggests that essential oils obtained from *Mentha longifolia, and Citrus reticulata* can inhibit *F. culmorum* growth at 500 µl/ml. Similarly, in Egypt, Sahab et al. (2014) evaluated the antifungal activities of some essential oils extracted from rocket seeds (*Eruca sativa*), rosemary (*Rosmarinus officinalis*) leaves, and tea tree (*Melaleuca alternifolia*) against *Fusarium* isolates obtained from maize. The authors reported that essential oil from rocket seeds and tea trees exhibited high antifungal activity and completely inhibited all *Fusarium* isolates growth at 0.1 %, and 0.4 % respectively, while rosemary essential oil showed moderated antifungal activity with reduced growth of *Fusarium* isolates (Sahab et al., 2014). At 0.995 a_w, cinnamon oil, clove oil, lemongrass oil, oregano oil, and palmarosa oil exhibited inhibitory effects on *F. graminearum* growth, and their ability to produce zearalenone (Velluti et al., 2004).

2.8.2 Biological Control

A good number of microbial species have demonstrated their ability to counter the growth and ZEA excretion in toxigenic *Fusarium* species. A study by Shude et al. (2021)

using antagonist yeast species isolated from leaf, flowers, anther, and/or stem of cereals crops, and weed plants from South Africa exhibited varying inhibitory effects on the mycelial growth of *Fusarium graminearum*. The Authors observed that most of the yeast antagonists (87.21%) that inhibited the growth of *F. graminearum* could maintain inhibition until 20 days' post inoculation (dpi); however, about 58.33 % (7 out of 12) of the antagonist yeast increased ZEA concentrations (Shude et al., 2021). Subsequently, acibenzolar-s-methyl was combined with yeast antagonist (*Paoiliotrema flavescens, and Pseudozyma* sp.) to test against *F. graminearum* on spring wheat: it was observed that the combination of yeast and acibenzolar-s-methyl treatment effectively reduced *Fusarium* Head Blight severity, and deoxynivalenol concentration compared to the sole treatments (Shude et al., 2021).

Another study conducted by Debbi et al. (2018) explored the potential of using *Trichoderma spp*. from Algeria as a biocontrol agent against *F. oxysporum.* f. *sp. lycopersici* (FOL), and *F. oxysporum* f. sp. *Radices lycopersici* (FORL) showed that *T. ghanense and T. asperellum* might reduce the severity of crown and root rot, and *Fusarium* wilt diseases by 53.1, and 48.3 % respectively.

2.8.3 Physical methods

2.8.3.1 Sorting

Intervention methods such as cleaning and hand-sorting of damaged, discoloured and shriveled kernels reduce ZEA levels in grains. In many African countries, such as South Africa, Ghana, Benin, Nigeria, Malawi, and Tanzania grains are customarily hand-sorted before cooking. However, this process is tedious and difficult to apply in medium-to-large-scale food processing industries. Currently, mechanized tools and UV light have

been used to segregate ZEA-contaminated products. In Tanzania, Aoun et al. (2020) explored and developed a low-cost sorting tool (Dropsort device), that can be used in Africa for reducing mycotoxin levels in grains. The dropsort, a low-cost sorter that separates grains based on kernel bulk density and 100-kernel weight, combined with size sorting was more effective in reducing fumonisin (another *Fusarium* mycotoxin) concentration to under 2 ppm, but could not reduce aflatoxin levels in maize grain to under 20 ppm (Aoun et al., 2020). However, the effect of the device on ZEA concentrations in maize has not been reported.

2.8.3.2 De-hulling

De-hulling is a widespread food processing technique used in Africa and has successfully been used to reduce mycotoxin concentrations in finished food. De-hulling is the removal of the outer coat of grains. For zearalenone, the toxin is largely restricted to the outer layers of grain and therefore if partitioned into various fractions, including germs, bran, and coarse and fine grits may reduce human and animal exposure to ZEA (Brera et al., 2006; Habschied et al., 2011; Schwake-Anduschus et al., 2015). In Malawi, Njombwa et al. (2020) evaluated occurrence levels of ZEA in dairy cattle concentrate feed and observed that 75% (83 out of 111) of corn bran tested positive for ZEA with the minimum, maximum, and median concentration levels of 100 µg/kg, 2400 µg/kg, and 240 µg/kg, respectively. In South Africa, corn bran, corn flour, corn germ, and corn grits were reported to be contaminated with ZEA at mean levels of 245.6 µg/kg, 31 µg/kg, 29.8 µg/kg, and 8.6 µg/kg, respectively, compared to mean value of 93.4 µg/kg for whole maize (Burger et al., 2013). The major limiting factor to this control technique in Africa is that the hull is used as a component in animal feeds. This may increase animal

exposure risk and invariably human intake of ZEA through the consumption of animal products.

2.8.3.3 Steeping/Fermentation

Being water-soluble mycotoxins, ZEA concentration can be partially reduced during steeping and /or fermentation. African indigenous fermentation process, mainly as spontaneous or in most cases the addition of yeast, is capable of affecting ZEA levels in traditionally fermented food and beverages. In Nigeria, Ezekiel et al. (2015) assessed the levels of ZEA in *Kunu-zaki* – a traditional fermented non-alcoholic maize-based beverage and *pito* – a traditional fermented alcoholic drink made from sorghum. It was observed that the traditional fermentation process used in preparing these drinks can reduce ZEA levels up to 76.2 % and 94.8 % for *kuku-zaki* and *pito* respectively. Previously in Botswana, Nkwe et al. (2005) analysed sorghum malts and their corresponding wort and beer samples for ZEA levels; the traditional wort (90 µg/kg) and beer (92 µg/kg) samples contain less ZEA than their raw malted sorghum samples (485 µg/kg). More recently, an effective reduction in the content of ZEA was observed in bread prepared by baking with the addition of yeast, ranging from 14.3% to 35.4% (Podgórska-Kryszczuk et al., 2022).

2.8.3.4 Radiation

Another physical method, generally recognized as safe and extensively reported technology used to decontaminate mycotoxins in food is irradiation (gamma radiation, electron beams, and X-ray). In recent years, ionizing radiation, as a physical, -cold process, has been investigated as a method for the degradation of ZEA and its associated fungi in food grains (Calado et al., 2020). In Egypt, Sebaei et al. (2020) examine the

reduction of ZEA in grains using gamma radiation and reported that ZEA was more easily degraded in wheat than in maize. The reduction ranged from 49 to 97 % in wheat, 25 to 51 % in yellow maize and 20 to 60 % in white maize of ZEA in ZEA-spiked samples with initial concentrations of 100 µg/kg. In Tunisia, an irradiation dose (gamma radiation) of 3 kGy and 10 kGy was sufficient to reduce 90 % of the natural fungal load and 32 % of ochratoxin A in sorghum respectively (Amara et al., 2022).

2.9. Potential food processing technologies to control ZEA in Africa

Although, most traditional operations of maize and other unit operation can reduce the concentration of mycotoxins including ZEA, complete removal or decontamination using these operations are time-consuming, not reliable and not suitable for large-scale food processing. Hence, additional processing technologies may be required for safe consumption. Cold plasma treatment is an emerging food safety intervention that has been explored for possible mycotoxin control.

2.9.1 Ozone

Ozonation, an environmentally friendly and generally recognized as safe technology is reported to be efficient in the decontamination of various mycotoxins, with the process effectiveness depending on ozone concentration, gas flow, exposure time, moisture content and physical characteristics of samples (Qi et al., 2016). The process is highly recognized for its ability to generate ozone gas easily from oxygen (pure or from the air) without leaving residues. The process has been applied to inactivate insects in grains, eliminate/reduce fungi spores and in recent times applied to reduce mycotoxin levels in grains (Ribeiro et al., 2021). Alexandre et al. (2019) observed 37.9 %, 55.7 % and 62.3 %

reduction of ZEA in naturally contaminated maize flour when exposed to 5, 10 and 60 min respectively. Qi et al. (2016) observed an 86 % reduction in naturally contaminated ZEA in maize when exposed to 100 mg/L of ozone for 100 min. In comparison to other detoxification methods, Ozone performed better in the degradation of ZEA than electron beam irradiation (Yang et al., 2020). Although ozone application is effective in degrading ZEA, the technology increased monounsaturated fatty acids and decreased polyunsaturated fatty acids such as linoleic, oleic, and α-linolenic fatty acids (Purar et al., 2022; Qi et al., 2016).

2.9.2 Dielectric barrier discharge

Dielectric barrier discharge is another non-thermal treatment technology employed to degrade mycotoxins in food. The technology can degrade up to 98.28 % of ZEA in food products at 50 KV for 120s (Huang et al., 2022). Dielectric barrier discharge treatment for ZEA degradation is more efficient in liquid food products and dry food samples compared to solid wet foods (Feizollahi & Roopesh, 2021). The authors further observed that dielectric barrier discharge generated from 85 % Argon (Ar) + 15 % O_2 resulted in higher degradation of ZEA compared to N_2 (Feizollahi & Roopesh, 2021).

2.10. Conclusion

This literature review has examined scientific information to explore the prevalence rate and concentrations of zearalenone and its associated producing fungi, their detection methods, current preventive measures, and legislations in food and feed from Africa. Although *Fusarium* fungi and their associated secondary metabolites severely impact grain quality and the economic well-being of livestock and humans, few studies have evaluated their presence in food production. *F. verticillioides and F. graminearum* are the

most commonly identified species in Africa's subregional blocks. Despite the relatively high prevalence rate of these fungi, the preventive and mitigation efforts applied so far are insufficient. Both pre-harvest and post ZEA decontamination measures need to be adopted by farmers in developing countries. While most countries do not know the status of zearalenone in their food supply chain, limited regulations exist to control its occurrence.

