UNIVERSITY OF GHANA

PREVALENCE OF MYCOTOXIGENIC FUNGI AND MYCOTOXINS (OCHRATOXIN A) IN DRIED COCOA BEANS

BY

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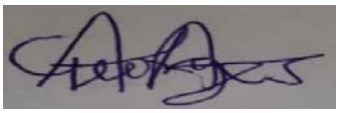
DECLARATION

I, the undersigned, ASHONG GEORGE AKWETEY, declare that I am the author of this thesis. I do declare that this work is the result of my own research work carried out in the Department of Plant and Environmental Biology, University of Ghana Legon under the supervision of Dr Ebenezer Owusu and Dr Paul A. Agyemang (Quality Control Company Limited, COCOBOD). It contains no material previously published by another person or material which has been accepted for the award of any other degree in any University, and references made in this work have duly been acknowledged.



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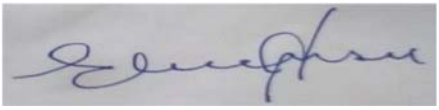

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DEDICATION

I dedicate this thesis to Almighty God for the strength and wisdom.

To my wife Joana and children.

Emmanuelle,

Imelda,

George Junior and

Jeanelle,

whose constant prayers, encouragement, sacrifice, and support that saw me through this work

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ABSTRACT

Concerns about levels of Ochratoxin A (OTA) detected in cocoa beans, its products, and the associated health issues with OTA contamination in cocoa is on the increase. This calls for the need for cocoa producing countries to research and obtain more data and information on the prevalence of Ochratoxigenic fungi present and OTA levels in dried cocoa beans, the raw material for chocolate and other cocoa products.

This work sought to determine the moisture content of cocoa beans sampled from farm gates/ societies from licensed buying companies (LBC's), determine the fungal loads and the occurrence of Ochratoxigenic fungi present as well as the Ochratoxin A levels in the cocoa beans sampled from four cocoa producing districts of the Central Region of Ghana; Agona Swedru, Assin Bereku, Assin Fosu and Breman Asikuma.

Hundred (100) samples of dried cocoa beans were obtained from twenty (20) licensed buying companies (Five societies from each LBC).

Moisture content of cocoa beans sampled was determined by The International Organization for Standards (ISO) method for moisture content determination.

Pour plate method was used in isolating the fungi after surface sterilization with 0.6 % sodium hypochlorite. Fungal identification was done using morphological characteristics such as spore size, shape, and structure as well as colour, using Leica D500 microscope connected to Leica Application Suite (LASEZ) version 2.1. Ochratoxin extraction and quantification by solvent extraction and HPLC-FLD respectively.

The method of extraction was partially modified and validated. The limit of detection (LOD) was found to be 1ng/Kg and the limit of quantification (LOQ) was 2ng/Kg.

A linear range from 1.0 -20 ng/mL and a correlation coefficient (R2) of 0.9997 were obtained for the entire range of studied concentrations.

Repeatability (RSD_r) and reproducibility (RSD_R) expressed as RSD were 5.5% and 9.8% respectively with mean recovery of OTA spiked at 2.5 and 5ng/kg in 10 replicates were consistent and more than 90%.

Cocoa beans obtained from Unicom in the Assin Fosu district recorded the lowest moisture content value of 7.05% and the highest moisture content of 9.86% for the cocoa beans obtained from Nyonkopa in the Assin Bereku district.

There were significant differences ($P \leq 0.05$) among the moisture content recorded for the cocoa beans obtained from the various LBCs.

Fungal isolation and identification revealed the presence of fifteen (15) species belonging to eleven (11) genera isolated on Malt Extract Agar and Potato Dextrose Agar from cocoa beans obtained from the four districts sampled. The following species were encountered; *Aspergillus* (*A. flavus, A. fumigatus, A. niger, A. parasiticus*), *Cladosporium* (*C. macrocarpum, C. sphaerospermum*), *Absidia corymbifera, Alternaria alternata, Byssochlamys nivea, Eurotium herbariorum, Mucor racemosus, Penicillium citrinum, Rhizopus stolonifer, Syncephalastrum racemosum a*nd *Talaromyces flavus.*

More species were isolated on Malt Extract Agar compared to Potato Dextrose Agar. *Byssochlamys nivea* and *Talaromyces flavus* were not isolated on PDA whilst *Mucor racemosus*, was also not isolated on MEA.

Although all the cocoa beans obtained from the twenty (20) LBCs in the Agona Swedru, Breman Asikuma, Assin Fosu and Assin Bereku districts each recorded the presence of *A. niger*. Ochratoxin A was not detected in any of the cocoa beans obtained from these sources.

Data obtained in this study indicated that most of the cocoa beans were not well dried to the recommended level of between 7% to 8% moisture content.

Although some few species of fungi were isolated on both PDA and MEA and identified, only one potential Ochratoxigenic fungu, *A. niger* was identified and Ochratoxin A was not detected in any of the cocoa beans samples from the four districts. This may be due to the absence of the conditions necessary for the growth of fungi and development of OTA.

CHAPTER ONE

GENERAL INTRODUCTION

Background

The history of Cocoa in Ghana dates to the 1870s when Tetteh Quarshie introduced the cocoa beans to Ghana. Through the efforts of the Basil Missionaries in the importation of more cocoa seeds, cocoa has spread to the forest zones of the Ghana and other West African states.

Cocoa farming is currently the backbone of Ghana's economy with many small-scale cocoa farmers making up about 60% of the country's agricultural base. In view of the importance of cocoa to the economy of Ghana, all factors that affect the quality of cocoa beans should be a matter of concern to policy makers.

The presence of mycotoxins in cocoa beans and chocolate products is emerging as an important public health issue and has created a need for more information about the occurrence of mycotoxigenic fungi in cocoa beans. Mycotoxigenic moulds and Mycotoxins have therefore attracted worldwide attention due to the significant effect or impact they have on human health, losses associated with the raw cocoa beans (Physical mouldiness observed on the beans and loss of weight) and their consequent national economic implications (Bhat and Vashanti, 1999).

Mycotoxins are by-product of some fungi, occurring naturally, and are of low molecular weight.

Mycotoxicosis is the pathological abnormities resuting from consuming food contaminated by these toxins.

Cocoa production begins with the harvesting of matured ripened cocoa pod, pod breaking, fermentation, drying and storage.

It is during these stages that Mycotoxigenic fungi and other fungi enter the product (Cocoa beans) and with the conducive environmental conditions, degrade the products. During the degradation process, some fungi (Mycotoxigenic) produce the by-product known as secondary metabolite (Mycotoxins).

In general, several species of fungi have been found to be associated with dried cocoa beans. These includes several species of *Aspergillus (niger, ochraceus, fumigatus), Fusarium (solani, oxysporum) Penicillium* sp*., Geotrichnum candidum* and some *Yeast* spp. (Anton and Rahmadi, 2008).

Mycotoxins associated with cocoa beans, which are incorporated into cocoa products (not removable in any of the production steps) include Ochratoxin A (OTA) and Aflatoxins (B1, B2, G1 and G2), referred to as AFB1, AFB2, AFG2 and AFG2. (Sánchez- Hervás et al. 2008; Mounjouenpou et al. 2008; Copetti et al. 2010, 2011a, 2011b; Maciel et al. 2018 Magalhães et al. 2011).

Some Aflatoxins such as AFB1 and AFB2 are known to occur naturally in food. Aflatoxins AFG1 and AFG2 are known to be hepatotoxic, mutagenic and teratogenic, and carcinogenic to humans and classfied as group 1 (IARC, 2012).

Species of the genus Aspergillus (*A. flavus, A. parasiticus*, and *A. nominus* are known to produce these toxins (Frisvad et al. 2005; Kostarelou et al. 2014).

According to WHO 2001; Pfohl- Leszkowicz and Manderville 2012, Ochratoxin A (OTA) is described as genotoxic, hepatotoxic, immunosuppressive and nephrotoxic. The International Agency for Research on Cancer (IARC 1993) classifies this as group 2B, possibly carcinogenic to humans.

Ochratoxin A (OTA) is produced by some *Aspergillus, Fusarium* and *Penicillium* species (Van der Merwe et al. 1993).

Bonvehí (2004) and Copetti et al. (2012), reported of the presence of aflatoxins (B1, B2, G1, and G2) and ochratoxins in cocoa products such as chocolate bars, nibs and cocoa powder.

The presence of these Mycotoxigenic fungi and their secondary metabolites render the product unwholesome and very unsafe for human consumption.

Cocoa bean samples with over 3% of beans having internally grown mould have been found to impart a musty flavour to cocoa liquor/Cake and unable to be removed during manufacturing processes, thus rendering the beans unusable.

Free Fatty Acid (FFA) levels in cocoa butter are also affected by fungal growth. This results in increased levels of fatty acids and lower the quality and value of the cocoa beans. (htt://www.codexalimentarium.org/).

Cocoa beans (seeds) are produced from the matured ripe fruits (Pods) from the commercial cash crop *Theobroma cacao*. Mossu (1992a) reported of 22 species of the genus *Theobroma*, with *Theobroma cacao* being the only commercially cultivated species. Opeke (1992) also reported of 20 known species of *Theobroma.* Recent study (*"Genus Theobroma". The Plant List, version 1, Royal Botanic Gardens, Kew and Missouri Botanical Garden. 1 December 2010. Retrieved 19 August 2018.)* also reported of 17 known species of the genus *Theobroma*. This leaves the number of known species of the genus speculatory. Some notable species include *T. angustifolium* DC, *T. bicolor* Humb. & Bonpl. - mocambo, and *T. grandiflorum* (willd. exspreng) K. Schum. Based on geographical, genomic and morphological characters 10 groups were proposed by researchers in 2008.

These groups are Amelonado, Cacao Guiana, Contamana, Criollo, Nacional, Curaray, Iquitos, Marañon, Nanay, and Purús. (Motamayor et al. 2008).

According to Beckett (1994). and Ardhana and Fleet (2003)., the genus *Theobroma* belongs to the family Malvaceae and grows naturally in the Amazon tropical rain forest regions and cultivated in the tropical regions. *Theobroma cacao* is a cauliflorous (lowers produced in clusters and directly on the tree trunk and older branches) and semi-deciduous evergreen plant.

Cocoa is an important ingredient in different kinds of foods such as cakes, biscuits, child-foods, ice- creams and sweets. It constitutes an inexpensive fat source with its principal raw material being chocolate (Tafuri et al. 2004).

About seventy percent (70%) of the world cocoa is produced in developing countries in West Africa (Ghana, Ivory coast, Nigeria, Togo etc.) and by smallholder farmers.

In Ghana, smallholding farms or families produce cocoa, and this involves over 800, 000 families or households. In response to world demand for the product, small farms and families have increased the production of this crop. Through government interventions and efforts by the Ghana Cocoa Board (COCOBOD), families have benefited greatly in terms of value for their products, which have improved the socioeconomic lives of many.

Apart from gold, other farm produce, forestry products and recently petroleum, cocoa has been central and consistent to Ghana's development, and the backbone to most of the economic reforms and poverty reduction strategies since the country's independence.

Compared to other agricultural commodities such as palm oil, rubber and others, cocoa beans are the most traded agricultural produce worldwide (Afoakwa, Quao, Takrama, Budu, and Saalia, 2013). Less resourced poor farmers (Bateman, 2015; Knudsen and Fold, 2011) produces more than 90 % of this product.

Cocoa has played key roles in the economy of Ghana and in the early years of independence, reserves accrued out of cocoa revenue were used for import substituted industrialization (ISI) strategy, a trade and economic policy that advocates replacing foreign imports with domestic production (Nelson, B. 2009 and Killick, 2008). Even after the discovery of oil, cocoa remains government's major source of income. Rimmer (1992), reported that most governments, including the colonial ones took cocoa taxes to build significant share of their revenue.

About thirty percent (30%) of total government revenue from 1957 to 1975 was obtained from cocoa. (Frimpong-Ansah, 1992). Between 1985 and 1987, this share decreased to 25% because of the government's strategy of moving away from export tax, demonstrating the importance of cocoa to the economy (Prichard, 2009).

According to the Global Agriculture Information Network (GAIN Report, 2012), the first quarter of 2011 cocoa beans and products export receipts were USS859.4 and this accounted for 61% of the total export earnings against USS682.5 million (48%) in the previous year. Ghana's economy depends on agriculture and this accounts for about 30% of its gross domestic product (GDP) and employs about 50% of its work force. Cocoa production accounts for about 10% of agricultural GDP (GAIN Report, 2012).

Today, cocoa constitutes one of the major export crops of the Ghanaian economy. Export of raw cocoa beans is of great economic importance in producing countries where, the cocoa is mostly traded internationally under very strict and high regulatory standard requirements on the physical and chemical properties of the cocoa beans (Adeyeye et al., 2010; Bateman, 2015). To meet the high-quality requirements for cocoa beans on the international market requires very strict and rigorous adherence to postharvest processing conditions and appropriate moisture content and aeration during storage.

Several criteria are used when considering the quality or grading of dried cocoa beans. These includes, flavour, food safety and nutritiveness (mycotoxin, chemical residues, physical characteristics (Consistency, yield of edible material), butter characteristics, colour potential, traceability, geographical indicators and certification. Manufacturers of cocoa products would usually consider the aforementioned factors when valuing a particular parcel or consignment before pricing that lot.

Flavour defects may be caused by prolonged fermentation, inadequate or too slow drying and fungal contamination.

Ochratoxin A contamination in cocoa beans is most worrying and presently there is no specific set out limits for cocoa in the European Commission. (2010). Commission Regulation (UE) No 105/2010).