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3. CHAPTER THREE

ZEARALENONE IN MAIZE

Zearalenone Prevalence and Concentration Levels in Maize and Traders' Awareness of Mycotoxins in Northern Ghana

Abstract

As food and feed raw materials are prone to several mycotoxigenic fungi, mycotoxin contamination in food needs to be continuously monitored. In this study, we determined the concentrations of zearalenone (ZEA) in 75 commercial maize samples from northern Ghana (Upper West, Upper East, North East, Savannah and Northern Regions) using HPLC. Traders' knowledge and awareness of mycotoxins were also evaluated. ZEA contamination was recorded in 33.3 % of the maize samples with concentration levels ranging from 0.61 ng/g to 3.05 ng/g. Awareness of mycotoxins contamination among maize sellers was high in the Northern Region (6 out of 10 traders) and Upper East Region (5 out of 10 traders). Younger traders (< 30 years) and those of more years in selling were more aware about the occurrence and knowledgeable of types of mycotoxins. This study provides preliminary insight into ZEA contamination in commercial maize grains and has shown only a fairly widespread awareness of cereal mycotoxin and handling practices among maize traders in northern Ghana.

Keywords: Zearalenone, traders' perception, maize, northern Ghana,

3.1 Introduction

Mycotoxins are secondary metabolites of fungal species including *Fusarium, Penicillium* and *Aspergillus*. Of the over 400 chemically diverse mycotoxins characterized in nature, zearalenone (ZEA), fumonisin, deoxynivalenol, ochratoxin A and aflatoxin are the major

metabolites of public health concerns due to their frequent occurrence in cereals and cereal-derived products (Daou et al., 2021).

Among the major *Fusarium* mycotoxins, ZEA exhibits the most potent endocrine disrupting activity, causing severe changes to mammary glands and organs of the reproductive systems (Eze et al., 2018). ZEA has been shown to significantly increase vulva area, and reduce serum immunoglobulin G level, superoxide dismutase content and antioxidant capacity in gilt (Song et al., 2021; Wu et al., 2021) and reduce nutrient absorption in gilt (Liu et al., 2020). Furthermore, higher levels of zearalenone in feed have also been correlated with feed refusal in livestock (Martina et al., 2002; Peter et al., 2002).

These adverse effects of ZEA on health make it imperative to develop monitoring systems that will reduce animal and human exposure to the toxins and also lead to the establishment of regulatory limits for ZEA in food crops. Public awareness, capacity building and behavioral change have been tested to be tools for reducing mycotoxin occurrence in agri-food (Adekoya et al., 2017; Lee et al., 2017; Muñoz et al., 2021).

Recent reports indicate a widespread occurrence of *Fusarium* mycotoxins in food and feed raw materials in developing countries (Rodrigues et al., 2011; Rodrigues & Naehrer, 2012) which predisposes animals and humans to the toxins. In Ghana, the prevalence of *Fusarium* mycotoxins (i.e., fumonisin, deoxynivalenol and zearalenone) in compound feed and maize ranged from 11-86 % (Kpodo et al., 2000; Rodrigues et al., 2011). Among the principal food crops grown and consumed in Ghana, maize (*Zea mays* L.) is exceptionally susceptible to ZEA contamination which makes it a ZEA toxicity risk cereal. Meanwhile, data on the prevalence of ZEA contamination in crops in the country

is unavailable and studies on the subject are poorly coordinated, unlike accounts on aflatoxin contamination in grains (Kortei et al., 2021a; Kortei et al., 2021b; Opoku et al., 2018). To appraise the importance of *Fusarium* toxins contamination in food in Ghana, comprehensive data is needed on the array of *Fusarium* mycotoxins that covers the prevalence, concentrations, geographic distribution and the range of food and feed sources that are affected. In the current study, we simultaneously investigated the levels of ZEA in maize and trader's awareness of mycotoxins contaminations in northern Ghana.

3.2 Materials and Methods

3.2.1 Study Area

Studies to detect the levels of ZEA and assesses farmers' awareness of mycotoxins in maize grains were carried out in the Northern part of Ghana. In general, climate of the entire country is tropical and strongly influenced by the West Africa monsoon winds. It is generally warm with variable temperatures all year round, masked by seasons and elevation. The northern part of the country lies between latitudes 8°N and 11°N and has a land area of 97702 km^2. Northern Ghana, consist of 5 regions: Northern, Savannah, Upper West, Upper East and North East. The area is typically dry and records one rainy season, which begins in May and lasts until September with erratic annual precipitation ranging from 400 to 1200 mm with intermittent drought.

The vegetation is characterized mainly by grassland with drought resistant trees such as shea, dawadawa, baobab and tamarind. Agricultural activity in the Northern part of Ghana is predominantly rainfed and smallholder basis with maize, rice, millet and sorghum been the major cereal crops grown in the area.

3.2.2 Sample Collection to detect ZEA levels in maize grains

Between April and May 2021, seventy-five maize samples (white maize and yellow maize harvested in 2020) were randomly purchased from 11 different central markets in five administrative regions in northern Ghana, namely, Northern, North East, Upper East, Upper West and Savannah Regions. Within each region, 15 maize samples were collected. At each sampling point (retail trade), about 8 to 10 small samples were randomly drawn from various sections of homogenized maize lot into plastic zip lock bags to a weight of 1 kg.

3.2.3 Moisture Analysis

Moisture content of maize kernels were determined using the method described in the American Association of Cereal Chemist international (AACC, 1999) with slight modification to the drying temperature (105 ^{o}C). Briefly, 35 g of maize kernels were milled using a Preethi kitchen blender (Trio-MG 158). Approximately, 2.0 g of each milled sample was transferred into aluminum moisture can and oven-dried at 105 ^{o}C for approximately 15 h to a constant weight. Moisture content was reported on dry matter basis.

3.2.4 Mycotoxin Analysis

3.2.4.1 Zearalenone Extraction and Purification

A sub-sample of 2.0 g of the milled maize was transferred into a 50 ml falcon tube and extracted for 21 min with a mixture of 5 ml water and 5 ml of 1 % acetic acid in acetonitrile on a rotary shaker. After extraction, 4.0 g of anhydrous magnesium sulphate and 1.0 g sodium chloride were added to the solution. The resulting mixture was

vigorously shaken and centrifuged for 35 min at 4300 rpm. Then 2.5 ml of supernatant were transferred to a reaction vial and evaporated inside a heating block flushed by a stream of nitrogen and stored until analysis. For chromatographic analysis, the samples were reconstituted with 200 µl of the mobile phase prior to HPLC analysis.

3.2.4.2 ZEA Quantification

Zearalenone separation and quantification were performed using High-Performance Liquid Chromatography system coupled to a fluorescence detector (HPLC-FD: Cecil-Adept binary pump HPLC with a Shimadzu 10AxL Fluorescence Detector). One hundred microliters (100 µl) of diluted eluate were injected into the HPLC system under the following conditions: 4.6 mm × 150 mm, 3.5 µm i.e., C_{18} reverse phase column (Agilent Eclipse Plus column); oven temperature of 40 ^{o}C; excitation wavelength of 225 nm and emission wavelength of 440 nm; mobile phase acetonitrile: water (50:50 v/v) at isocratic elution flow rate of 1 ml/min. The analytical method had a limit of detection (LOD) and limit of quantification (LOQ) of 0.1 ng/g and 0.5 ng/g, respectively. The calibration curve for this method was linear, with an R^2 of 0.99.

3.2.4.3 Method Validation

The analytical method was validated with the following parameters: linearity, limits of detection and quantification (LOD and LOQ), and recovery (Table 3.1). To assess the precision of the analytical method, recovery experiments were carried out, using European Union (EU) maximum permitted level for ZEA in maize intended for direct human consumption, (100 µg/kg, n =3). Linearity of the analytical method was determined by assessing the coefficient of determination (R^2) from scatter plot. LOD and

LOQ were evaluated by injecting standard solution until the signal to noise ratio (S/N) reached 3 and 10, respectively.

Table 3.1: Validation of analytical method for the determination of ZEA in raw maize

Validation parameter	Results
Linearity (R^2)	0.99
LOD (µg/kg)	0.1
LOQ (µg/kg)	0.5
Recovery at 100 µg/kg (%)	98

R^2, coefficient of determination; LOD, limits of detection at 3 baseline noise; LOQ, limits of quantification at 10 baseline noise

3.2.5 Assessment of Traders Knowledge of Fungi and Mycotoxin in maize grains

A cross-sectional descriptive study was carried out at the sampling markets using 10 randomly selected retailers from whom maize kernels were sourced. Information was collected on traders' ability to identify fungi on maize kernels, training they received on mycotoxin mitigation and storage conditions of maize kernels.

3.2.6 Statistical Analysis

All data were analyzed using Statistical Package for Social Sciences (IBM SPSS® Version 18). For ZEA concentration, mean, median and range were calculated for each region, with proportion of samples above the LOD (0.1 µg/kg). Moisture content was presented using boxplots. Multinomial logistic regression was used to evaluate the association between demographic variables (age, years of selling and education) and awareness of mycotoxins at 95% confidence interval (CI). The last subgroup in each

category was used as a reference in the model analysis. All other information from short answered questionnaires were summarized in percentages.

3.3 Results

3.3.1 Zearalenone Contamination of Maize grains? Kennels?

The results on the occurrence of zearalenone in maize kernels collected in the northern parts of Ghana are shown in Table 3.2. Out of the 75 samples collected, ZEA was found in 33.3 % of the samples (25/75), although generally at low levels. The contamination levels ranged from 0.61 to 3.05 ng/g with mean concentration of positive samples of 1.50 ng/g.

Table 3.2: Occurrence of zearalenone in maize kernels collected from Northern Ghana

Region	No. of positive samples (%)	Min (ng/g)	Max (ng/g)	Mean (ng/g) [a]
Upper East	6 (40)	0.63	2.02	1.24±0.10
North East	3 (20)	1.36	2.47	1.90±0.20
Upper West	4 (26.7)	0.86	2.03	1.25±0.15
Northern	6 (40)	1.02	2.31	1.40±0.10
Savannah	6 (40)	0.61	3.05	1.61±0.10
P-value				0.723

[a] Mean value represents average of positive samples

3.3.2 Moisture Content of Grains

Grains from Upper East generally had high moisture content (10.16-12.33 %), followed by North East (10.13-11.79 %), Upper West (7.49-9.39 %), and then Northern (7.27-10.76) Region. Maize samples from Savannah Region exhibited high variation in moisture content (Figure 3.1).

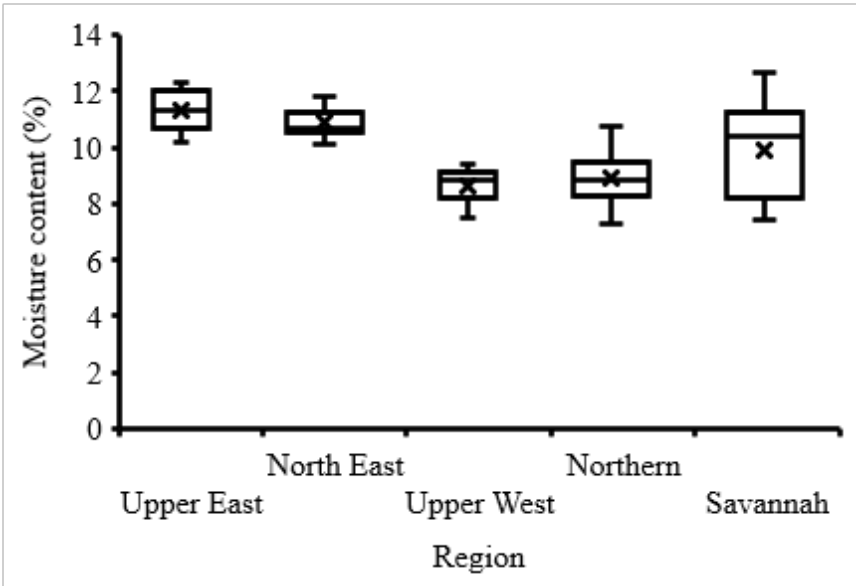

Figure 3:1: Moisture content of maize kernels.

3.3.3 Maize traders Awareness Study

In the present study, 54% of all maize traders answered yes, the question "Have you heard about mycotoxins?". Knowledge and awareness of mycotoxins contamination among maize sellers was high in the Northern Region (6 out of 10 traders) and the Upper East Region (5 out of 10 traders) and low in Savannah region (Figure 3.2).

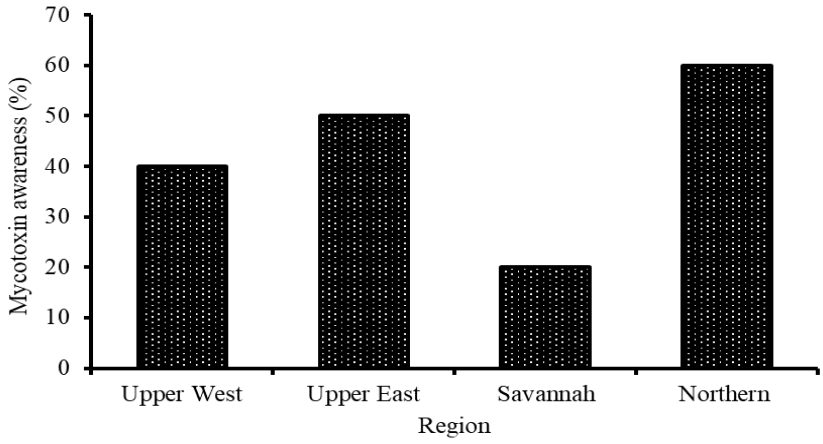

Figure 3:2: Awareness of mycotoxins by maize traders in four regions in Northern Ghana (*Question: Have you heard about mycotoxins*)

3.3.3.1 Traders' Awareness and Knowledge about Mycotoxins

Of the 41 maize traders who participated in the current study, more than half (53.7%, n=22) of the traders have no knowledge on mycotoxins contamination of maize grain. Awareness and knowledge among traders age 30 years and above were relatively high (Table 3.3). Traders with more years in selling and/or of higher education (Tertiary) were more aware and knowledgeable in the occurrence and types of mycotoxins.

Table 3.3: Traders' awareness and knowledge about mycotoxins

Variable [a]	Have heard of mycotoxins		Mycotoxins known to affirmative		
	Category (N)	Affirmative response (%)	Aflatoxin	Fumonisin	Zearalenone
Age	<30 (9)	3 (33.3)	2	1	0
	30-49 (14)	8 (57.1)	6	2	0
	40-49 (6)	2 (33.3)	2	0	0
	>50[b] (12)	6 (50.0)	6	0	0
Years of selling	<5 (4)	2 (50.0)	2	0	0
	5-10 (16)	3 (18.8)	3	1	0
	10-15 (14)	9 (64.3)	9	2	0
	>15[b] (7)	5 (71.4)	5	0	0
Education	Primary (7)	2 (16.7)	2	0	0
	Secondary (12)	7 (58.3)	7	0	0
	Tertiary (7)	5 (71.4)	7	4	0
	None [b] (15)	5 (33.3)	5	0	0

N; number of respondents in each category

3.3.3.2 Multinomial Logistic Regression

In a multivariate logistic regression model, it is shown that younger traders (age group < 20) and traders with more years in selling maize are more likely to be aware of mycotoxins occurrences in maize (Table 3.4). Although not significant (P > 0.05), traders with higher level of education had increased awareness of mycotoxin occurrence compared to the reference group.

Table 3.4: Multinomial logistic regression model of awareness to mycotoxins among maize traders in Northern Ghana (*Question: Have you heard about mycotoxins*)

Variable [a]	Category	Odd ratio	95% CI	P-value
Age	<30	0.013	0.000-0.506	**0.020**
	30-49	0.267	0.024-3.007	0.267
	40-49	0.084	0.004-2.000	0.084
	>50[b]			
Years of selling	<5	1.052	0.021-52.089	0.980
	5-10	0.402	0.029-5.486	0.494
	10-15	0.027	0.002-0.474	**0.014**
	>15[b]			
Education	Primary	0.249	0.017-3.592	0.308
	Secondary	5.776	0.522-63.919	0.153
	Tertial	14.196	0.795-253.578	0.071
	None [b]			

a: reference category is NO; b: the parameter was set to zero because it is redundant

3.3.3.3 Training received on mycotoxin mitigation

Table 3.5 shows that about 85 % of the traders did not receive any form of training on fungi and mycotoxins mitigation although a significant number of them are willing to attend and receive training on mycotoxins mitigation techniques.

Table 3.5: Training on Mycotoxin Mitigation

Question	Response	Percentage
Received trained on fungi and mycotoxin mitigation techniques	Yes	14.6
	No	85.4
Wiliness to attend training on fungi and mycotoxin control	Yes	73.2
	No	26.8

3.3.3.4 Handling and Storage Practices

Descriptions and summary statistics of the variables included in the handing and storage model are presented in Table 3.6. More than 80 % of the traders acknowledge maize

damage caused by insect pests (73.5 %) and rodents (16.3 %) during storage. Polyethylene (73.2 %) and jute sacks (26.8 %) were the dominant storage sack used by traders with significant number of the trader's storing maize in cement block storage rooms (73.2 %). Additionally, majority of the traders did not further dry their maize before storage.

Table 3.6: Handling and Storage practices (n = 41)

Parameter	Response category	Percentage
Further drying	Yes	4.9
	No	95.1
Storage period	<3 months	73.2
	3-6 months	26.8
	>6 months	0
Storages sacks	Jute sack	26.8
	Polyethylene sack	73.2
	Triple layer hermetic sack	0
Storage structure type	Cement block	73.2
	Wooden structure	7.3
	Local mud	19.5
Challenges in maize storage	Insect/pest infestation	73.5
	Rodents	16.3
	Mold growth	10.2

3.4 Discussion

Mycotoxin contaminated grains are involved in numerous morphological and physiological changes in animals and invariably human health. Among the major mycotoxins regulated globally, ZEA is known to carry the most potent endocrine disrupting activity. Unfortunately, maize is highly susceptible to ZEA contamination (Eze et al., 2018). In the present study we evaluated ZEA levels in maize across northern Ghana. We found the contamination prevalence across northern Ghana to be 33.3 % and at generally low (0.61 to 3.05 ng/g) concentration level. While data on aflatoxin and

fumonisin in maize grains from Ghana and their associated health risk are relatively abundant in literature (Hudu et al., 2021; Kortei et al., 2021; Opoku et al., 2018), data on ZEA are much less available. Rodrigues et al. (2011) found ZEA in 11 % (178 ng/g to 310 ng/g) of 18 test feed samples from Ghana. To the best of our knowledge, this study is the first report of the concentration levels of ZEA in raw maize grains from Ghana. In other African countries, Ekwomadu et al. (2021) demonstrated the occurrence of ZEA in 25 (50 %) of 50 samples of commercial maize with levels ranging from 0.74 to 38.0 µg/kg and average concentration of 12.8 µg/kg in South Africa. Similarly, Mahdjoubi et al. (2020) reported 23.3 % (7/30) ZEA contamination in maize samples from local markets in Algeria out of which one sample had levels which was 579 µg/kg above the maximum permitted level established by EU. In Nigeria, Olopade et al. (2021) analyzed 60 samples of maize, sorghum and millet and recorded 75 %, 90 % and 85 % of maize, sorghum and millet to be contaminated with ZEA with maximum concentrations of 16 µg/kg, 20 µg/kg and 396 µg/kg, respectively. The widespread ZEA contamination across Africa is apparent but the prevalence and concentration vary greatly among countries and localities. The present prevalent values and concentration levels are relatively lower than previously reported in Ghana for compound feeds and the other African countries. None of the samples were contaminated with levels above EU regulatory limits (European Commission, 2006). The variations could be as a result of differences in environmental conditions and agronomic practices that promote or inhibit growth of the *Fusarium.*

On average, moisture levels were low in maize samples collected across the study areas. The wide use of jute and polypropylene sacks for storing maize as well as the sampling time for this study might be an obvious cause as it is generally reported that jute or

polypropylene bags allows rapid loss of moisture in stored maize grains (Baoua et al., 2014; Manu et al., 2019; Ng'ang'a et al., 2016). Moisture content previously investigated in market maize from Northern Region of Ghana by Manu et al. (2019) showed low moisture levels, most of which were below 13 %. Similarly, another study from Upper West Region showed that moisture content of maize grains was low, ranging from 9.39-10.38 % (Alhassan & Patrick, 2018). Moisture contents of grains are critical for mycotoxin accumulation. The low prevalence and levels of ZEA observed in this study from those that reported high ZEA levels in maize may partly be attributed to the adequate drying of grains, resulting in relative low moisture content of the maize kernels.