In Ghana, the government controls all the major aspects of Cocoa production, pricing and export of the commodity. Ghana Cocoa Board (COCOBOD) was established in 1947 to carry out this function. It started with many subsidiaries with a large workforce. During the 1980s and the 1990s, COCOBOD was restructured significantly through the Cocoa Sector Rehabilitation Project (CSRP), Sponsored by the World Bank. This brought the number of subsidiaries to five (5) and also reduced the workforce significantly. Presently COCOBOD is made up of: The Cocoa Marketing Company (CMC), Quality Control Company limited (QCC), Cocoa Research Institute of Ghana (CRIG), Seed Production Unit (SPU), and the Cocoa swollen Shoot Virus Diseases Control Unit (CSSVDCU) (Essegbe A. G. O. and Ofori-Gyamfi E. 2012).

The buying of cocoa from farmers was for some years monopolized by the Produce Buying Company (PBC), which was a subsidiary of COCOBOD. Another change which occurred during the cocoa sector reforms on the internal purchasing of cocoa was to break the monopoly enjoyed by PBC (Shepherd and Onumah 1997: 41).

This however, brought in private competition and led to the introduction of Licensed Buying Companies (LBC's) These firms have been licensed by government to purchase dried cocoa beans from the farmers/societies in the cocoa growing areas (Vigneri M. and Santos P. 2007). There are about twenty- seven (27) private cocoa buying firms (LBC). Notable amongst these cocoa buying companies include Agroecom, Nyonkopa, Royal Commodities (ROCO),

Produce Buying Company (PBC), a former subsidiary of COCOBOD which is still the leading buying company, Kuapa Kokoo, Adwumapa, Olam and Armajaro.

In Ghana, the major cocoa farming areas are located in the following regions; Ashanti, Ahafo, Bono East, Central, Eastern, Western, Western North and Volta regions, with the main buying season beginning from October and a minor one in July (Clark, Nancy L 1994).

To further create more jobs and revenue from cocoa, Ghana Cocoa Board (COCOBOD) in September 2019 signed a memorandum of understanding with the China Development Fund and Genertec International Corporation to establish a cocoa processing plant at Sefwi Wiawso in the Western region of Ghana, with funding from the China Development Bank (CDB) and the Sino-African Fund), and will be operated by COCOBOD and Genertec through a Public Private Partnership (PPP).

The project holds great potential to contribute significantly to the improvement of the Ghanaian economy, and particularly, for the local economy of the Sefwi-Wiawso area.

JUSTIFICATION

Global concern about health implications of the consumption of food contaminated with mycotoxin is on the increase whilst consumption of cocoa and cocoa products has increased all over the world (Narjis *et al*. 2017).

Irrespective of the country in which cocoa beans are produced, they are susceptible to contamination by filamentous fungi (Minifie, 1980, 1999; Ardhana and Fleet, 2003; Schwan and Wheals, 2004).

The presence of filamentous fungal species such as *Aspergillus* and *Penicillium* in dried cocoa beans predisposes the cocoa beans to mycotoxin contamination, especially Ochratoxin A and Aflatoxins. These mycotoxins are known to be carcinogenic and therefore have significant public health concerns (Pitts and Hocking, 1997; Richard *et a*l 2007; Samson et al*.,* 2004) works by Chaytor and Saxy (1981), Bonvehi S. (2004) and Mounjouenpou *et al.,* 2007) recorded the contamination of cocoa beans by mycotoxins.

It has therefore become imperative for cocoa producing countries to develop post-harvest treatment guidelines to maximally reduce Aflatoxin and OTA contamination of their cocoa beans. This can be achieved by knowing the fungal species associated with their cocoa and the mycotoxin loads.

Cocoa producing countries like Ivory Coast (Manda *et al.,* 2017), Cameroon (Niemenak et al. 2014), Brazil (Copetti et al. 2011a) have done similar works to determine the quality of their cocoa beans and some cocoa products with regards to Mycotoxigenic fungi and the mycotoxin levels. Brazil have gone further to set limits of mycotoxin for their products.

It has become essential for Ghana, the second leading producer of cocoa and the highest ranked cocoa premium, to monitor the mycotoxigenic fungal diversity and loads, and constantly evaluate the mycotoxin loads in its cocoa beans. This will serve as basis for similar investigations in other cocoa growing areas of the country. It will also allow results to be compared within the country in terms of regional variations in climatic conditions as the central and volta regions of the country have less rains and longer dry periods as compared to the forest zones of Western, Brong Ahafo, and Ashanti regions.

This assessment when done on yearly basis by the Cocoa Quality Control Company (QCC) of Ghana COCOBOD, will enable CCOBOD to assess fungal diversity and levels OTA in cocoa beans.

Information about Ghana's cocoa beans concerning mycotoxins levels when added to other product quality requirements such as moisture content, free fatty acid (FFA) levels, percentage of internally moulded beans, slat beans, etc., would add more value and increase the confidence of the buyer. This work seeks to add knowledge to quality assessment in the area of fungal species diversity and quantification and mycotoxin loads, as there are more issues of mycotoxin loads in cocoa beans and products arising globally due to health implications of mycotoxins.

GENERAL OBJECTIVE

This project was aimed at determining the prevalence of mycotoxigenic fungi and mycotoxins such as Ochratoxin A. in dried cocoa beans

SPECIFIC OBJECTIVES

The specific objectives for this study were to.

1. Access the moisture content of cocoa beans obtained from various Licensed Buying Companies in four districts viz - Agona Swedru, Bremen Asikuma, Assin Bereku and Assin Fosu in the Central region of Ghana.

2. Determine the occurrence of fungal species associated with cocoa beans obtained from various LBCs in the four districts.

3. Determine the occurrence of Ochratoxin producing fungi *A. niger* from cocoa beans obtained from various LBCs in the four districts

4. Assess ochratoxin contamination of cocoa beans obtained from various LBCs in the four districts

CHAPTER TWO

LITERATURE REVIEW

2.1 Brief History of Cocoa production in Ghana

Palm, rubber and other forestry production used to be the main agricultural industries until the introduction of Cocoa into the Gold Coast, now Ghana.

Tetteh Quarshie, a master blacksmith and a farmer, born in 1842 to Ghanaian parents, took an expedition to Fernado Po (Bioko in the Equatorial Guinea) in 1870 and upon returning after six years brought in some cocoa beans (Amelonado) and this begun the cocoa revolution in Ghana.

There have being many claims to who introduced cocoa to Ghana, the first was the British Governor Sir William Brandford Griffith (1880 – 1885) who claimed cocoa was introduced in to the Gold Coast by his father Sir W. Brandford Griffith (Simpson's "Gold Coast Men of Affairs", p.208).

The Basel missionaries also claimed to have introduced cocoa into Ghana and have in their dairies to have carried out trials on cocoa beans. The question of who introduced Cocoa to the Gold Coast aroused and was investigated by the British Governor of the Gold Coast at the time (1919-1927), Sir Gordon Guggisberg. He concluded that it was not likely that responsible officers as Mr. Gerald C. Dudgeon, Superintendent of Agriculture, the Director of Agriculture, the Late Mr. W.S.D. Tudhope, would report Cocoa been brought in the Gold Coast by Tetteh Quarshie without any exhaustive inquiry having been done previously.

A fact the Gold Coast Board of Education recognized and attributed Tetteh Quarshie's name to Cocoa in the Gold Coast (Simpson's "Gold Coast Men of Affairs", p.208).

He planted some seeds at Mampong successfully in 1879 and gave out some of the seeds to friends and relatives who planted them. Other close farmers also did some planting. The Basel Missionaries came into cocoa cultivation by importing large quantities of planting materials and setting up farms. This also led to the spread of Cocoa to countries like Nigeria and Sierra Leone (Simpson's "Gold Coast Men of Affairs", p.208).

Export of Cocoa in Ghana begun in 1891 with the official export of two Bags in 1893. Ghana had led the world Cocoa production from 1910 to 1980s as the world's largest exporter until this position was taken by the Ivory Coast. However, Ghana still boasts of the highest quality (Premium) cocoa in the world (Ephson 1936).

2.2 Current state of Cocoa production in Ghana

Ghana is the second world's largest cocoa producing country with an estimated market share value of about 20 percent and well known for its standard of high-quality cocoa beans.

The country once led the world cocoa production between the 1910 and the 1980's. Several factors led to this position being taken by the Ivory Coast. This was largely due to wide bush fires which was fueled by very unfavorable environmental conditions (Drought) and diseases incidence mainly the Black Pod diseases caused by the fungi *Phytophthora palmivora* and *P. megakrya*, and the cocoa swollen shoot diseases (CSSD) caused by the cocoa swollen shoot disease virus (CSSDV).

Dormon et al. (2004), came out with the following findings that resulted in low yields of Cocoa in Ghana. The first was the cheating by the licensed buying companies, which some time back was monopolized by Produce Buying Company (PBC), smuggling to neighboring Ivory Coast due to low prices paid to farmers and awards at farmer s'day not given equitably.

Other socio-economic factors included low producer prices, farmer's inability to buy fairming inputs, lack and high cost of labour and poor road networks. Inadequate crop management: inability to replant new crops to replace old, dead and diseased plants, over shading (No pruning), and inadequate control of weeds contributed greatly to reduced yields. Other pests and diseases also affected farmers. Some examples are mistletoes, termites, capsids and stem borers.

Despite this take over by the Ivory Coast, Ghana still leads in production of the best (Highest Premium) cocoa in the world. Various governmental interventions through COCOBOD have helped in achieving high quality cocoa and increase in productivity although there have been some inconsistencies in production figures over the years.

One very important intervention and strategy which government adopted in the 2000's was to pay farmers (producers) a very good share of the world market prices. With increases in the world market price of cocoa over the period, this gave producers higher prices for their produce. This brought about increase in farm sizes and numbers in households going into cocoa production.

Other governmental programs which also brought about the cocoa revolution in Ghana initiated in the early 2000's was the provision of improved crop varieties to farmers, subsidizing and removal of import duties on farm inputs and fertilizer and production/training of more extension officers to provide free disease and pest control services to cocoa farmers. This was achieved through previous governments (1980's to 1990's) efforts in restructuring Ghana COCOBOD, significantly removing 1000's of ghost names from its payrolls, removal of nonperforming subsidiaries and other functions played by COCOBOD which did not fall under their mandate e.g., road construction ("The Ghana Cocoa Story". Ghana Cocoa Board. 2016. Retrieved April 20, 2018).

In recent times, 2012/2013 to 2018/2019 cocoa growing seasons, cocoa production has seen ups and downs increment in production (In thousands of tons) In 2012/2013 and 2013/2014 production year, there was an increase of about 62 thousand tons in production, i.e., from 835 to 897 thousand tons. During the 2014/2015 production year, there was a sharp decrease from 897 to 740 thousand tons and increased to 778 thousand tons in 2015/2016 and upward to 969 thousand tons in 2016/2017 production year. Again, the country recorded a sharp decrease of about 61 thousand tons in 2017/2018 and a further 5 thousand tons in 2018/2019, bringing production in that year (2018/2019) to 900 thousand tons (Shahbandeh M. 2021).

2.3 Regional Production and economic importance

Cocoa production was centered in six regions (Western, Ashanti, Central, Brong Ahafo, Eastern and Volta) of the country before the creation of new regions. Production in Ghana is mainly by smallholder farmers and (GLSS 2014) reports that 800,000 families or households are involved in this production.

Production in the Eastern region, which used to be the center of cocoa production in Ghana, has seen a decline in production over the period from 2002 to 2011. A reduction from 12 to 9% of national production even though there was about 20,000 tonns increase in production. The center of production has gradually shifted to the West. The western region increased its production from 53% in 2002 to 56% in 2011. It also continues to be the leading production region in the country with Enchi, the country's largest producer in 2002 and the leading producing district until date (Vigneri and Kolavalli, 2018).

Production in the Brong Ahafo regions has also seen steady increases exceeding 1000 tonns in 2010/2011 production year (Gockowski 2012).

2.4 World Production, Processing and Consumption of Cocoa

On the world market, Ghana's cocoa attracts a premium between three (3) to five (5) percent compared to that produced in the Côte d'Ivoire, the world's current largest producer of cocoa beans. According to ICCO 2017 figures for 2015/2026 production year, Africa (West Africa; Côte d'Ivoire, Ghana, Nigeria, Cameroon and Togo) produced about 73% of the world's cocoa, 17% by the Americas and 10% by Asia and Oceania regions. Out of this, Côte d'Ivoire produced about 40% followed by Ghana with about 20% of the world's production. Indonesia, Ecuador, Cameroon, Nigeria and Brazil followed with 8%, 6%, 5%, 5%, and 4% respectively. With Peru, Dominican Republic, Columbia and other countries producing the remaining 12%. The world's production according to this report was approximately 4000 (Thousand metric tonnes).

In 2018/2019 and 2019/20 crop year, Ivory Coast increased production from 2,154 to 2,180 (in 1000 metric tons) whilst Ghana's production also went up from 812 to 850 (1000 metric tonnes)

Although Africa produces more of the world's cocoa, it is the lowest processor and consumer of this product. Europe, the largest cocoa beans processor, grinds about 40% of the world's annual harvest. Thirteen percent (535,000 tons) processed in the Netherlands and 40,000 tons (1%) processed in Switzerland. Brazil and Malaysia process about 5% each of the world's cocoa.

The Americas process about 22%, Asia and Oceania 21% with Africa processing 19% of the world's annual harvest. Out of the total processed beans in Africa, Ghana processes about 5%of this product.

Europe tops the world with the consumption of cocoa and cocoa products. They consume about 46% of the world's production. This is followed by the Americas with 32%. Asia and Oceania 18%. and Africa consumes about 4% of the world total production although it produces about 74 % (ICCO 2017).