Although insect pest infestation of maize grains was not quantified in this study, majority of the traders who responded to the survey instrument indicated high insect infestation of stored grains (Table 3.6). This phenomenon might be related to the wide use of jute and polypropylene sacks as storage bags: literature published in recent years find's positive correlation between the use of jute and/or polypropylene sacks and high insect pest infestation (Bakhtavar et al., 2019; Manu et al., 2019; Ng'ang'a et al., 2016). Furthermore, although mycotoxin levels, particularly aflatoxins, are highly correlated with higher degree of insect infestation and damaged kernels, this was not investigated in this study.

The high percentage of traders with no knowledge and training on the occurrence of mycotoxins and their harmful effect on human and animal health forms part of major limitations that may hinder the fight against mycotoxins. As part of measures to minimize mycotoxins level in food crops, particularly aflatoxins, the government of Ghana has proposed a certified grain traders' model where traders without certificate would not be

allowed to sell grains. Similarly, more than 90 % of farmers and traders were reported to have limited knowledge on the occurrence of aflatoxins and its associated health effect (Awuah et al., 2009; Jolly et al., 2006). Therefore, in order to reduce the negative impact and exposure risk of mycotoxins on health, it is imperative to increase awareness and bridge knowledge gaps among traders. The large majority of traders willing to be trained on fungi and its associated mycotoxin control measures, will make it easy to institute intervention program that may include sorting, insect and rodent control, post-harvest handling and storage conditions.

3.5 Conclusion

For the first time, this study provides preliminary insight into ZEA contamination of commercial maize grains as well as trader's awareness and handling practices of mycotoxin in northern Ghana. ZEA was found in 33.3 % of maize samples collected (25/75) and were present at generally at low levels.

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4. CHAPTER FOUR

FUSARIUM SPECIES OCCURRENCE

Mycotoxigenic *Fusarium* species on commercial maize kernels in northern Ghana

Abstract

The fungal genus *Fusarium* contains many toxigenic pathogens of maize with associated yield losses, reduction of grain quality, and accumulation of mycotoxins in harvested grains. To identify the various *Fusarium* species in commercial maize grains, a survey of 50 maize samples, collected from 11 market centres in the five regions in northern Ghana were identified based on morphological characteristics, sequence analysis of the internal transcribed spacer region, and polymerase chain reaction using species specific primers. *F. verticillioides* was the most prevalent species in the studied samples: 43.5 % from Upper East Region, 23.9 % from North East Region, 15.2 % from Upper West Region, 13.0 % from Savannah Region, and 4.3 % for Northern Region. Other fungal species found were *F. equiseti* and *F. solani*. More of the *Fusarium* isolates were found in white maize (609 isolates from 27 samples) compared to yellow maize (225 isolates from 23 samples).

Keywords: Northern Ghana, commercial maize, *Fusarium* species, pathogen, CTAB method

4.1 Introduction

The fungal genus *Fusarium* contains many important field and storage pathogens of cereals with associated grain yield and quality losses (Lobulu et al., 2021; Strunk & Byamukama, 2016). The estimated yield losses associated with *Fusarium* diseases may

be more than 50% (Nathawat et al., 2020; Schjøth et al., 2008), while the most harmful groups cause mycotoxin contamination during storage (Kankolongo et al., 2009; Mesterházy et al., 2022; Mudili et al., 2014). *Fusarium* spp. infection primarily results in the mycotoxins zearalenone, fumonisin, and trichothecenes contamination.

Consumption of *Fusarium* mycotoxins could be life threatening in humans and animals such as reported recently in sub-Saharan Africa (Biomin, 2019). It was previously noted in Africa that multiple *Fusarium* metabolites contamination in feed and feed raw materials is very common (up to 95%) where more than twice the prevalence rate of *Aspergillus* mycotoxins was recorded (Gruber-Dorninger et al., 2018). In Ghana, the prevalence of *Fusarium* toxins fumonisin, trichothecenes and zearalenone in feed and feed commodity stood at about 89 %, 50 % and 11 % a decade ago (Rodrigues et al., 2011).

Among the principal cereal crops grown in Ghana for human and industrial usage, maize (*Zea mays* L.), is exceptionally susceptible to *Fusarium* infections. The crop is a widely cultivated food crop for human consumption (72.09 %) and feedstock formulations (26.99 %) with estimated national output of 3.06 million tons in 2019 (MoFA-IFPRI, 2020; MoFA-SRID, 2021). Maize production in the Savannah zone has more than doubled (55.1% increase from 2006 to 2016) compared to the Coastal zone (29.1%) and Forest/Transition zone (24.0%) over the same period (MoFA-IFPRI, 2020). Culturally, the crop per capita consumption is high in northern Ghana (MoFA-IFPRI, 2020).

Despite these potentials of maize cultivation in northern Ghana, data on the occurrence of *Fusarium* spp. in the area is lacking. Across the country generally, data on *Fusarium* are

very limited (Korley et al., 2022; Kpodo et al., 2000) which makes it difficult to appraise the importance of the fungal infection and or contamination in the maize value chain. In the present study, we provide a baseline data on the diversity of *Fusarium* spp. in commercial maize kernel across the five regions of northern Ghana and to start a collection of variable accessions for future reference using DNA sequence analysis tools.

4.2 Materials and Methods

4.2.1 Sample Collection

A total of 50 maize samples were randomly purchased from small-scale commercial maize sellers in the northern part of Ghana between April and May 2020. Most of the samples contained a mixture of varieties and therefore were broadly categorized into white and yellow maize based on the colour of the pericarp. There was no information regarding the length of the storage of the grains. At each sampling point, 8 to 10 incremental samples were randomly drawn from various sections of homogenized maize into plastic zip-lock bags to a weight of about 1 kg.

4.2.2 Isolation and characterization of *Fusarium* Species

4.2.2.1 Fungi Culture and Isolation

For *Fusarium* spp. recovery, maize kernels (100 kernels) from each sample were randomly taken and washed with sterile distilled water to remove attached dirt and dust, surface sterilized in 3.5 % NaClO solution for 3 min, rinsed several times in distilled water and dried on sterile filter paper to remove excess water. The dried kernels were subsequently placed on RBS growth medium containing 10 g glucose, 2 g peptone, 0.5 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 20 g agar, 10 ml triton-X 100 and 1 ml of 0.5 mg/ml

chloramphenicol in 1 L distilled water (autoclaved at 105 ºC for 15 minutes) at a density of 10 kernels per 90 cm Petri dish. The Petri dishes with maize kernels were left at room temperature for 7 days for fungi growth. All fungi colonies were transferred to potato dextrose agar (PDA, Oxoid) amended with 1 ml of 0.5 mg/ml chloramphenicol, and incubated at room temperature for 14 days.

4.2.2.2 Single Spore Isolation

Single spore isolates were obtained from 14 days old cultures using the procedure described by Opoku et al. (2011). To obtain spore suspension, 14 days old cultures were individually flooded with 10 ml sterile distilled water, culture materials (either macroconidia, microconidia or both depending on the species) were scrape with an L-shaped sterile rod and then filtered through four-layer sterile gauze swab (MicroTech) to remove mycelia. Spores' concentrations were then estimated using hemocytometer (Neubauer Improved, Germany) and adjusted to approximately 10 spores/µl. Three microliter of the spore suspension was placed on water agar (15 %) and observed for germinating spores after 12-24 h incubation. Single germinating spores were transferred on PDA (Oxoid) growth media.

4.2.2.3 Morphological Identification of *Fusarium* Species

Fusarium species identification was based on the morphological characteristics outlined by Leslie & Summerell (2006). Pure cultures were grown at 30 ºC on PDA for two weeks. Macroscopic characteristic including colony appearance, color and pigmentation were described. Growth rate and colony diameters of cultures were measured from colonies grown for 5 days at room temperature. Microscopic characters such as

development of sporodochia, macro- and microconidia and chlamydospores, where available, were observed from three weeks old culture.