2.5 The Ghana Cocoa Board (COCOBOD)

The Ghana Cocoa Board (COCOBOD) mainly controls the Cocoa sector in Ghana and oversees all the aspects of cocoa production and marketing in Ghana. COCOBOD, formerly Ghana Cocoa Marketing Board (CMB) was established by ordinance by the then colonial masters in 1947 with a starting capital Gh¢ 2700 (¢27 million) being Ghana's share of the net profit of the West African Produce Control Board.

It however traces its formation further back to the cocoa hold-up of 1937 ("CocoaMarketing.com". Archived from the original on 2010-02-16. Retrieved 2010-01-22.).

In order to carry out this mandate, several subsidiaries of COCOBOD were set up to oversee specific aspect of Cocoa production through to marketing of the product. These subsidiaries include the Produce Buying Company (PBC), The Cocoa Marketing Company Ghana Limited, The Cocoa Research Institute of Ghana (CRIG) Seed Production Unit etc.

Since its formation, COCOBOD had gone through several reforms and transformations to meet the modern trends in the Cocoa production and marketing industries quality and quantity demands of the product. Some of the transformation or Reforms was also geared towards enhancing the livelihood of farmers, getting better earnings from their proceeds from the export of the product then paying over stretched staffs of COCOBOD and its subsidiaries.

Between 1980s and the 1990s, COCOBOD was significantly restructured through the Cocoa Sector Rehabilitation Project (CSRP) sponsored by the World Bank. This included the reduction in the size of COCOBOD by re-organizing some of the subsidiaries to enhance productivity and quality of products.

Currently, COCOBOD operates with five subsidiaries. Three of the subsidiaries, Cocoa Research Institute of Ghana (CRIG), Cocoa Swollen Shoot Virus Diseases Control Unit (CSSVDCU), and the Seed Production Unit (SPU) ensure that farmers get quality planting materials, control diseases and pest and to improve on yield.

The remaining two subsidiaries, Quality Control Company Limited (QCC) and the Cocoa Marketing Company Limited are responsible for overseeing the main organizational goal of exporting good quality, high Premium cocoa to the world market. Through the efforts of these subsidiaries, Ghana Cocoa boasts of 4%-6% price premium on its cocoa on the global market.

One very important link between the farmers and COCOBOD, which ensures that Cocoa beans move from the farmers in the hinterlands to the Cocoa Marketing Company (CMB), is the Licensed Buying Companies (LBCs). The buying of Cocoa from the hinterlands from farmers or societies, bulking and haulage to the warehouses of Cocoa Marketing Company affects the overall production and export of quality Cocoa to the global market.

The largest, sole Licensed Buying Company (LBC), Produced and Buying Company Ghana Limited (PBC), used to be a subsidiary of COCOBOD. To bring competition into the effective buying, bulking, packaging and haulage of Cocoa to the Cocoa Marketing Company and as part of the reforms was privatized in June 1993 (Appiah-Kubi · 2001). This broke the monopoly in the buying of Cocoa in Ghana and was guided by a legal framework spelt out in the regulations and guidelines for the privatization of the internal marketing of Cocoa.

There are about twenty-seven (27) Licensed Buying Companies operating in the system, the numbers remain unstable due to the competitive nature of the work. Some companies have folded up with new ones coming up. Between the period 2001 and 2010, 11 LBCs delivered about 96.4% of cocoa to the Cocoa Marketing Company.

Some of the current operating companies include PBC, Olam Ghana Limited, Agroecom Ghana Limited, Akuafo Adamfo Marketing Co. Limited, Cocoa Marchants, UNICOM, Best Link Blobal, Tradeco, Fedco, Royal Commodities (ROCO), ELIHO, Kuapa Kokoo, Nyonkopa, Adikanfo, PRESTIGE, Transroyal Ghana Limited. PBC still remains the largest LBC controlling nearly about 35% of the market shares.

2.6 Post Harvest Processes and cocoa quality

Pod Harvesting and Breaking

Mature cocoa trees produce pods throughout the season and each pod contains about twenty (20) to forty (40) seeds (Minifie 1980).

Mucilaginous pulp surrounds the seeds in a riped pod. Ripped pods are harvested regularly every two to three weeks. This does not include pods which are not fully riped and those over-riped. Harvested pods are gathered on farms and collected to a central location for pod breaking.

Rotten, diseased and injured pods (during harvesting) are excluded during the gathering. Harvested pods are not allowed on farms for more than five (5) days to prevent more ripped pods from getting rotten. These measures are taken to eliminate pods infected with moulds from getting into the fermentation stage.

Fermentation

Fermentation in cocoa beans is a process in which the tannins in cocoa beans are oxidized giving the cocoa beans a palatable flavor that eliminates the sourness. It is also a modification in physical composition of the bean when bean colour is changed from slaty, slaty-violet, through to violet, violet –brown and finally the desired brown colour (Niemenak, et al. 2014).

There are other biochemical processes that takes place to give the cocoa beans the desired flavour and colour. Over fermentation can result in the development of moulds, loss of chocolate flavours, off flavours due to putrefaction and increase in pH values of the cocoa beans.

Scientifically, spectrophotometry can be used to monitor the process of fermentation by measuring the colour of the beans. Over fermented beans are darker and under fermented beans looks purplish. This can help to eliminate under or over fermented cocoa beans before they are processed, saving time and money.

The development of flavour precursors and colour of cocoa beans begins at the process of fermentation. This includes the action of microorganisms on the cocoa pods and enzymatic actions on proteins, carbohydrates and polyphenols in the cocoa beans. Fermentation results in changes in pH and temperature, thus influencing enzyme activities (Hansen et al. 1998; Biehl et al. 1990).

During the fermentation process, acetic acid, lactic and citric acids are produced by bacteria and this inhibits the growth of Ochratoxigenic fungi and therefore prevention of the development of OTA. After harvesting the pods, they are heaped on the farms for over 24hours.

The pods are then broken or cut open and the beans taken out to begin the fermentation process. The process can be carried out in wooden boxes, holes or the beans can be heaped on the farm on cut plantain leaves as mat.

The fermentation method depends on the climate, cocoa variety, quantity of beans or the size of the farm and the locally available technology. Large farms adopt the box method and small farms use the heaping method also known as the traditional method. The process can also be altered or influenced by the addition of micro-organism starter cultures, changing of pulp to beans ration, aeration and number of turnings.

For the traditional method (heaping) which is widely practiced in Ghana, after 24hours, the heap is turned and recovered to ensure that all beans are fermented properly. This is repeated after every 24 hours for between 3 to 5 days. Farmers cut few beans at intervals of the process to observe colour changings as means of tracking fermentation progress and deciding on the endpoint.

Some measures adopted to prevent fungal contamination include the cleaning of cutting material or equipment, baskets and platforms. Removal and discarding of black beans and germinating beans, husks and placenta. The fermenting heap must also be prevented from rain and direct sunlight. This method is standard and used in all parts of the country, giving a uniform colour to its cocoa beans.

Elsewhere in Cameroon, different smallholder farmers perform different fermentation practices that affect their cocoa bean quality and loss of revenue. Brazil and South East Asia mainly practice the box method of fermentation with a bed depth of about 40 to 100cm for between 500 to 2000kg of wet beans.

The assumption however is Cocoa beans fermented between 4 to 6 days with two days' intervals of opening and turnings gives a well fermented bean (Sadoux, 1961; Rohsius et al. 2006) A brown bean colour after cut test without moulds, with low astringency and bitterness, the absence of off-flavours such as smoky notes and excesses acidity indicates a well-fermented and well dried bean. Handlaer (1980) and Niemenak (2006) reported that there is a change of bean colour (slaty to slaty-violet, violet, violet brown to brown) during the fermentation process. A slaty bean indicates an unfermented bean whilst a violet colour indicates under fermented bean. A change from violet to brown indicates a well fermented bean.

A random cut test is performed in most cases to check the level of fermentation of beans.

The pH of the beans also determines the level of fermentation of beans. Unfermented beans have a pH of between 5.5-5.8 and a well fermented beans have a lower pH of between 4.75 to 5.19.

Hansen et al. (1998) reported that enzymes have short periods of action during the fermentation process whereas some enzymes such as endoprotease and glycosidase are active throughout the whole fermentation process. Aminopeptidase, invertase and polyphenol oxidase are strongly inactivated, and carboxypeptidase are partly inactivated.

Drying

The drying stage comes next after the fermentation process. Polyphenol oxidase, which is still active during this stage of drying converts polyphenols to quinone. Quinones with free amino and sulfhydryl undergo condensation reactions leading to brown polymers (Biehl and Ziegleder 2003a).

The two most important achievements of this process are to arrest the process of fermentation which leads to over-fermentation of the cocoa beans and to gradually reduce the water content in the beans to about 7.5% to 8.0% moisture content. This reduces the pH, astringency and bitterness of the cocoa beans as well as giving it its characteristic brown colour and flavour (Afoakwa et al. 2008; Merkus, 2014).

The low moisture content of the beans also allows the beans to be stored and transported to local processing destinations and the international market without getting contaminated by bacterial and mycotoxigenic and other moulds, insects, polycyclic aromatic hydrocarbons (PAH) and finally the beans getting deteriorated by combination of these factors.

Cocoa beans are best dried under direct sunlight but depending on the weather conditions, artificial means of drying can be used in place of sun drying or supplement sun drying. The artificial methods of drying include wood fuelled kiln dryers and direct fuelled burners (Gasoline). Sun drying is widely used in Ghana and is the preferred method of drying compared to other Artificial methods of drying.

Giacometti et al. (2015) reported that off-ground (Step-up) sun dried cocoa beans gives the best flavour profile, with low bitterness, astringency and sourness whilst Afoakwa et al. 2008, also reported of a more pronounced chocolate flavours using the above method of drying.

Artificially dried beans are characterised by off-flavours notes such as hammy, rubber, smoky or gasoline notes (Bernaert et al. 2012).

Any method of drying employed must be thorough with reduced moisture content of 8.0%. It must also be done within the required hours / days (6 to 10 days for sun drying) as too slow drying results in longer days of drying, and mouldiness of beans and hence the likely increase in OTA levels.

Fast drying on the other hand at higher temperatures below the required days, i.e., 6 days (mainly achieved by artificial means of drying) will also result in faster rate of water loss from the shells as compared to that of acids from the nibs or cotyledon into the shells. This leaves a higher concentration of acids in the nibs after drying (Acidic beans) and also inhibiting other reactions that lead to the formation of other cocoa flavours.

Excessively dried cocoa beans also become brittle, breaking easily and increasing the waste beans proportion and FFA formation (Lipolysis) whilst incompletely dried or rain-soaked beans is characterised by high mouldy beans, highly concentrated strong-smelling carbonyl compounds.

CAOBISCO/ECA/FCC, (2015) recommends that drying should be done off-ground or drying surfaces /equipment should be elevated and should be away from sources of contaminations. If possible, drying platform placed directly on the sun.

It also recommends that the beans should be well spread on the drying platform and should not exceed 6cm thick and should be turned about 5 to 10 times each day while removing any defective beans (shrivelled, flat, mouldy, black, germinated, small/or fused together and insect damaged). Sun-dry when possible should replace and complement with well-designed and maintained artificial drying when necessary. Cocoa beans should be covered at night and during rains and kept from smoke.

Levels of dryness is also an important quality control measure used in the grading of cocoa beans both on the local and international market. Moisture content above 8% is not allowed.

Storage

After cocoa beans with mucilage had undergone the heat generated, chemical and biochemical reactions (fermentation) and dried either with sun or with artificial means, the next very important step is the storage of the cocoa beans.

Dried cocoa beans after production are stored at different stages of the production chain and inner transportation, before finally transported to the main exporting site, (various QCC warehouses at the various ports in Ghana) stored for varied periods of times before exporting to the processing countries.

Within the periods of transportation and storage of cocoa beans, the beans have high risk of damage and deterioration due to the characteristic high temperatures and relative humidity and the moisture content of the product.

The longer the storage period, the higher the risk and vice versa. The cocoa beans if not properly dried to the required moisture content, transported and stored under required conditions and the required quality control measures taken becomes another access point for mycotoxigenic fungi infestation, rapid development of internal moulds and increase in mycotoxin levels.

Beetles, mites and moths (*Ephestia cautella* moths' larvae, storage pest in tropical countries of origin and closely related *Ephestia eluttella*, storage at warehouses at destinations in countries with cooled temperatures), and fat degradation. These further puts the integrity, quality and commercial value of the product at risk.

Different organisms deteriorating and damaging cocoa beans in store requires different levels of relative humidity to commence and continue their growth and development. Critical levels of moisture content recommended for dried cocoa beans is 7%. Bacteria and fungi (including Mycotoxigenic types) respectively require MC levels of 8% and above and relative humidity levels of above 90% and 70% for their growth. Mites require a relative humidity of 60% and above whilst insects (depending on their class and species) require the range of 30-50% RH.

In Ghana, dried cocoa beans from the farm gates or societies move (sold) to the LBCs in jute bags. The bags are re-opened bucked and re-dried to the required moisture content. The dried cocoa beans are bagged according to batches with the needed information such the name of the LBC, the district and region of origin and batch number inscribed on the bags and packed on wooden pallets (well treated with wood preservatives).

New jute sacks are used to bag cocoa beans and sewn properly to be able to withstand packing on tracks for onward transportation to the warehouses, offloading and re-parking in warehouses and stacked for fumigation and other phytosanitary investigations or inspections and finally loading into vessels for shipment.

Postharvest (on farm) processes greatly affect the physical and physiological integrity of the freshly harvested cocoa and can compromise the quality of the cocoa beans. After these processes, the cocoa beans do not respire any longer due to the destruction of the living seed (embryo) by the heat-generated process of microbial fermentation. At this stage, the cocoa bean structure becomes more porous and easily penetrated by any deterioration and damaging factor.

This becomes another access point when seeds are not properly dried and stored, leaving the cocoa beans at high risk of damage and deterioration from biotic and abiotic factors. These factors interact and therefore an uncontrolled factor can lead to the development of other factors. The factors can be divided in to three; Chemical, Physical (abiotic) and pathogens (biotic). The physical is further categorized in to primary and secondary. The primary physical factors include temperature and relative humidity, and changes in these factors results in condensation and surface moisture or water, which becomes secondary physical factors.

In most cases, the biological factors are carried over from the postharvest processes and have become inactive due to the dryness of the product.

The primary physical factors initiate the formation of the secondary conditions and this is followed by absorption of water by the product (dry cocoa beans), resulting in increased moisture content of the product in storage. This condition triggers the growth of biological factors such as internal and external fungi which later gives way to attack by other biological factors such as insect, mites, etc.

2.7 Moulds and their health effects

Moulds are microscopic fungi with large taxonomically diverse fungal species. They can be found in the divisions Zygomycota and Ascomycota (Hibbett et al. 2007). There are thousands of known species of moulds, which have diverse life-styles including saprotrophs, mesophiles, psychrophiles and thermophiles and a very few opportunistic pathogens of humans. (Ryan, 2004). Typically, moulds secrete hydrolytic enzymes, mainly from the hyphal tips. These enzymes degrade complex biopolymers such as starch, cellulose and lignin into simpler substances which can be absorbed by the hyphae. In this way moulds play a major role in causing decomposition of organic material, enabling the recycling of nutrients throughout ecosystems.. Moulds can also grow on stored food for animals and humans, making the food unpalatable or toxic and are thus a major source of food losses and illness (Malloch, 1981). Many strategies for food preservation (salting, pickling, jams, bottling, freezing, and drying) are to prevent or slow mould growth as well as growth of other microbes.

Many moulds also synthesize mycotoxins and siderophores which, together with lytic enzymes, inhibit the growth of competing microorganisms. These mycotoxins can pose serious health risks to humans and animals. Some studies show that exposure to high levels of mycotoxins can lead to neurological problems and in some cases, death (Empting, 2009). Prolonged exposure to mycotoxins may be particularly harmful. Research on the health impacts of moulds has not been conclusive (Money, 2004). These toxic properties may be used for the benefit of humans when the toxicity is directed against other organisms. For example, penicillin adversely affects the growth of Gram-positive bacteria (e.g. *Clostridium* species), certain spirochetes and certain fungi (Blood and Studdert, 1999).

2.8 Moulds Associated with cocoa

In general, several species of fungi have been found to be associated with dried cocoa beans. These include several species of *Aspergillus* (A. *niger,* A. *ochraceus,* A. *fumigatus*), *Fusarium* (*F. solani, F. oxysporum*) *Penicillium* sp., *Geotrichnum candidum* and some Yeast spp. (Anton and Rahmadi 2008),

Bunting (1928) and Dade, (1928) first documented six (6) fungal species causing internal mouldiness of cocoa beans in Ghana. These included four (4) species of *Aspergillus*, *Mucor sp* and *Penicillium* species. Other works such as Hughes 1952, 1953 and Piening, 1962 also listed several species of fungi associated with dried cocoa beans. Abitey (1982) isolated twenty- one (21) species including 12 speciesof *Aspergillus*. Fapohundal et al. (2018), identified 6 fungal species viz *Aspergillus tamanii, A. niger, A. flavus*, *A. japonicas*, *Fusarium chlamydosporum* and *Syncephalastrum racemosum* on cocoa beans.

In some specific areas of Nigeria; Ado, Emure, Ise, Ikere in Ekiti states, cocoa beans in storage were sampled between July – December and the following fungal species were isolated consistently from cocoa beans which have grown mouldy; *Aspergillus flavus*, *Aspergillus niger*, *Botryodiplodia theobromae*, *Mucor* spp., *Neurospora* spp. *Penicillium* spp., *Phytophthora palmivora*, *Rhizopus* spp. (Fagbohun et al. 2011).

In Cote d'Ivoire, the leading producer of cocoa in the world, cocoa grows in 13-forested zones with annual production of about 1.45 million tons in 2012/2013, 1.8 million in 2017 and a projection of 2.18 million tons in 2019/2020.

Cocoa beans sampled from three (3) different zones, namely, Central: Gagnoa (Tehiri), East: Abengourou (YakasséFeyassé); and south – west: San Pedro (Gabiadji), revealed the presence of ten (10) fungal genera. These include *Aspergillus* (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillu sterreus*, *Aspergillus candidus*, and *Aspergillus glaucus)*; *Alternaria, Acremonium, Aureobasidium, Bipolaris, Cladosporium, Exophiala, Fusarium (Fusarium solani and Fusarium oxysporum), Penicillium and Scytalidium* (Manda et al. 2017).

2.9 Mycotoxigenic moulds associated with Cocoa

Mycotoxigenic moulds are moulds that produce mycotoxins as secondary metabolites. Ochratoxin A is the major mycotoxin associated with cocoa (Mounjouenpou et al. 2007).

Although several moulds have being found to be associated with cocoa, not all are mycotoxigenic. Some specific moulds lead to the elaboration of mycotoxins including Ochratoxin A (OTA) and Aflatoxins (AFB1, AFB2, AFG1 and AFG2) in the cocoa beans.

Aspergillus parasiticus, *A. flavus* and *A. nomius* are known to produce Aflatoxins whilst *Aspergillus* species (*Aspergillus carbonarius, Aspergillus niger, Aspergillus westerdijkiae, Aspergillus steynii,* and *Aspergillus ochraceus* and other species are known to produce ochratoxins. (Taniwaki et al. 2003; Visagie et al. 2014). Some Fusarium and Penicillium species also produce Ochratoxin A (OTA) (Van der Merwe et al. 1993).

2.10 Ochratoxin A (OTA)

Ochratoxin A ($C_2OH_{18}ClNO_6$) (7-(L-β-phenylalanyl-carbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin) is a group of mycotoxins which was discovered after Aflatoxins and was originally isolated from the fungus *Aspergillus ochraceus* (Van der Merwe et al. 1965). It is the most important and consist of a polyketide-derived dihydroiso-cumarin moiety linked through the 12-carboxy group of phenylalanine. Other forms of Ochratoxin are the Ochratoxin B and Ochratoxin C (Luster et al. 1987; Weidenborner, 2001) which are not chlorinated and that makes them comparatively less than OTA.

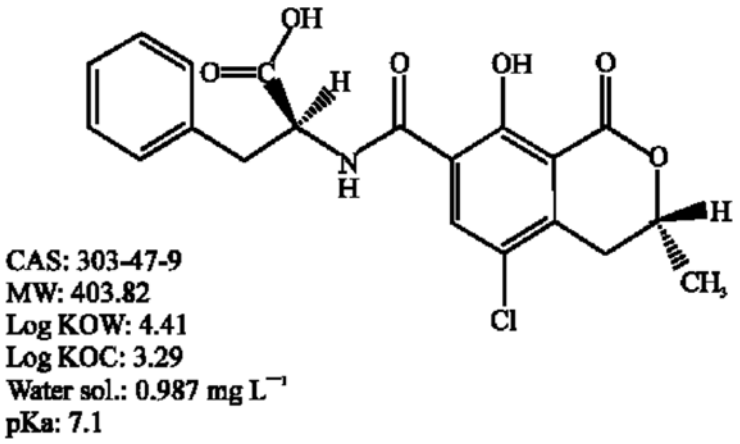

Figure: Chemical structure, molecular weight, water solubility and other properties of Ochratoxin A (Dall'Asta et al. 2008; Hussein and Brasel, 2001; Mortensen et al. 2006)

Ochratoxin A was isolated and characterized before it was proven in the late 1970s to cause kidney disease outbreaks in poultry (Hamilton et al. 1977) and pigs (Krogh, 1978b). It is produced by several species of *Aspergillus* and *Penicillium* and is a common food-contaminating mycotoxin.

Contamination of food commodities, such as cereals and cereal products, coffee beans, dry vine fruits, wine and grape juice, spices and liquor ice, dried cocoa beans and cocoa products occurs worldwide.

Frisvad et al. (2004) and Perrone et al. 2007 have reported several *Aspergillus* species associated with the production of OTA. These include *A. ochraceus, A. cretensis, A. flocculosus, A. pseudoelegans, A. roseoglobulosus, A. sclerotiorum, A. steynii, A. sulphurous,*

A. westerdijkiae, section Circumdati sensu stricto species including A. auricimus, A. elegans,

A. insulicola, A. melleus, A. ostianus, A. petrakii and *Neoppetromyces muricatus*.

Two *Penicillium* strains are also widely reported to produce OTA, these are *P. verrucosum* and *P. nordicum*. Although they are similar species, they can be distinguished by the colour on their reversed plate on Yeast Extract Sucrose Agar (YES) and their habitat preference (Bragulat et al. 2008; Larsen et al. 2001). *P. nordicum* is also known to produce less OTA compared to *P. verrucosum*. Specificity of different strains of fungi in a given food with regards to OTA production is not known (Pardo et al. 2004)

Other *Penicillium* species known to produce OTA include *P. brevicompactum, P.*

chrysogenum, P. crustosum, P. cyclopium, P. olsonii, P. viridicattum, and a few other species (Rosa et al. 2006; Vega et al. 2006).

Different *Aspergillus* and *Penicillium* species and or strains produce OTA at different environmental conditions (Aziz and Moussa, 1997; Callaghan et al. 2006). The occurrence of OTA producing fungi in contaminated food does not necessarily indicate the presence of OTA in that food product or material.

OTA are produced under varying environmental conditions such as temperature, presence of oxygen, water activity level (α_w) and the availability of some metal ions (Amezqueta et al. 2009; Astorreca et al. 2007; Kokkonen et al. 2005).

The water activity range for ochratoxigenic fungi is around 0.81 α_w and 0.83 α_w (Lindbald et al. 2004; Cairns-fuller et al. 2005). In OTA producing *Aspergillus* species, temperatures between 20ºC to 30ºC favours production (Pardo et al. 2004; Callaghan et al. 2003) whilst *Penicillium* species can produces OTA at temperatures as low as 5ºC (Centre for Food Safety, 2006). The composition of the media or substrate also affects OTA production. Glucose and sucrose availability in substrates is known to promote OTA production (Muhlencoert et al. 2004).

Dried Cocoa beans of West African origin and some tropical countries like Brazil have also been established with problems with OTA. In a recent study, (Pires, Vargas, Gomes, and Vieira, 2019) the presence of OTA was established in 123 samples of cocoa beans sampled from five states in Brazil. Using High Performance Liquid Chromatography with Fluorescence Detector (HPLC-FLD) after immunoaffinity column clean-up, mean levels of OTA contamination of 1.2 µg/kg was established with none of the samples exceeding the maximum limit established by the Brazilian legislation. (ANVISA 20011), A maximum limit (ML) of 10 µg/kg for total AFS (Aflatoxins) and 10 µg/kg for OTA in cocoa beans.

Also, in Côte d'Ivoire, (Manda et al. 2017) which is the world's leading producer of cocoa, cocoa pods were collected and sorted into four categories based on quality (intact, pricked, rotten and injured) and were subjected to the dried cocoa beans production steps (i.e., pod opening, fermenting and drying) and levels of OTA were determined.

Average levels of 0.16 μg/kg (intact pod), 1.10 μg/kg (rotten pods), 1.56 μg/kg (injured pods) and 1.51 μg/kg (pricked pods), indicating some levels of OTA in the final dried cocoa beans from that country were detected.

Ochratoxin A is formed during the storage of crops and due to its long half- life, it builds up in the food chain. Orally ingested OTA has a shorter half-life as compared to intravenously injected OTA (Marquardt et al. 1992).

OTA is classified as group 1 carcinogen (IRAC, 1987). Structure activity studies suggest that the toxicity of OTA may be linked to its isoroumarin moiety and lactone Carbonyl group.

Later (IRAC 1993) reported OTA as genotoxic, mutagenic, immunosuppressive, and teratogenic, classifying it as a group 2B and as possible carcinogenic to human.

Multiple mycotoxins can be present in one sample. The co-occurrence and variations in levels of OTA and Aflatoxins was detected in 5% of 130 cocoa beans sampled in Bahia (Brazil). It is also known that the consumption of contaminated food with different types of mycotoxin may increase health risk due to its additive effects (Speijers and Speijers 2004; Smith et al. 2016). Sedmíková et al. (2001) also reported that in the same sample, the mutagenic activity of AFB1 can be increased by the presence of OTA.

The committee on Toxicity of Chemicals in Food, Consumer products and Environment (COT) concluded that OTA is genotoxic carcinogen and therefore there should be a reduction in the level of detection to make it technologically attainable (COT 1997).

The set provisional intake by the joint expect committee on Food Additives of the WHO and FAO set a provisional maximum intake of 100ng/kg weight (bw)whiles the daily intake proposed by the Scientific Committee on Food of the European Union is 5ng/kg (bw) (WHO 1996).

On the European markets, Foodstuffs are subjected to regulations and directives. EU regulations 1881/2006 stipulates set limits or levels of 5 µg/kg and 3 µg/kg in cereals and processed cereal –based products respectively, 5 µg/kg and 10 µg/kg in roasted and ground coffee respectively, 5 µg/kg in raisins and 2 µg/kg in spices and wine. An acceptable level of 120ng/kg body weight weekly intake of OTA has also been set and accepted by the European Food Safety Authority (EFSA) in 2006.