4.2.3 Molecular characterization of *Fusarium Species*

4.2.3.1 DNA Extraction (CTAB method)

Genomic DNA of 75 representative *Fusarium* isolates based on morphological differences (growth rate, mycelium structure, spore form and color) were extracted using modified CTAB extraction method described by Lee et al. (1988). Small fraction of mycelium was scraped off the surface of 14-day old culture and grinded with 500 µl of CTAB buffer (2 % CTAB, 100 mM tris-HCl; pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2 % PVP and 0.2 % beta mercapto-ethanol) in a 1.5 microcentrifuge tube using Kontes' pestle. The tube was incubated for 60 min at 65 ^{o}C in a heating mantle. After incubation, 500 µl of chloroform: isoamyl alcohol (24:1) was added, vortex for 1 min and centrifuge at 2500 g for 20 min. The upper aqueous fraction was reextracted with 500 µl of chloroform: isoamyl alcohol. After centrifugation, the supernatant was then transferred into 2.0 ml microcentrifuge tube containing 300 µl of 100 % ice-cold isopropyl. The supernatant was kept in a freezer overnight (approximately 14 hours) and DNA was pelleted at 2500 g for 3 min. The pellet was washed with 500 µl of ice-cold 70 % ethanol and centrifuged at 2500 g for 3 min. After removal of the supernatant the DNA pellet was air dried for 1 h and resuspended in 50 µl of molecular water. After extraction, the DNA concentration was measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

4.2.3.2 Molecular Identification

4.2.3.2.1 Internal Transcribed Spacer (ITS) Amplification

Prior to species-specific amplification, a control PCR was carried out on all the DNA samples using the generalized primers (ITS4 and ITS5, Table 4.1) (White et al., 1990) to ensure all samples contained fungal DNA that were amplifiable. These PCRs were performed in a volume of 50 µl reaction containing 1 µl of DNA, 1 µl of 10 µmol each of primer: forward and reverse (ITS4 and ITS5), 5 µl of 2x PCR standard buffer (New England Biolabs: pH 8.9, 1X tartrazine, 1X xylene cyanol, 0.05 % Tween® 20, 0.06% IGEPAL® CA-630, 5 % glycerol, 20 mM Tri-HCl, 22 mM KCl, 22 mM NH_4Cl, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.625 U One Taq® DNA polymerase). The thermal cycling conditions were set at 94 °C for 75 s for initial denaturation, followed by 40 cycles of 15 s at 94 °C, 15 s at 50 °C, 45 s at 72 °C and final extension of 72 °C for 4 min before cooling to 4 °C using peqSTAR thermal cycler (Germany). Amplicons (800 bp) were resolved on 1.5 % agarose gel stained with ethidium bromide.

4.2.3.2.2 Species-specific Amplification

Identification of isolates using species specific primers (Table 4.1) was done using PCR conditions of Goertz et al. (2010). Each reaction mixture (50 µl) for PCR contained 4 µl of DNA, 2 µl of 10 µmol each of primer: forward and reverse, 25 µl of 2x PCR standard buffer (New England Biolabs: pH 8.9, 1X tartrazine, 1X xylene cyanol, 0.05 % Tween® 20, 0.06% IGEPAL® CA-630, 5 % glycerol, 20 mM Tri-HCl, 22 mM KCl, 22 mM NH_4Cl, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.625 U One Taq® DNA polymerase). The PCR conditions were as follows: 95 ºC for 2 min (initial denaturation); 35 cycles of 95 ºC for 30 s, 60 ºC for 40 s and 72 ºC for 50 s and final extension of 72 ºC for 10 min. PCR

amplicons (7 µl) were separated by electrophoresis on ethidium bromide (250 µl) stained agarose gel (1.5 % of 1X TBE w/v) for 35 min at 90 V and visualized on a Gel Doc 1000 system under UV light.

4.2.3.2.3 DNA sequencing and analysis

To identify the 16 ITS amplified DNA that could not be amplified with any of the species specific primers, we send the 13 of the 16 ITS amplified DNA together with 13 of the VERT amplified DNA for sequencing at Inqaba Biotechnology (Pty) Limited, Pretoria, South Africa. Quality control of illumine reads was performed with GENtle software v.1.9.4 by cleaning and trimming sequences. Nucleotides were aligned in Clustal Omega (Madeira et al., 2022). Nucleotide sequences of each gene loci (ITS and VERT) were compared to other sequences available in the National Centres for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 24 August 2022). TCS haplotypes network was constructed in PopArt 1.7 (Leigh & Bryant, 2015). DnaSP software was used to determine the nucleotide and haplotype diversities of the sequences (Librado & Rozas, 2009). Maximum likelihood phylogenetic tree based on the ITS sequences obtained in this study and sequences retrieved from GenBank (Table ….) was drawn using Molecular Evolutionary Genetics Analysis (MEGA X) software v.11 (Kumar et al., 2016).

4.2.4 Statistical analysis

Statistical analysis was performed using GenStat statistical software version 11.1 (VSN international Ltd). Mean number of infected kernels per region were analyzed using One-

way Analyzes of Variance (ANOVA). The Fisher's unprotected LSD test was used to compare differences between means when the ANOVA was significant ($P < 0.05$).

Table 4.1: PCR primer pairs used for the identification of *Fusarium* species

Target species	**Primer name**	**Sequence**	**PS (bp)**	**Reference**
GP	ITS 4	TCCTCCGCTTATTGATATGC		White et al.
	ITS 5	GGAAGTAAAAGTCGTAACAAGG		(1990)
FG	Fg16F	CTCCGGATATGTTGCGTCAA	400–500	Nicholson et
	Fg16R	GGTAGGTATCCGACATGGCAA		al. (1998)
FP	PRO1	CTTTCCGCCAAGTTTCTTC	585	Mulè et al.
	PRO2	TGTCAGTAACTCGACGTTGTTG		(2004)
FC	Fc01F	ATGGTGAACTCGTCGTGGC	570	Nicholson et
	Fc01R	CCCTTCTTACGCCAATCTCG		al. (1998)
FS	AF330109CF	AAAAGCCCAAATTGCTGATG	332	Demeke et al.
	AF330109CR	TGGCATGTTCATTGTCACCT		(2005)
FV	VERT-1	GTCAGAATCCATGCCAGAACG	800	Patiño et al.
	VERT-2	CACCCGCAGCAATCCATCAG		(2004)

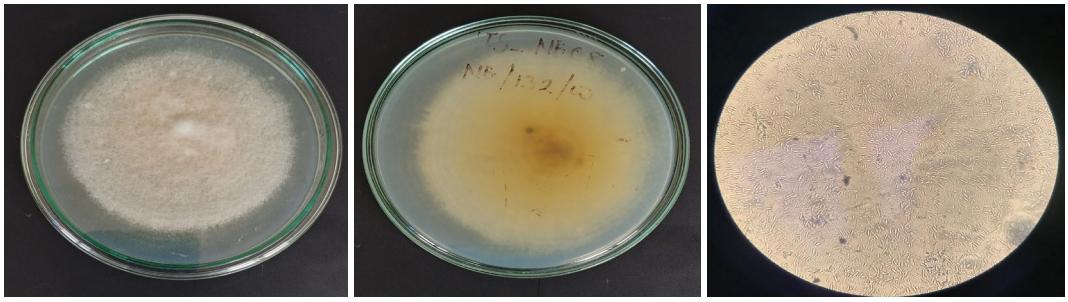
PB: Product size; **GP**: General primer; **FG**: *Fusarium graminearum*; **FP**: *Fusarium proliferatum*; **FC**: *Fusarium culmorum*; **FS**: *Fusarium sporotrichioides* and **FV**: *Fusarium verticillioides.*

4.3 Results

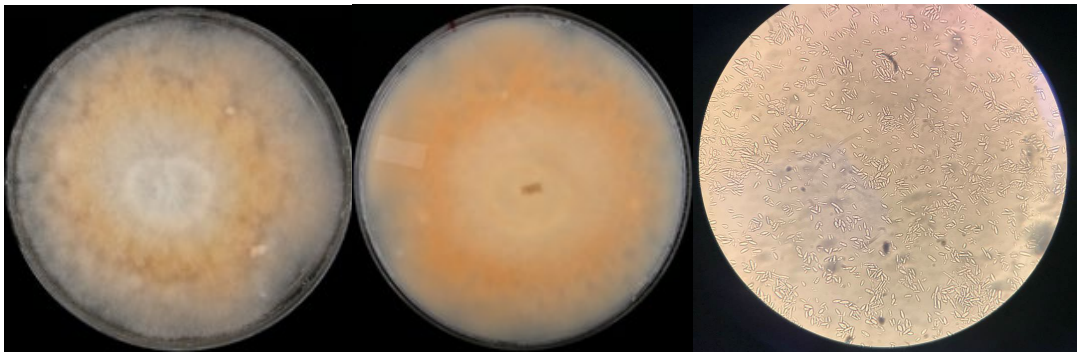
4.3.1 Morphological identification

4.3.1.1 Incidences of *Fusarium* species in Maize Kernels

Based on morphological assay (Figure 4.1; colony color and structure), *Fusarium* species were detected in all maize samples, except in samples from the Northern Region where the fungi growth was detected in only four out of the ten maize samples. Of the 1000 kernels analyzed for each region, the frequency of infected kernels ranged from 0.9 % to 36.3 % (Table 4.2).



Front side | Back side | Spores under microscope



Front side | Back side | Spores under microscope

Figure 4:1: Representative colonies formed on PDA and conidial morphology characteristics

Table 4.2: Incidence of *Fusarium* species recovered from maize kernels

Region	Mean incidence (%)	One-way ANOVA summary statistic			
		Source of variation	ss	df	p-value
Northern	0.09[a] (0.9%)	Region	783.428	4	< 0.001
Savannah	0.68[b] (6.8%)	Residual	1415.460	495	
Upper West	1.56[c] (15.6%)	Total	1289.888	499	
North East	2.38[d] (23.8%)				
Upper East	3.63[e] (36.3%)				

Values within a column that have no superscript in common are significantly different at $p < 0.05$.

4.3.1.2 *Fusarium* species recovered

Three *Fusarium* species were identified in the maize kernels. The predominant species was *Fusarium verticillioides*, isolated from 92 % of the maize samples (Figure 4.1). Other *Fusarium* species that were infrequently isolated were *Fusarium* equiseti (3 out of the 50 samples) and *Fusarium* solani (2 out of 50 samples). More of these *Fusarium* species was detected in white maize (609 isolates from 27 samples) compared to yellow maize (225 isolates from 23 samples) (Table 4.3).

Table 4.3: Mean distribution of *Fusarium* species base on maize colour (t-test)

Region	Maize colour		p-value
	White maize	Yellow maize	
Northern	0.10	0.08	0.73
Savannah	1.18	0.18	< 0.001
Upper West	2.02	1.10	0.001
North East	3.14	0.60	< 0.001
Upper East	4.48	2.78	< 0.001

4.3.2 Molecular Identification

4.3.2.1 Internal Transcribed Spacer Primer

A total of 75 isolates of the *Fusarium* species, including 70 *F. verticillioides*, 2 *F. solani* and 3 *F. equiseti* (from the morphological assay), was amplified using ITS primers (Table 4.4). The initial control PCR (ITS4 and ITS5) showed that DNA of fifty-six (56) of the *F. verticillioides* and all the *F. solani* and *F. equiseti* was amplifiable (Table 4.4). *Fusarium* isolates such as *F. solani* and *F. equiseti*, for which species-specific primers were not available, could not be identified further and were not included in the species-specific identification. Furthermore, their ITS amplicons though amplified could not be sequenced and were therefore typed morphologically.

4.3.2.2 Use of species specific primers

Using the specific primer pairs of VERT1/2, FG16 F/R, FC01-F/R, PR01/2 and AF330109CF/R, 45 out of the 56 amplified ITS DNA were confirmed to be *F. verticillioides*. None of the DNA samples could be amplified for *F. graminearum, F. culmorum, F. sporotrichioides* and *F. proliferatum* (Table 4.4).

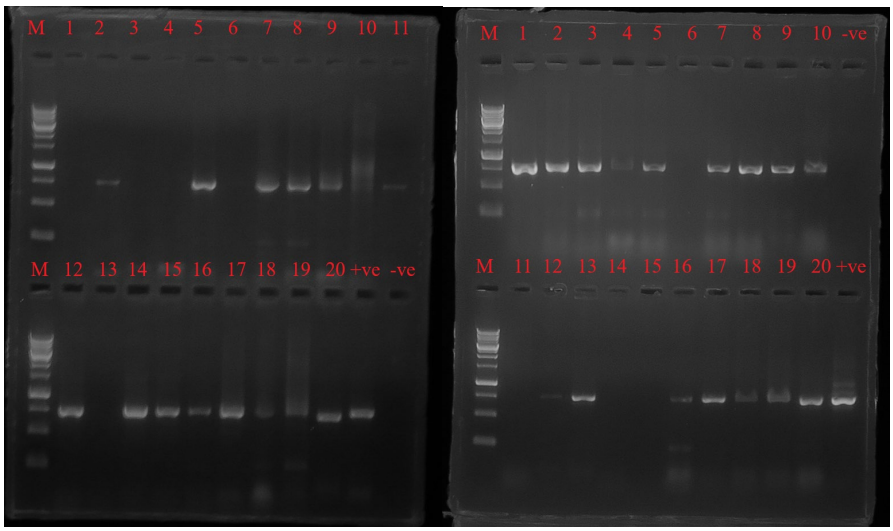


Figure 4:2: Picture of the gel with the amplification of the PCR product

Table 4.4: PCR amplification status of isolates used in molecular analysis

S/N	Isolate code	Origin	ITS4 ITS5	Species-specific primers				
				VERT1 VERT2	FG 16F FG16R	FC01-F FC01-R	PR01 PRO2	AF33F AF33R
1	NE 11	North East	+	-	-	-	-	-
2	NE 7	North East	+	+	-	-	-	-
3	NE 13	North East	+	-	-	-	-	-
4	NE 12	North East	+	+	-	-	-	-
5	NE 9	North East	+	+	-	-	-	-
6	NE 6	North East	+	+	-	-	-	-
7	NE 18	North East	+	+	-	-	-	-
8	NE 2	North East	+	+	-	-	-	-
9	NE 16	North East	+	+	-	-	-	-
10	NE 4	North East	+	+	-	-	-	-
11	NE 10	North East	+	+	-	-	-	-
12	NE 5	North East	+	-	-	-	-	-
13	NE 19	North East	+	+	-	-	-	-
14	NE 8	North East	+	-	-	-	-	-
15	NE 17	North East	+	-	-	-	-	-
16	NE 1	North East	+	+	-	-	-	-
17	NE 15	North East	+	-	-	-	-	-
18	NE 13	North East	+	+	-	-	-	-
19	NE 15	North East	-					
20	NE 17	North East	-					
21	NE	North East	-					
22	UE 8	Upper East	+	+	-	-	-	-
23	UE 23	Upper East	+	+	-	-	-	-
24	UE 18	Upper East	+	+	-	-	-	-
25	UE 15	Upper East	+	+	-	-	-	-
26	UE 16	Upper East	+	+	-	-	-	-
27	UE 12	Upper East	+	+	-	-	-	-
28	UE 13	Upper East	+	+	-	-	-	-
29	UE 14	Upper East	+	-	-	-	-	-
30	UE 17	Upper East	+	+	-	-	-	-
31	UE 19	Upper East	+	+	-	-	-	-
32	UE 10	Upper East	+	+	-	-	-	-
33	UE 11	Upper East	+	+	-	-	-	-
34	UE 20	Upper East	+	+	-	-	-	-
35	UE 24	Upper East	+	+	-	-	-	-
36	UE 21	Upper East	+	+	-	-	-	-

S/N	Isolate code	Origin	ITS4 ITS5	Species-specific primers VERT1 VERT2	FG 16F FG16R	FC01-F FC01-R	PR01 PRO2	AF33F AF33R
37	UE 4	Upper East	+	+	-	-	-	-
38	UE 5	Upper East	+	-	-	-	-	-
39	UE 7	Upper East	+	-	-	-	-	-
40	UE 9	Upper East	+	+	-	-	-	-
41	UE 22	Upper East	+	+	-	-	-	-
42	UE 1	Upper East	+	+	-	-	-	-
43	UE 2	Upper East	+	+	-	-	-	-
44	UE 3	Upper East	+	+	-	-	-	-
45	UE 30	Upper East	-					
46	UE 31	Upper East	-					
47	UE 32	Upper East	-					
48	UE 25	Upper East	-					
49	SR 7	Savannah	+	+	-	-	-	-
50	SR 8	Savannah	+	+	-	-	-	-
51	SR 6	Savannah	+	+	-	-	-	-
52	SR 3	Savannah	+	+	-	-	-	-
53	SR 5	Savannah	+	+	-	-	-	-
54	SR 2	Savannah	+	+	-	-	-	-
55	SR 4	Savannah	+	+	-	-	-	-
56	SR 1	Savannah	-					
57	UW 7	Upper West	+	+	-	-	-	-
58	UW 10	Upper West	+	+	-	-	-	-
59	UW 9	Upper West	+	+	-	-	-	-
60	UW 15	Upper West	+	+	-	-	-	-
61	UW 5	Upper West	+	-	-	-	-	-
62	UW 13	Upper West	+	+	-	-	-	-
63	UW 8	Upper West	+	+	-	-	-	-
64	UW 12	Upper West	+	+	-	-	-	-
65	UW 14	Upper West	+	-	-	-	-	-
66	UW 6	Upper West	-					
67	UW 11	Upper West	-					
68	UW 2	Upper West	-					
69	NR 1	Northern	+	-	-	-	-	-
70	NR 2	Northern	+	-	-	-	-	-
71	NR 2	Northern	+	-	-	-	-	-
72	NR 10	Northern	+	+	-	-	-	-
73	NR 5	Northern	-					

S/N	Isolate code	Origin	ITS4 ITS5	Species-specific primers VERT1 VERT2	FG 16F FG16R	FC01-F FC01-R	PR01 PRO2	AF33F AF33R
74	NR 8	Northern	-					
75	NR 9	Northern	-					

+/-: indicate the presence/absence of the PCR product; **AF33F**:AF330109CF; **AF33R**: AF330109CR

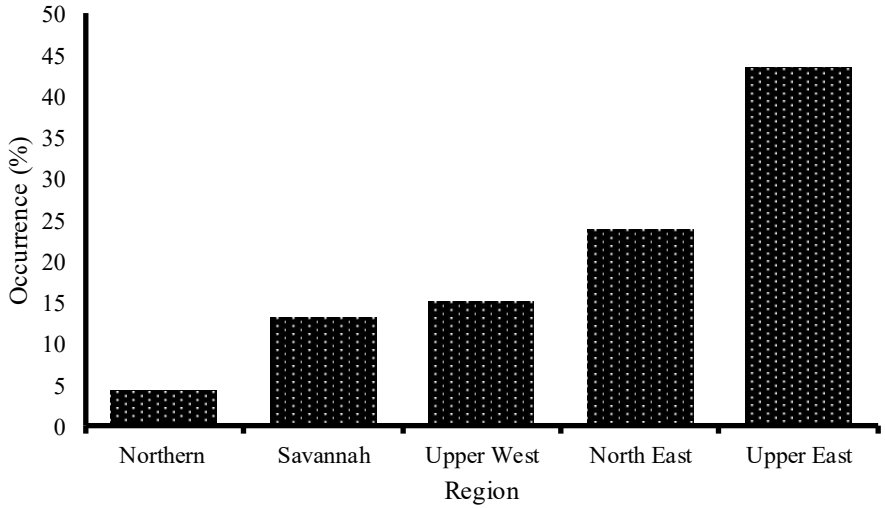


Figure 4:3: Occurrence rate of *F. verticillioides* in Northern Ghana.