A commission of five of the European Communities Commission (ECC.) Regulation (UE) No 105/2010 set up in February 2010 to modify regulation (EC) N 1881/2006 on fixing of maximum contents for certain contaminants in foodstuffs with regard to ochratoxine A. did not set any maximum acceptable limits for OTA in cocoa and cocoa –derived products. This was as a result of very low levels of OTA in those products which did not contribute to any OTA exposure significantly.

This notwithstanding, the presence of inconstant levels of OTA have been detected in cocoa beans sampled from different cocoa producing countries in West Africa and other different regions. (Burdaspal et al. 2003), Bonvehi (2004), Amézqueta et al. (2005), Dembele et al. (2007), Dongo et al. (2008), Gilmour et al. (2008), Copetti et al. (2010). Currently, the EU is considering introducing regulatory limits of OTA in cocoa and cocoa products, and cocoa production countries have been edged to enforce preventive measures to reduce OTA.

OTA is taken up passively throughout the gastrointestinal tract and actively in the kidney and is known to cause a number of toxic effects in animal species. Highest amounts of OTA could be found in the blood and it is distributed in adipose tissues, muscles, liver and the kidney, in an increasing order.

The most sensitive and notable effect is kidney damage, but the toxin may also have effects on fatal development and on the immune system. Contrary to the clear evidence of kidney toxicity and kidney cancer due to ochratoxin A exposure in animals, this association in humans is unclear, however effects on kidney have been demonstrated (Sauvant 2005).

2.11 Grading Criteria for Cocoa Bean quality

CAOBISCO, the European Cocoa Association (ECA) and the Federation of Cocoa Commerce (FCC) are committed to working towards more sustainable cocoa which complies with such requirements for consumer, manufacturer and farmer benefit. Quality cocoa beans are needed to be processed in to safe cocoa products on the international market.

Several criteria are used when considering the quality or grading of dried cocoa beans which include flavour, food safety and wholesomeness, physical characteristics (Consistency, yield of edible material), butter characteristics, colour potential - "Colourability", traceability, geographical indicators and certification. Manufacturers of cocoa products would usually consider the aforementioned factors when valuing a particular parcel or consignment before pricing that lot.

2.12 Aspects of Cocoa Beans Quality affected by moulds

Flavors (Mouldy Off– Flavors)

Mouldy off- flavours are mouldy or musty flavours or odour which is given to liquor/ cocoa paste and finally to the final cocoa product (Chocolate) as a result of the presence and growth of internal moulds in dried cocoa beans.

Mouldiness in cocoa beans caused by *Aspergillus* and *Penicillium* can result in mouldy-off flavour by the production or deposition of carbonyl compounds, 2-arels, methyl ketones and

2,4-dienals. The carbonyls are dissolved and remain in the oil after the beans have been pressed. The same effect can also be caused in cocoa powder by metabolizing trichloranisol present in packed cocoa products (Erkmen, and Bozoglu, 2016). The effect of these compounds cannot be removed by any of the processing stages and is carried into the final product, thereby giving it an unacceptable or unpalatable taste. At least three percent of cocoa beans with internal moudness have been found to impart a musty flavour to cocoa liquor/Cake, which could render a whole consignment of cocoa beans unusable. The presence of these moulds is detected in cocoa beans by the cut test which involves dividing the cotyledon into two and observing the presence or absence of moulds.

Prolonged fermentation and too slow drying or inadequate drying contribute to mould growth in cocoa beans and subsequently result in flavour defects. There are about 600 (Six Hundred) different compounds (Alcohols, Aldehydes, Carboxylic acids, Esters, Ketones, and Pyridines) identified as flavour–active compounds.

The exact aroma of cocoa arises from complex biochemical and chemical reactions which occur during the processing of cocoa pods (Post harvest processes). Other factors such as the genotype, chemical composition of seeds, environmental conditions, cultural farming practices and some stages of manufacturing have also been found to affect the flavour of cocoa liquor and the final cocoa product.

The interaction between the chemical which may be present and, the biochemical processes which takes place during the post-harvest processes and how they affect the flavour of cocoa has not been fully understood (Aprotosoaie et al. 2005).

The compound, 3-Methylphenol is known to produce musty and phenolic odour which is perceived (Sensory perception) repulsive (Bonvechi, 2005). The Phenol, 2-methoxyphenol is also known to possess aroma-damaging properties and produces undesirable taste. Cocoa beans of high quality should contain very less quantities (Jinap et al. 1998).

Food Safety and Wholesomeness

It is important that raw materials of food and food products, including cocoa beans must be of high safety standard for consumption. Therefore, cocoa beans, butter, chocolates and other cocoa products should not contain impurities that are proven injurious to the health of consumers.

In view of this, cocoa beans and product producers have to ensure that consumers get product which are safe and wholesome and meet both the local and international legislative requirements and standards and enforced prior to and at the point of entry of raw materials into the respective processing countries.

In Ghana the core mandate of ensuring that quality cocoa beans are produced and shipped to other parts of the world is entrusted in the hands of the Quality Control Company (QCC) of the Ghana Cocoa Board (COCOBOD).

Aside quality control measures set up by producing countries for their products to meet the world market standards or specifications, some organizations have been established to set standards for food safety management whose core mandate are to identify and control contaminations at any point of product supply and sells.

Codex Alimentarium Commission is one of such organization set up in 1963 by Food and Agricultural Organizations (FAO) and World Health Organization (WHO) with the mandate of developing global food standards, guidelines and code of practice to ensure that health of consumers is protected and unbiased food trade practices. It also seeks to promote and coordinate quality control programs and activities by member states (htt://www.codexalimentarium.org/).

Another of such organization is the International Organization for Standardizations (ISO), which is a network of standard institutes from 164 countries with a central office in Geneva, Switzerland. ISO 22000 is made up of standards centering on different parts of food safety management.

In Europe, an integrated approach to food safety and proper monitoring was established in EC Regulation 178/2012 and its amendments. This includes the setting up of EFSA (European Food Safety Authority) and also RASFF (Rapid Alert System for Food and Feed), concerned with sharing of information to facilitate restrictions or withdrawal of unsafe food from the market. There is also a guideline published for safe manufacturing practices in the cocoa, chocolate and confectionary industry (CAOBISCO, 2011) and (Syndicate du chocolat. 2012).

The main food safety concern for the cocoa industry includes Allergies, Dioxins and PCBs, Bacterial, Foreign matter, Mineral oil and hydrocarbons, Heavy metals, Pesticide residues, Polycyclic aromatic Hydrocarbons, and Mycotoxins including Ochratoxins A (OTA).

Free Fatty Acid (FFA) Level

Fungal growth in cocoa bean may cause spoilage by changing the composition of its constituent and resulting in increased levels of free fatty acids (FFA) in cocoa butter. High levels of FFA are considered as one of the factors that reduce the commercial value of raw cocoa beans since high levels of FFA above certain values cause off flavours. It influences negatively on the hardness of cocoa butter, affecting its processing quality (crystallization properties). This results in poor quality chocolate (affecting its bloom and tempering and also the flavour). Whole healthy, properly fermented and well dried cocoa beans within reasonale time of drying and proper storage conditions will have a fat composition of FFA less than 1% and not greater than 1.3% (Jonfia-Essien and Navarro, 2010). High levels of FFA mostly arise because of the degradation of triglycerides making up the cocoa butter by the action of the enzyme lipase.

The seed contains lipase which is activated during germination but does not result in high levels of FFA in content in cocoa butter. Microbial lipase mostly produced by moulds because of poor post harvest practices, use of beans from diseased pods, very slow drying after fermentation (clustered beans not properly detached from the placenta) are some causes of increase in FFA content. Other factors like, high levels of broken seeds due to improper pod braking, insect damaged seeds, high moisture content during storing and unfavourable weather conditions (tropical west African weather conditions), will also result in cocoa beans and butter with high FFA content. One of the most common fungal species associated with bean spoilage is *Aspergillus fumigatus*. This fungus is very harmful on the tissues of the cocoa beans and allows the passage of other fungi such as *A. niger, A flavus, Penicillium, Eurotium*, and *Mucor*. However, these fungi do not produce mycotoxins. Other Lipase producing moulds such as *Geotrichum, Cladosporium, Monillia* and some species of yeasts, also causes the breakdown of oil (Lipolysis) increasing the free fatty acid levels in cocoa beans. An acceptable maximum limit of FFA content of 1.75% of oleic acid equivalent was set by 73/241/EEC (EEC, 1973). This standard is also set in the UE (Directive 2000/36/EC) (EU,2000) and in codex standard for cocoa butter (86-1981, Rev.1-2001) (Codex Alimentarius 2001). Cocoa butter which meets this standard or better, 1% or below is a good indication of cocoa beans prepared and stored under acceptable standard at the country of origin.

CHAPTER THREE

MATERIALS AND GENERAL METHODS

3.1 Study Site

Cocoa producing districts in the Central Region of Ghana was used for this study. The Central Region of Ghana is demarcated into seven (7) cocoa growing districts namely Cape Coast, Assin Breku, Assin Foso, Agona Swedru, Breman Asikuman, Twifo Nyinase and Twifo Praso.

Four out of the Seven Cocoa producing districts of the Central Region of Ghana, i.e., Agona Swedru, Breman Asikuma, Assin Breku and Assin Fosu were used for these studies.

3.2 Materials

Dried cocoa beans (Cocoa) were used for this study

3.3 Sampling

Two hundred grams (200g) each of dried cocoa beans samples were collected randomly from five (5) Societies (Farm Gates) each from five licensed buying companies in each of the four selected cocoa districts of the Central Region.

These samples were bulked in to twenty based on LBC's and their moisture content, fungal loads, and ochratoxin A levels were determined.

One hundred grams was used for Mycotoxin analysis at the Quality Control Company (QCC Limited), whilst the remaining 100grams was used for fungal isolation, identification and enumeration and moisture content analysis at the Department of Plant and Environmental Biology of the University of Ghana, Legon. Samples were stored in the cold room at -10ºC until ready for analysis.

A total of hundred samples (n=100) were collected for analysis. The samples were extracted and scrutinized in triplicate.

3.4. Chemicals and Reagents for OTA analysis

HPLC grade acetonitrile, Ethyl acetate, methanol, phosphate buffered saline (PBS), and glacial acetic acid were purchased from Merck (Germany). Double distilled water was used for the preparation of solutions. Analytical standards of Ochratoxin A (OTA) standard concentration (stock) 10.05 ug/mL were purchased from Biopure Company and stored at 2ºC. The laboratory glassware used were soaked and kept at 10% (v/v) nitric acid (Merck, Germany) overnight and rinsed several times with ultrapure water before use.

3.5 Methods

3.5.1 Moisture Content Determination

A modified International Organization for Standards (ISO) method was employed for moisture content determination (according to Hamid and Lopez (2000).

About 10 g of cocoa beans samples was grind and placed in a pre-weighed glass Petri plates (W1) with a lid and re-weighed (with sample) to the nearest mg (W2).

Petri plates with contents were put in an oven at 103 ± 2°C for 16 hours. The Petri plates with contents were transferred from the oven to a balance room into a desiccator, allowed to cool and were reweighed (W3). The results were calculated, and the mean values recorded.

Calculations

Moisture of Cocoa samples were calculated as follows using oven drying procedures:

%Moisture (wt/wt) = wtH_2O in sample /wt of wet sample× 100 [2]

%Moisture (wt/wt) = wt of wet sample − wt of dry sample / wt of wet sample × 100 [3]

%Moisture (wt/wt), MC= (W2-W1) - (W3-W1)/ (W2-W1) × 100

3.5.2 Isolation and Enumeration of fungi by pour plate method

Ten grams (10g) of cocoa bean sample was weighed into a conical flask containing 0.6 percent of sodium hypochlorite and then shaken for two minutes on an orbital shaker. The samples were washed twice in sterile distilled water and homogenize for 3 min in a laboratory homogenizer. One gram (1g) each of the homogenized samples were weighed into a McCartney tube containing nine 9.0 mL of sterilized 0.1 % peptone water. The contents were allowed to stand for 30 minutes and were shaken to obtain a uniform homogenate. Serial dilution was done in sterilized 0.1% peptone water to a concentration of 10^{-2} (0.01g/ml).

One millilitre (1mL) aliquot each of uniformly mixed dilutions were transferred into six Petri plates (three plates for each media). Twenty millilitre of each molten media, Potato dextrose Agar (PDA) and Malt Extract Agar (MEA) were added to each plate, there were three replicates of each.

The mixtures were shaken and allowed to set, tuned upside down and incubated aseptically at 25°C for 3-7 days. Fungal colonies obtained from the plates were counted and recorded and the various colonies were subcultured onto PDA and MEA plates and incubated at 25°C for 7 days. Pure cultures obtained were stored at 5°C awaiting to identification.

3.5.3 Fungal Identification

Detailed characterization and identification of isolates were conducted at the Department of Plant and Environmental Biology, University of Ghana Legon. The identification was based on the cultural and morphological characteristics described by Pitt and Hocking (1997), Pitt (2000), Samson et al. (2002), Samson et al. (2004), Frisvad et al. (2004). Pitt and Hocking (2009).

Leica D500 microscope connected to Leica Application Suite (LASEZ) version 2.1 (2012) was used in the observation of cultural and morphological characteristics and taking of photographs of the samples.

3.5.4 Mycotoxin Extraction and Quantification

Sample Preparation for OTA Analysis

Twenty samples were prepared based on the number of licensed Buying Companies (LBC's) sampled in the four cocoa districts demarcated for the studies. Twenty grams of each sample collected from the five societies from each LBC was bulked, (100g Sample) for OTA analysis. Hundred-gram (100g) samples from each licensed buying company (LBC) was milled with a laboratory blender and kept in Erlenmeyer flask fr future analysis.