4.3.2.3 DNA sequencing and phylogeny

Eight amplicons of the ITS and seven of the VERT amplicons were successfully sequenced and used in further molecular analyses. The sequences of the ITS were approximately, 441 to 530 bp while the sequences of the VERT amplicons were 513 - 708 bp after editing parsimony-uninformative characters of raw nucleotide sequences. Among ITS sequences, five were distinct; sequences from ITS_UE01a, ITS_UE05, ITS_NE14 and ITS_NE11 were found to be identical (Figure 4.4). Haplotype, GH 4 had the highest number of mutations. Other summary statistics of the nucleotides are presented in Table 4.4.

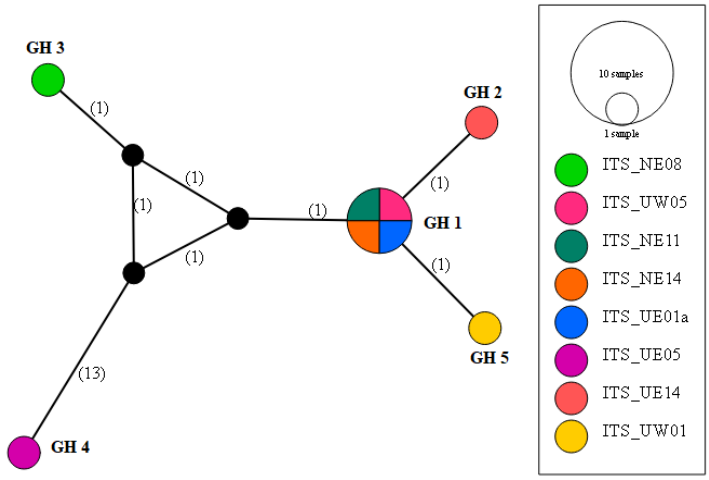


Figure 4:4: TCS network of different haplotypes of obtained ITS sequences. Sizes of cycle represent haplotype frequency in the dataset and the numbers represent mutational steps. Colors indicate the proportion of individuals sampled in different populations within the study area.

However, six distinct sequences were found from the seven nucleotide sequences for *F. verticillioides* (VERT) isolates. Sequence VERT_UE08 and VERT_UE16 were found to be identical (Figure 4.5). Samples from the Upper West Region had the highest number of mutations. Other summary statistics of the nucleotides are presented in Table 4.5.

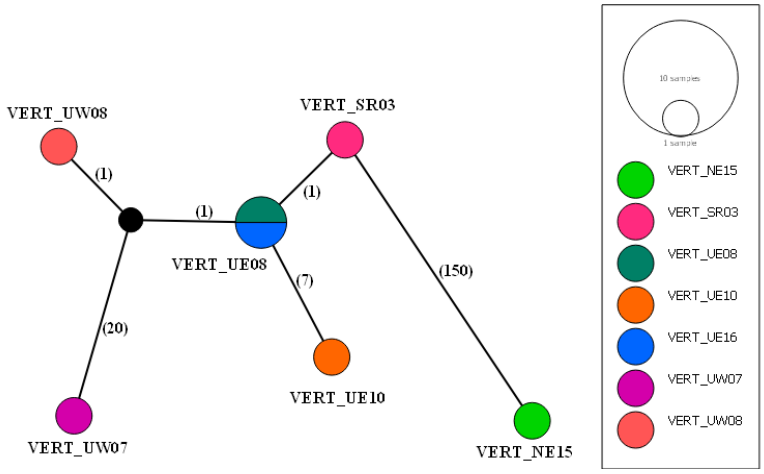


Figure 4:5: TCS network of different haplotypes of obtained VERT sequences. Sizes of cycle represent haplotype frequency in the dataset and the numbers represent mutational steps. Colors indicate the proportion of individuals sampled in different populations within the study area.

Table 4.5: Neutrality test statistics and DNA polymorphism data for F. *verticillioides* based on VERT and ITS

	VERT		ITS	
	S	P-value	S	p-value
Tajima's D	-1.653	$p < 0.05$	-1.725	$p < 0.05$
Fu and Li's D	-1.686	$p < 0.05$	-1.819	$0.10 > P > 0.05$
Fu and Li's F	-1.862	$p < 0.05$	-1.998	$0.10 > P > 0.05$
Fu's Fs	2.951		0.745	
Haplotype diversity	0.952		0.786	
NHap probability	0.278		0.307	
Segregating site	157		17	

4.3.2.4 Phylogenetic relationship

4.4.3. Sequence analysis and phylogenetic study of the ITS and VERT region

The phylogenetic tree drawn with the sequences of the ITS region of isolates obtained from this study and those retrieved from the genebank is shown as Figure 4.6. Of the five resolved clusters observed, sequences of the current study (GH 1-5) clustered among themselves, forming one group with strong bootstrap value 76 %. Maximum likelihood tree showed that nucleotides of this study are not closely related to those retrieved from other countries. Among the sequence GH1-5, GH4 and GH3 are seen have strong relationship with bootstrap value of 100 %.

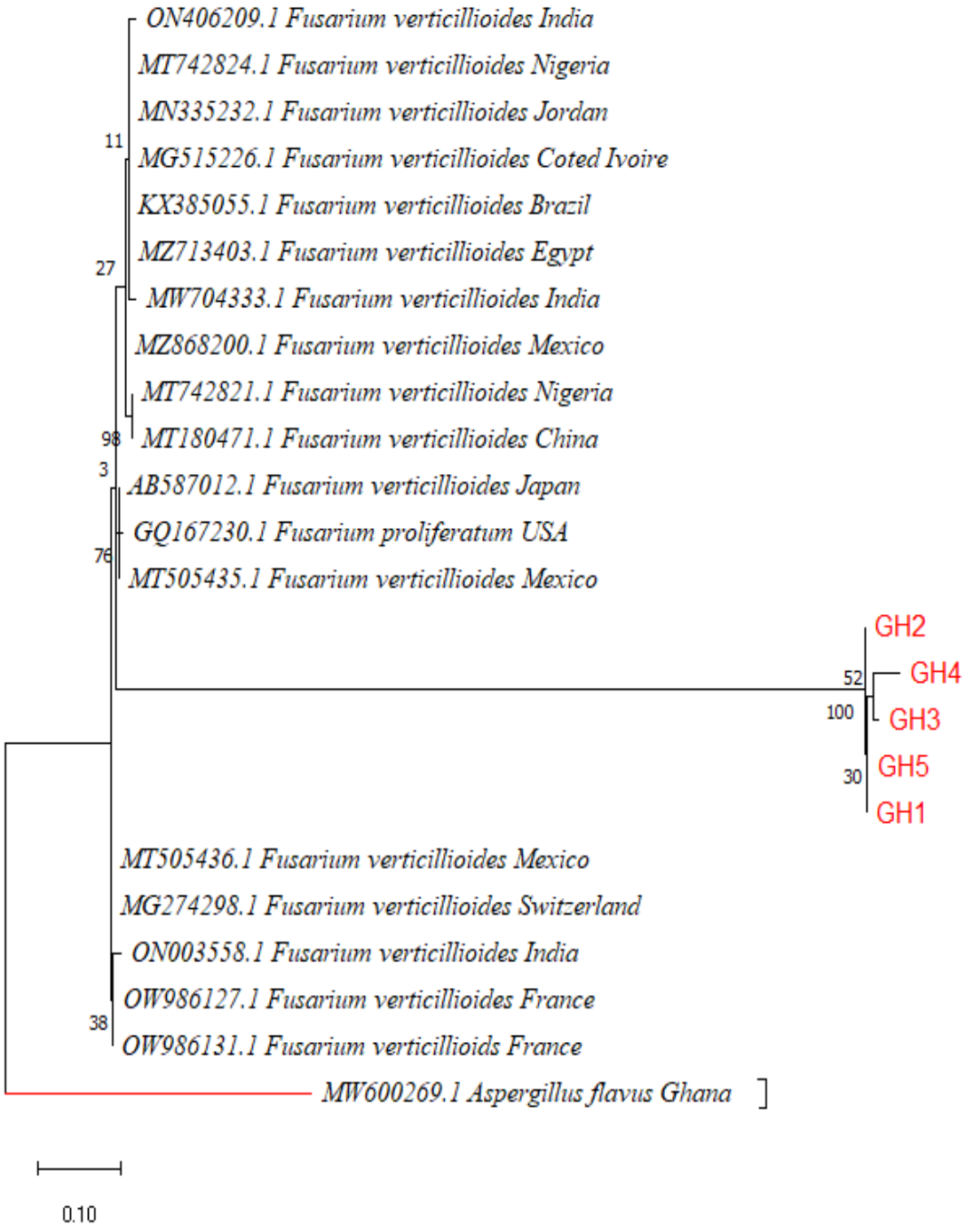


Figure 4:6: Maximum likelihood tree of ITS nucleotides and their related sequences retrieved from NCBI database.
GH1-5 presents nucleotide of the current study in haplotype analysis. *Aspergillus flavus* was used as outgroup. Bootstrap values are shown as percentage of 1000 replicates.

4.3.2.5 BLAST search results

Based on comparison of genomic regions of ITS and VERT loci, all the isolates were confirmed as *F. verticilliodes*. The nucleotide sequences of the VERT isolates were 91.77 to 100 % identical to *F. verticillioides* sequences deposited in NCBI GenBank repository (Table 4.6).