Sample Clean up and Extraction of OTA from ground cocoa beans

Ochratoxin A (OTA) extraction was done using liquid/liquid separation using a separating funnel based on modified standard protocol discribe by Tabata et al. (2008).

Ten grams (10g) of the milled sample was weighed into 250mL centrifuge tube. The sample was spiked with 0.5mL of 50ppb of OTA. Ten milliliters of 20%NaCl and 1mL of H_3PO_4 were added to the sample. A hundred millilitre of ethyl acetate was added and the content was homogenized (IKA^{R}T25 digital ultra-Turrax Homogenizer) for 3mins at 17000rpm.

The mixture was centrifuged (Centurion Scientific K3 Series) for 5mins at 2500rpm and 20mL of the upper layer was transferred into a separation funnel.

Twenty millilitres of 20% NaCl was added and the mixture shaken vigorously for about 1min and the lower layer was discarded.

Forty millilitres (40mL) of 2% $NaHCO_3$ was added to the upper layer in the separation funnel and shaken vigorously for about 1min and the lower layer was drained into a conical flask A. twenty millilitres (20mL) of 2% $NaHCO_3$ was again added to the upper layer in the separating funnel and shaken vigorously for 1min. The lower layer was drained into conical flask A and the upper layer discarded.

Thirty millilitres (30 mL) of ethyl acetate was added to the content in conical flask A and was followed by 8mL of 4M HCl. The mixture was transferred into the separation funnel and shaken vigorously by hand for 1min.

The lower layer was drained into conical flask B and the upper layer into conical flask C. The content in conical flask B was transferred into the conical flask and 30mL of ethyl acetate was again added and shaken for a min.

The lower layer was discarded, and the upper layer added to conical flask C. 10gm of $NaSO_4$ was added to the mixture in conical flask C, shaken and filtered using a Whatman filter paper and evaporated to dryness using Rotary Evaporator (Heidolph Rotary Evaporator).

The residue was dissolved with 5mL of the mobile phase i.e., H_2O/ACN/CH_3COOH (51:47:2 v/v/v) using a water bath sonicator (Bandelin Sonorex digitec).

The mixture was filtered using Millex PTFE 0.45mm (Millipore, USA) into glass amber vial and analyzed on HPLC-FLD

HPLC Detection and Quantification

Ochratoxin A was detected and quantified using HPLC-FLD.

HPLC Parameter.

Shimadzu HPLC system (Kyoto, Japan), autosampler (SIL-10AF), pump (LC-20AT), column oven (CTO – 10ASvp) temperature set at 40°C and a flow rate of 1mL/min was used with a fluorescence detection (RF – 10Axl)) set at 333nm excitation and 460nm emission (Plate 1).

A reverse phase Shimadzu Shim-pack VP-ODS (4.6 ×250 mm, 5.0 μm) column was employed. The mobile phase used for OTA analysis was water: acetonitrile: acetic acid (51: 47: 2).

OTA standard (1ppb, 2ppb, 5ppb, 10ppb and 20 ppb) was used for construction of a five-point calibration curve of peak areas versus concentration (ppb). The injection volume was 20 μL for both standard solution and sample extracts.

HPLC was performed according to the general criteria for good manufacturing practices (GMPs). Triplicate analysis was conducted on each sample while solvent blanks was intermittently injected into columns (column washing) to eliminate peak splitting or tailing during the analysis (Irakli et al. 2017).

Plate 1: Shimadzu HPLC-FLD system (Kyoto, Japan), Autosampler (SIL-10AF) at Quality Control Company (QCC) of COCOBOD, Tema Laboratory (x1/15).

Statistical Analysis.

All the measurements of cocoa samples were repeated three times and the data was statistically analyzed by using the software IBM SPSS Statistics, version 20. Regression analyses were applied to find out the coefficient of determination ($R2$). The calibration curves used for quantification were calculated by least-squares method. Samples with a concentration of Ochratoxin A higher than limit of detection were considered positives, while the samples with concentrations lower than limit of detection were considered negative.

Method Validation.

The validation of the partially modified analytical method for the analysis of Ochratoxin A in Cocoa beans was performed as described in document (European Commission, 2015. No. SANTE/11945/2015.)

The method was tested for validation performance parameters and criteria in terms of limit of detection (LOD), limit of quantification (LOQ), linearity, precision (repeatability and reproducibility) and accuracy (trueness).

The LOD was found to be 1ng/Kg and resulted by considering a signal-to-noise ratio of 3 (three) with reference to the background noise obtained from the blank cocoa beans sample.

The LOQ of the method was 2ng/kg with a signal-to-noise ratio greater than 10 (S/N >10).

The linear range was from 1.0 – 20 ng/ml and a correlation coefficient (R^2) of 0.9997 was obtained for the entire range of studied concentrations.

Repeatability (RSD_r) and reproducibility (RSD_R) expressed as RSD were 5.5% and 9.8% respectively.

Spiking cocoa beans at different levels to establish the validity and the reliability of the analytical method used was carried out as recovery assay (accuracy). Cocoa beans were spiked with OTA at levels of 2.5ng/kg and 5ng/kg in ten (10) replicates.

The samples were extracted, cleaned-up and analysed following the method as described. The mean recoveries of OTA spiked at these levels were found to be consistent and more than 90% recovery (Table 1).

Table 1. Mean recoveries of OTA spiked at different levels

Spiking Level (ppb)	(n[a]=10) Recovery (%)	RSD (%)
2.50	93.00	5.50
5.00	110.00	8.70

a = number of replicates
RSD = Relative Standard Deviation

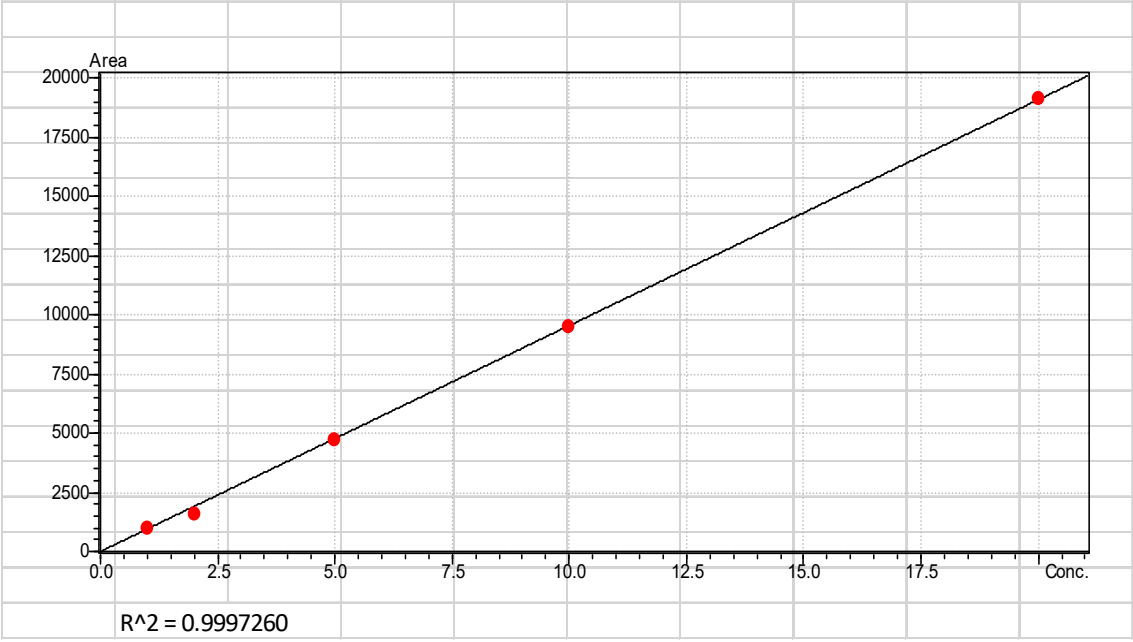

Figure 1. Calibration curve for OTA standards

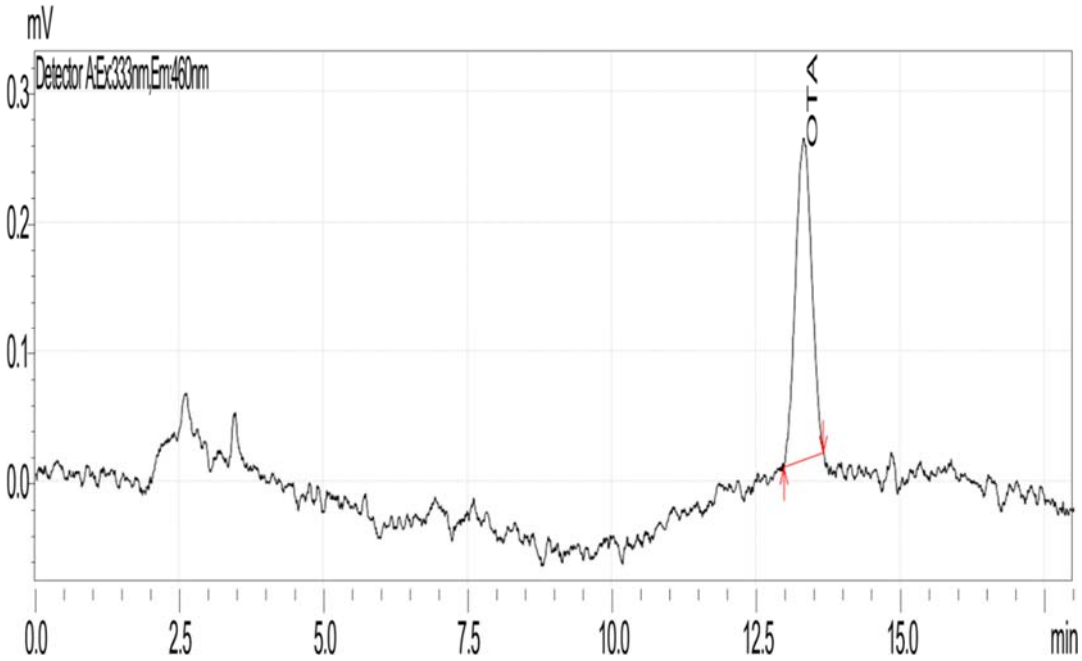

Figure 2: HPLC-FLD Chromatogram of OTA Standard 5ppb

CHAPTER FOUR

RESULTS

4.1 EXPERIMENT 1

Fungal profile of dried cocoa beans samples obtained from five licenced buying Companies (LBCs) in the four cocoa districts

The districts and the LBCs that cocoa beans were sampled were Agona Swedru (Transroyal, Agroecom, Olam, Nyonkopa and Royal Commodities), Bremen Asikuma (Agroecom, Olam, Nyonkopa, PBC, and Royal commodities), Assin Bereku (Kuopa Kokoo, Nyonkopa, Atlas, Olam, Agroecom) and Assin Fosu (Adinkanfo, Transroyal, Fedco, Unicom and Olam).

Fifteen (15) fungal species belonging to 11 genera (*Aspergillus, Cladosporium, Absidia, Alternaria, Byssochlamys, Eurotium*, *Mucor, Penicillium, Rhizopus Syncephalastrum* and *Talaromyces*) were resident on the dried cocoa beans (Table 2, Plates 1 -15). Of the 15 fungal genera encountered, 14 were isolated on PDA, whilst 13 genera were were isolated on MEA media (Table 3).

On both media used for fungal growth, the occurrence of *Aspergillus* species predominated (26.6%), followed by *Cladosdosporium* (13.3%) and the remaining 9 genera were represented by 6.6% each.

Table 2: Fungi Isolated from Dried Cocoa Beans obtained from the various Licensed Buying Companies in the four districts in the Central Region on PDA and or MEA media

Fungi Isolates	Media	
	PDA	MEA
Absidia corymbifera (Cohn) Sacc. & Trotter	+	+
Alternaria alternata (Fr.) Keissler.	+	+
Aspergillus flavus Link.	+	+
Aspergillus fumigatus Fres.	+	+
Aspergillus niger van Tieghem	+	+
Aspergillus parasiticus Speare.	+	+
Byssochlamys nivea westling		+
Clasdosporium macrocarpum Preuss	+	+
Clasdosporium sphaerospermum Penzig	+	+
Eurotium herbariorum (Wiggers) Links	+	+
Mucor racemosus Fres.	+	
Penicillium citrinum Thom	+	+
Rhizopus stolonifer (Ehrenb.) Lind.	+	+
Syncephalastrum racemosum Cohn.	+	+
Talaromyces flavus (Klocker) Stolk &samson.		+

Plates of Photographs showing some of the fungal species isolated from dried cocoa beans obtained from the various Licensed Buying Companies in the four districts in the Central Region.

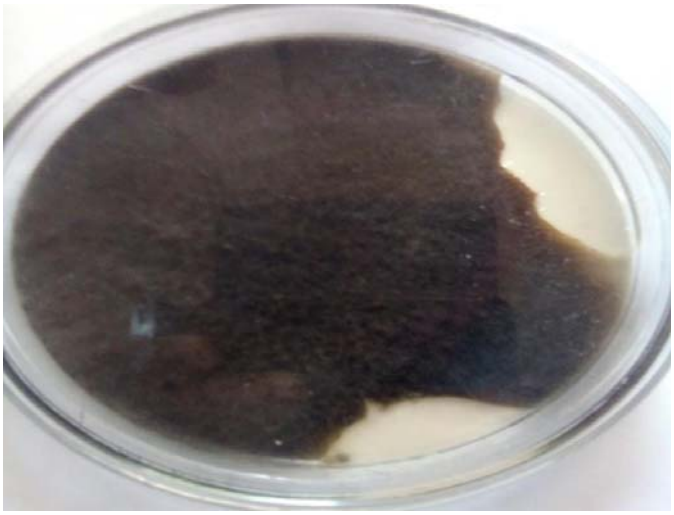

Plate 2(a) Colony of *Alternaria alternata* (Fr.) Keissler, growing on Malt Extract Agar after a week of incubation at 25^{o}C. Black to olivaceous-black and suede-like to floccose.

Plate 2(b): *Alternaria alternata* (Fr.) Keissler. Microscopically branched acropetal chains (blastocatenate) of multicellular beaked conidia (X100 magnification).

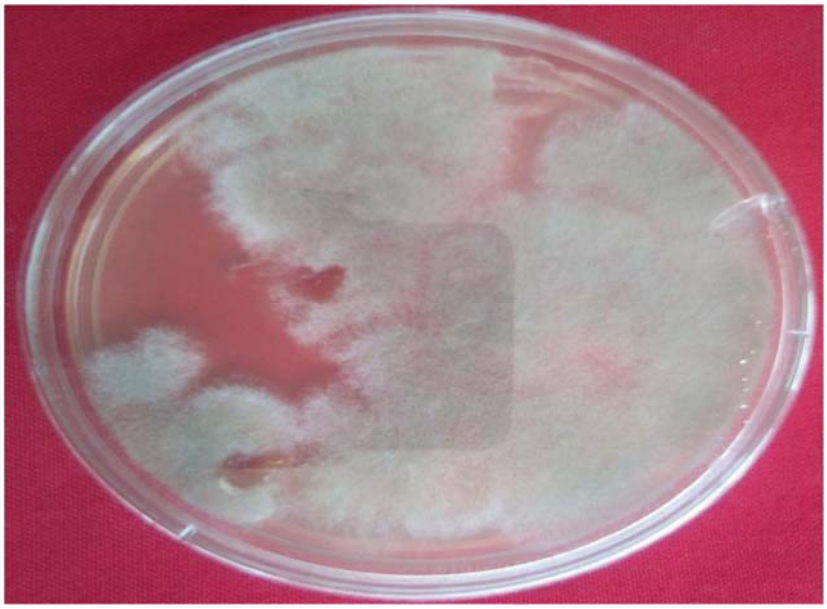

Plate 3(a) Colony of *Absidia corymbifera* (Cohn) Sacc. & Trotter, growing on Malt Extract Agar after 7 days of incubation at 25^{o}C (Floccose, light grayish, growing rapidly).

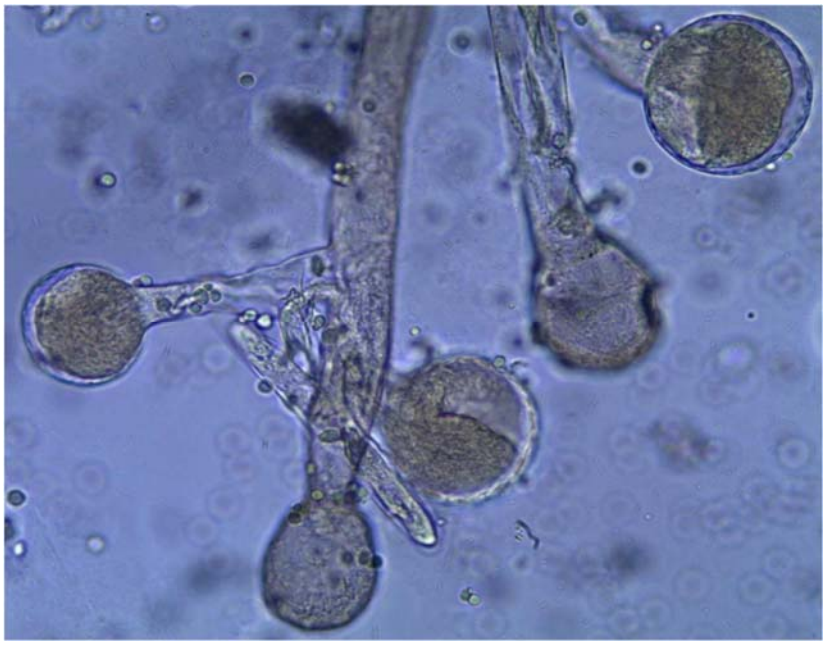

Plate 3(b): *Absidia corymbifera* (Cohn) Sacc. & Trotter Sporangiospore with terminal sporangium (x100 magnification)

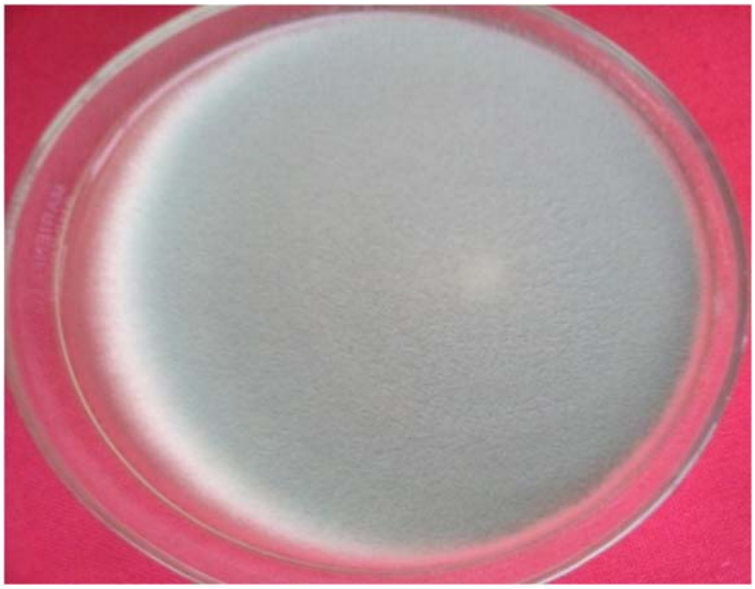

Plate 4(a) Colony of *Aspergillus fumigatus* Fres., growing on Malt Extract Agar after a week of incubation at 25ºC. A dense felt of dark green conidiosphore intermixed with aerial hyphae bearing conidiophore.

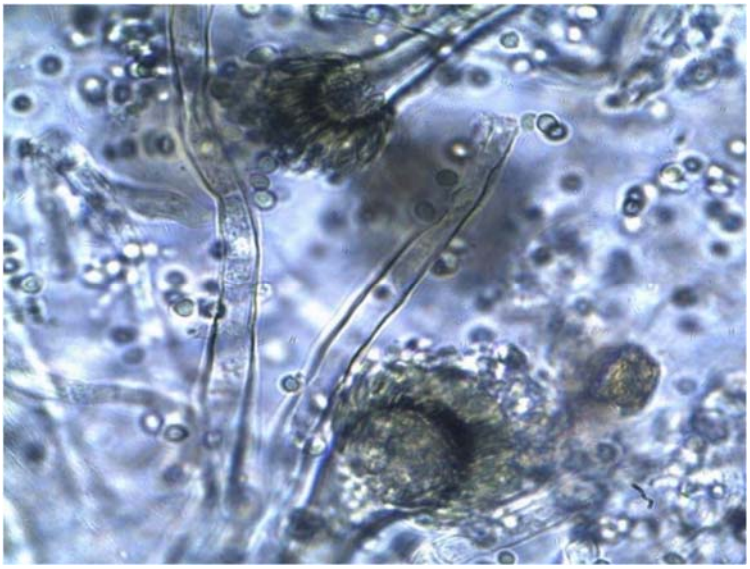

Plate 4(b): High power magnification (x100) of *Aspergillus fumigatus* Fres. Conidium bearing phialides directly on vesicles and chains of conidia.

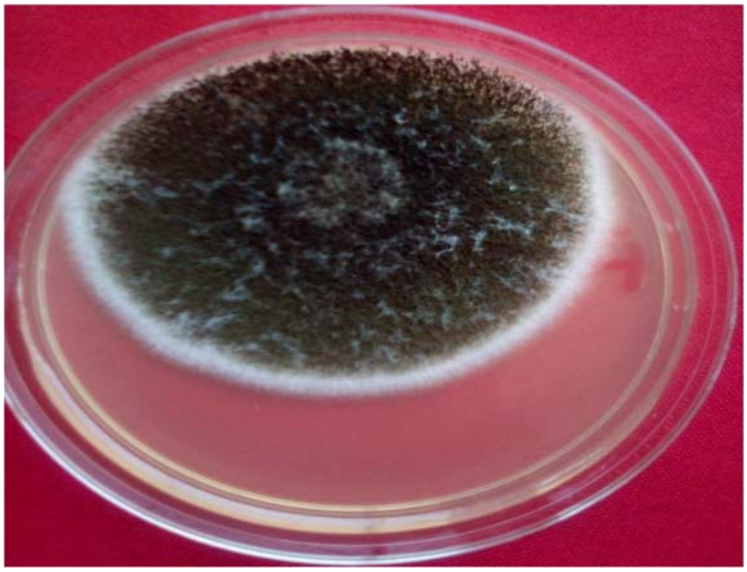

Plate 5(a) Colony morphology of *Aspergillum niger* van Tieghem, growing on Malt Extract Agar after 7 days of incubation at 25^{o}C. Compact white basal felt with a dense layer of black conidiophore.

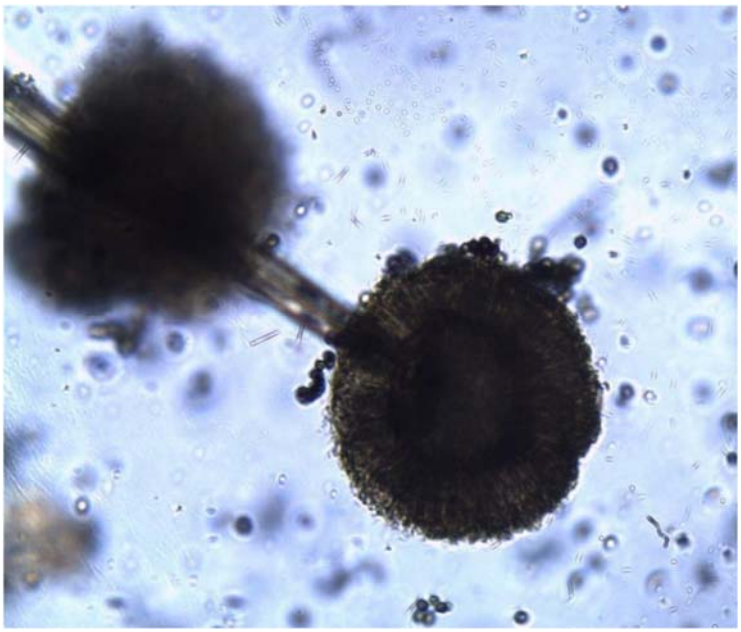

Plate 5(b): High power magnification (x100) of Globose conidial heads of *Aspergillus niger* van Tieghem with rosettely-arranged conidiosporangia.

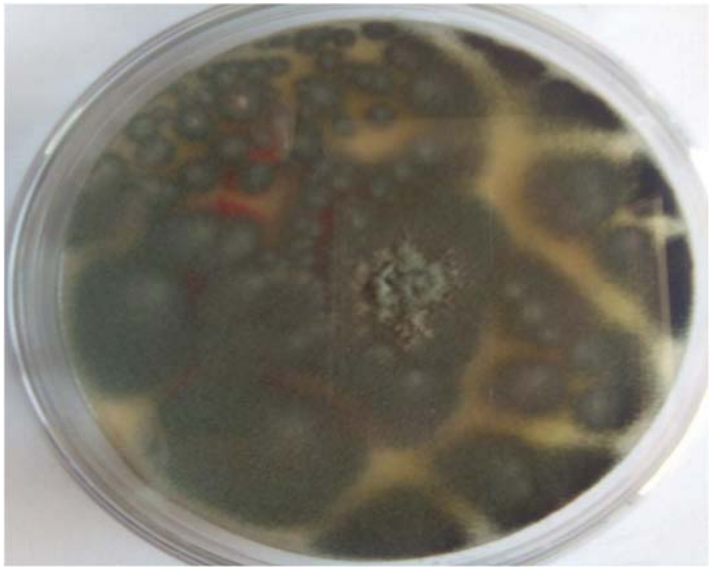

Plate 6(a) Colonies of *Aspergillus parasiticus* Speare, growing on Malt Extract Agar after 7 days of incubation at 25^{o}C, consisting of a dense felt of green conidiophose.