Table 4.6: Comparison of *Fusarium verticillioides* nucleotide to reference genes in GenBank (ITS)

Isolate code	strain	GenBank Accession (ITS)	Country	% Identity	Source
NE15	*F. verticillioides*	AY249379.1	USA	91.77	Zea mays
	F. verticillioides	HQ165877.1	China	91.77	Cornea
SR03	*F. verticillioides*	AY118115.1	USA	97.12	-
	F. verticillioides	AY249379.1	USA	97.45	Zea mays
UE08	*F. verticillioides*	AY118114.1	USA	99.56	
UE10	*F. verticillioides*	AY249379.1	USA	99.28	Zea mays
	F. verticillioides	HQ165877.1	China	99.49	Cornea
	F. verticillioides	AJ575185.2	USA	99.22	Zea mays
UE16	*F. verticillioides*	HQ165877.1	China	100	Facial lesion
	F. verticillioides	AY249379.1	USA	99.85	-
	F. verticillioides	AJ575185.2	USA	99.81	Zea mays
UW07	*F. verticillioides*	HQ165877.1	China	94.66	Facial lesion
	F. verticillioides	AJ575185.2	USA	94.46	Zea mays
	F. verticillioides	AY118114.1	USA	98.97	
	F. verticillioides	AJ575185.2	USA	98.85	Zea mays

4.4 Discussion

The fungal genus *Fusarium* contains major pathogen of cereal plants and their grains, characterized by severe crop loss and accumulation of mycotoxins such as zearalenone, fumonisin, and trichothecenes. At higher concentrations, mycotoxins are not only harmful to human, but are also involved in the modulation of gut microbiota composition

(Liew & Mohd-Redzwan, 2018). In the present study, we identified and characterized *Fusarium* spp. from commercial maize kernels from northern Ghana. The results showed that F. verticillioides was the most frequently occurring *Fusarium* species on maize. This is consistent with reports by earlier workers (Korley et al., 2022; Kpodo et al., 2000). Recently in Africa, the occurrence of mycotoxigenic *Fusarium* species has gain widespread attention. In Uganda and Angola, Wokorach et al. (2021) and (Alaro, 2021) respectively evaluated maize kernels for *Fusarium* spp. infestation and reported high incidence of *F. verticillioides*. Maize kernels collected from various households in Kenya were reported to have high infestation of *F. verticillioides* (Kagot et al., 2022). In Nigeria, commercial maize kernels were screened for *F. verticillioides* contamination; the maximum occurrence rate of the fungus was 28.2 % (Adetayo et al., 2022). Aasa et al. (2022) evaluated the incidence of filamentous fungi in some food commodities from Ivory Coast and found higher prevalence of *F. proliferatum, F. graminearum,* and *F. culmorum*.

Till now, the susceptibility of the different variety of maize to *Fusarium* species is less understood. In a recent study involving white and yellow maize in Egypt, Hussain et al. (2018) reported higher incidence of *F. verticillioides* in white maize (11.81%) than in yellow maize (3.13%), quite contrary to what was reported from Mexico where more *F. verticillioides* was recovered from yellow maize kernel (39.29%) than white maize kernels (25.98) (Montes et al., 2009). In our study, compared to yellow maize *F. verticillioides* recovery was significantly higher in white maize. However, Multiple pieces of evidence suggest that natural occurring phenolic compounds such as ferulic acid and tocopherol may have inherent selectable trait for controlling *Fusarium* species

growth (Dambolena et al., 2012; Ferrigo et al., 2021; Martínez-Fraca et al., 2022; Picot et al., 2013). Although the concentrations of these phenolic compounds were not determined in this study, the relative high levels of bioactive phenolic compounds, including ferulic acid, in yellow maize may partly account for the low isolation rate of *Fusarium* species in yellow maize (Mora-Rochin et al., 2010; Picot et al., 2013; Singh et al., 2019).

Environmental factors and host-specific profile have strong impact on the occurrence of specific *Fusarium* chemotype and invariably the types of mycotoxins they accumulate. In tropical countries including Ghana, high temperature and precipitation favour *Fusarium* growth. *Fusarium verticillioides*, is typically found in warmer maize growing regions (Basler, 2016). High level of fumonisin production have been found mainly in *F. verticillioides* and *F. proliferatum* strains (Waalwijk et al., 2008). Clusters of fumonisin biosynthetic (*FUM*) gene activity and expression are high in maize (Cao et al., 2022). *Fusarium verticillioides* are not prolific ZEA producers. We believe that the high level of *F. verticillioides* observed, the main fumonisin producer (Yli-mattila & Sundheim, 2022), might have contributed to the low levels of zearalenone record in this study (Chapter three).

In order to confirm the amplified *F. verticillioides* isolates, and to identify the isolates that none of the species-specific primer could amplify, we respectively sequenced seven and eight of the VERT and ITS amplicons. Nucleotide BLAST of the partially sequenced genes in this study confirm the isolates to be *F. verticillioides*. Haplotype analysis showed that the genetic variations between the VERT sequences (haplotype diversity of 0.952; segregation site 157) was higher than the ITS sequences (haplotype diversity of

0.786; segregation site 17). This is supported by their distinct haplotype numbers, i.e., six haplotypes from seven VERT isolates and 5 haplotypes from eight ITS isolates.

4.5 Conclusion

Ghana's climatic conditions favour the cultivation of maize throughout the country. Since investigations into the occurrence of *Fusarium* in Ghana are limited, an extended investigation was conducted to monitor the occurrence of *Fusarium* species (ZEA producing once) in maize from northern Ghana. *Fusarium verticillioides* species was proven to be widely spread, with high recovery rate in Upper East and North East regions of Ghana. To a lesser extent, few strains of *Fusarium equiseti* and *Fusarium solani* were morphologically identified. Additionally, this investigation shows that a nationwide evaluation of maize cultivars resistance to *Fusarium verticillioides* is needed as well as their impact on grain yield and other *Fusarium* mycotoxin occurrence.

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5. CHAPTER FIVE

GENERAL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 General Summary and Conclusion

Although mycotoxigenic *Fusarium* species and their associated secondary metabolites have serious impacts on sustainable agriculture and economic well-being of livestock and humans, few studies have evaluated their presence in food produce or determine the diversity of *Fusarium* in Africa. Furthermore, data on ZEA occurrence and evidence of high dietary exposure in individual countries are woefully inadequate for use in establishment of maximum permitted levels in food and feed raw material. As a result many of these countries have adopted the European Commission and Codex Alimentarius Commission standards for ZEA.

In Ghana, as detailed studies on *Fusarium* mycotoxins, particularly ZEA are very limited, few publications studied *Fusarium* spp. prevalence rate using morphological methods. To provide a more comprehensive assessment of zearalenone and its associated producing fungi (*Fusarium* species) in maize kernels from northern Ghana, this thesis measured the levels of zearalenone (ZEA) in commercial maize kernels from northern Ghana, assessed traders' knowledge and awareness of mycotoxins, and isolated and characterized *Fusarium* species (ZEA producing once) in the maize kernels.

ZEA was found in 33.3 % of the samples (25/75), although generally at low levels. The contamination levels ranged from 0.61 to 3.05 ng/g with mean concentration of positive samples of 1.50 ng/g. None of the samples were contaminated with levels above EU regulatory limits.

Multinomial logistic regression was used to understand the relationship between traders' demographics (age, number of years of selling and education) on mycotoxin occurrences (Lee et al., 2017). The results showed that older traders (> 30 years) and those of more years in selling were more aware about the occurrence and knowledgeable of types of mycotoxins. Awareness of mycotoxins contamination among maize sellers was high in the Northern Region and Upper East Region. Moisture content of the maize kernels showed significant variations between the various region. Maize kernels from Savannah Region exhibited high variation in moisture content.

We isolated and characterized resident *Fusarium* species in maize kernels using morphological (colony color and structure and shape of microconidia) assay. Based on these results, we selected seventy-five isolates and characterized them molecularly using ITS and species-specific primers. PCR reactions amplified 61 of the selected *Fusarium* isolates for ITS primers of which 45 were amplified with VERT primers. To further identify and confirm the PCR amplicons, we sequenced 13 *Fusarium verticillioides* amplicons and all the 13 amplicons from the ITS reactions. The sequenced data further confirms the accuracy of the all the *F. verticillioides* amplicons and identified 8 of the ITS amplicons as *F. verticillioides*. Majority of these isolates were from maize kernels collected from Upper East region, and were predominantly in white maize. Thus, we expand knowledge about the wide spread of *F. verticillioides* in maize kernels.

5.2 Limitations, Recommendations and Future Perspectives

In the course of this research, it was realized that nitrogen fertilization levels may affect the levels of *Fusarium* infestation and their mycotoxins production in maize. Nitrogen fertilization rate that far exceeds crop requirements can provide a high risk of mycotoxin

contamination in maize kernels. Like other mycotoxins (example, aflatoxin, fumonisin), zearalenone concentration levels is also affected by storage practices, as well as environmental conditions. However, there was no information regarding the storage duration. As a results, these factors were not considered in this work. For this thesis, market-ready grade maize kernels were used for analysis. It is worth-mentioning that the samples were collected during the dry season (from January to May), and may not be representative of samples in the wet season.

In mycotoxins risk management, prevention is a key point for success. It is vital that food and feed marketers know the contamination levels and the associated risks in order to have a preventative approach. Since this thesis assess traders' knowledge and awareness of mycotoxins, it was event that significant number of maize markets are not aware and did not receive any formal training on mycotoxin prevention methods. It is recommended that traders work with strategic partners to generate new knowledge, build capability and develop training, and practical solutions to help mitigate risk at vulnerable points in the food supply chain.

Another possible roots to eliminate the impact of *Fusarium verticillioides* (the predominant *Fusarium* species isolate in this study) on crop production and food supply value chain is to breed cultivars that are more resistant to *Fusarium verticillioides* attack, since this particular specie is endemic in Africa. However, the maize cultivars being promoted and planted in northern Ghana have not been evaluated for *Fusarium* and other fungi resistance. This could be considered in future studies. Though the levels of ZEA in the maize kernels were generally low, the frequency of *F. verticillioides* detection was

relatively high (about 36 %). Therefore, further investigations should be conducted to further unravel the role of *F. verticillioides* in maize kernel.