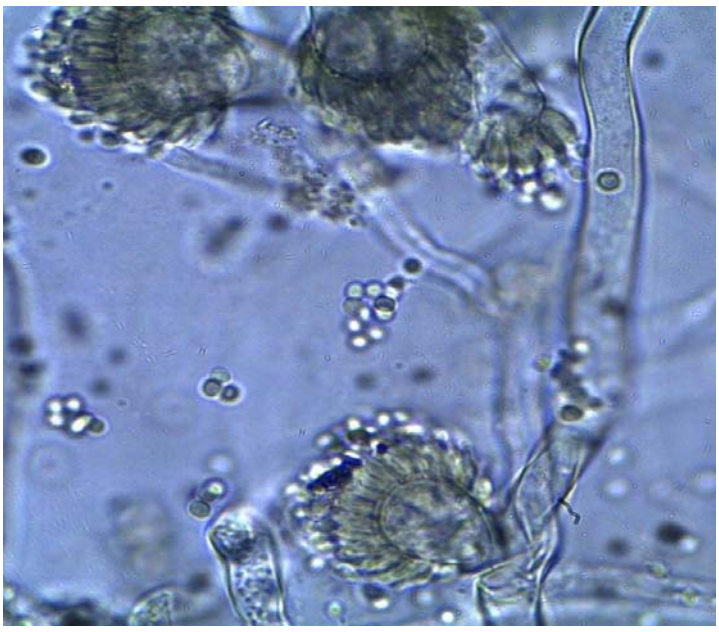

Plate 6(b): *Aspergillus parasiticus* Speare. Phialides directly borne on subglobose vesicles, conidial heads and conidia (x100 magnification)

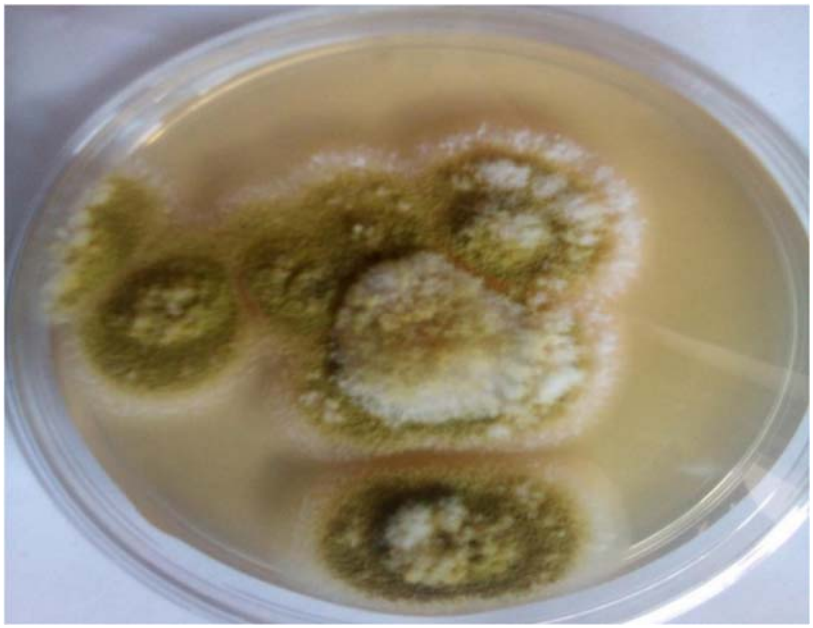

Plate 7(a) Colonies of *Aspergillus flavus* Link, growing on Malt Extract Agar after a week of incubation at 25^{o}C, consisting of dense felt of yellow-green conidiophores.

Plate 7(b): High power magnification (x100) of *Aspergillus flavus* Link. Conidial head and tip of conidiophores. Vesicle globose to subglobose with phialides directly borne on the vesicle.

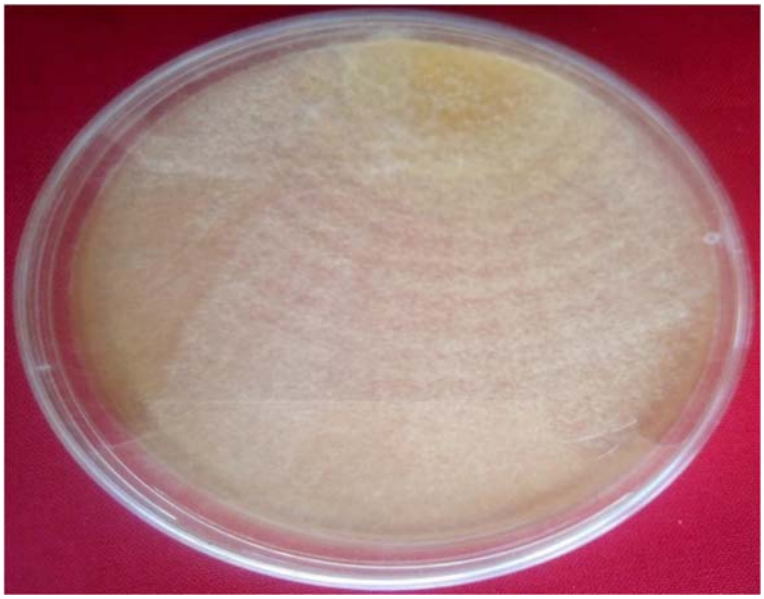

Plate 8(a) Colony of *Byssochlamys nivea* Westling, growing on Malt Extract Agar after a week of incubation at 25ºC. White ascomata in the basal felt obscured by floccose to funiculose overgrowth spreading broadly.

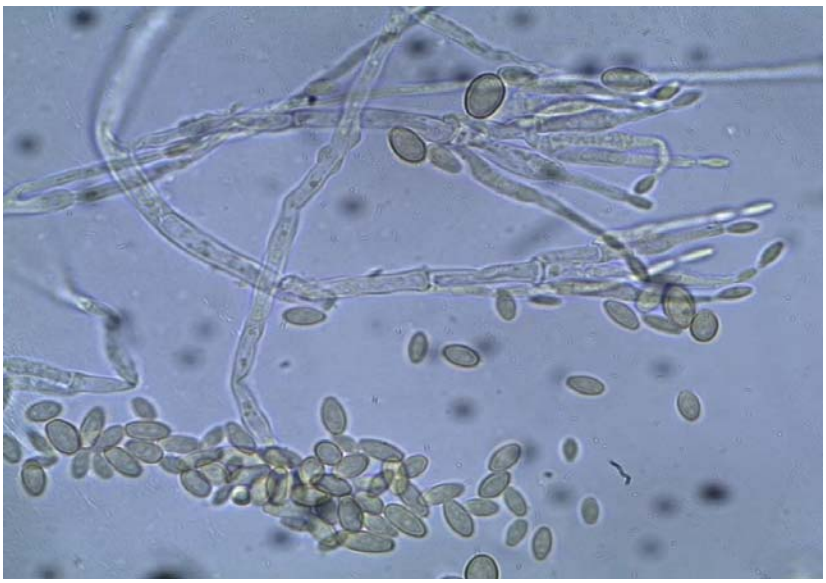

Plate 8(b): *Byssochlamys nivea* Westling. Conidial structure and ascospores (x100 magnification)

Plate 9(a) Colony of *Clasdosporium macrocarpum* Preuss, growing on Malt Extract Agar after a week of incubation at 25^{o}C. Velvety and covered with greyish aerial mycelium.

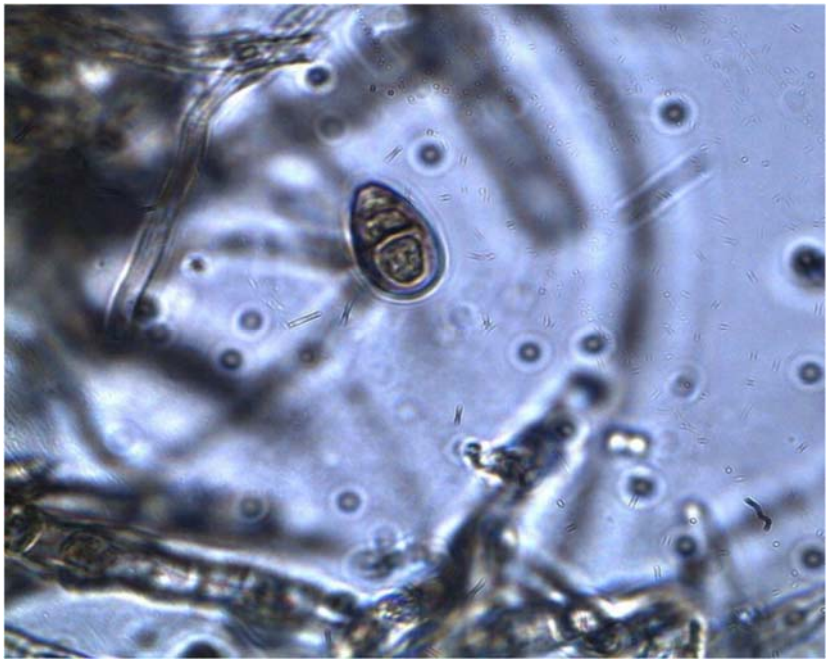

Plate 9(b): Conidiophores and conidia of *Cladosporium macrocarpum* Preuss. (X100 magnification)

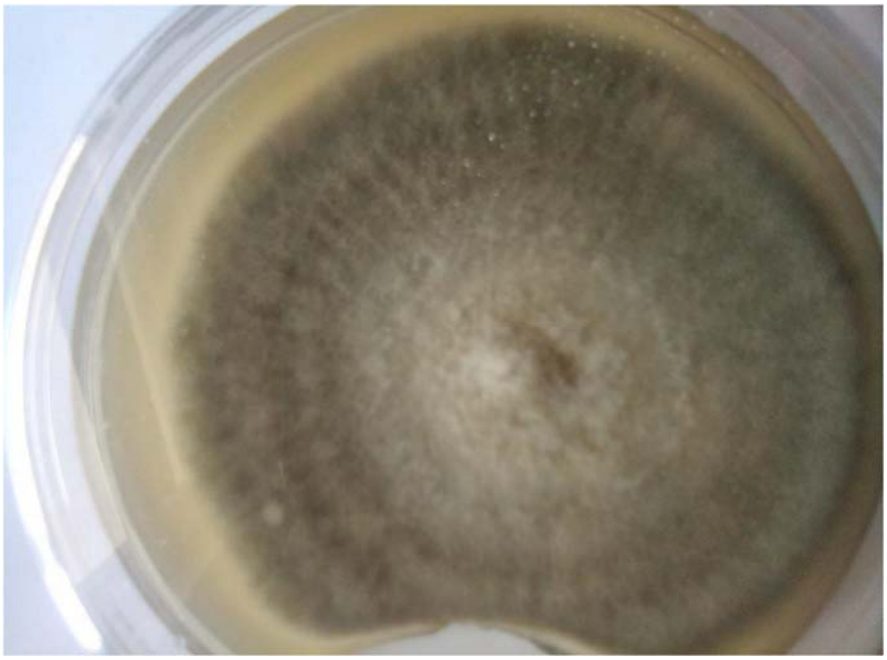

Plate 10(a) Colony of *Cladosporium sphaerospermum* Penzig, growing on Malt Extract Agar after a week of incubation at 25^{o}C. Velvety olive-green to olivaceous-brown.

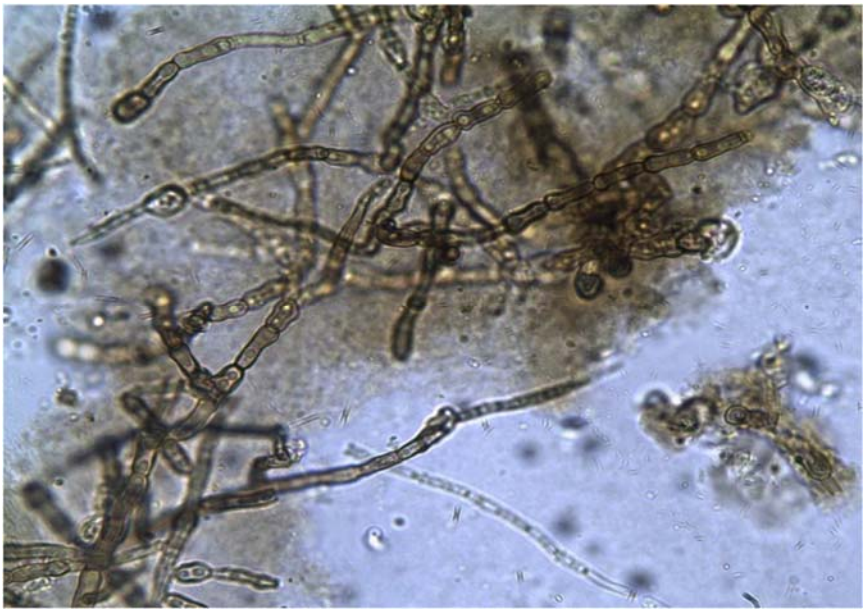

Plate 10(b): High power magnification (x100) of Acropetal conidia and Conidiophores of *Cladosporium sphaerospermum* Penzig.

Plate 11 (a): Colony of *Eurotium herbariorum* (Wiggers) Link, growing on Malt Extract Agar after a week of incubation at 25^{o}C. Spreading broadly and irregularly, and characterized by broad zones of dull green to gray-green.

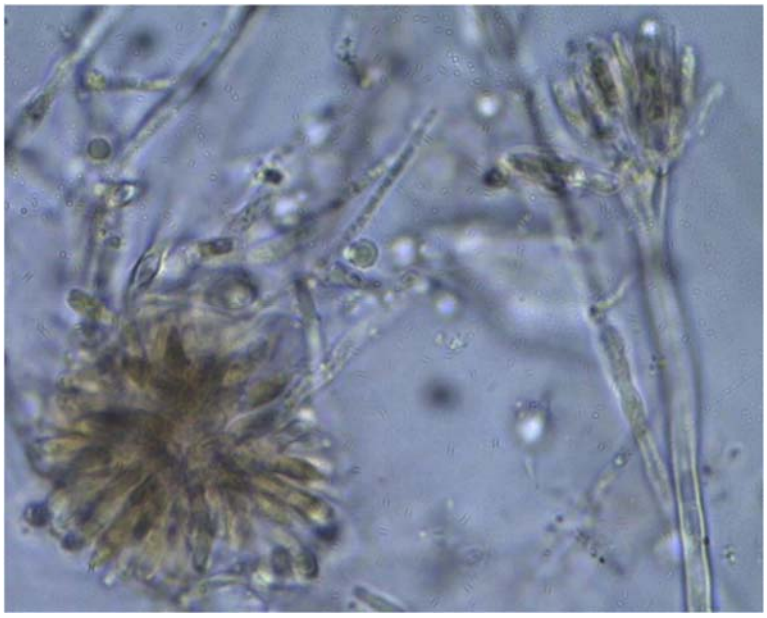

Plate 11 (b): High power magnification (x100) of *Eurotium herbariorum* (Wiggers) Link. Conidial structures showing conidiophores and conidia.

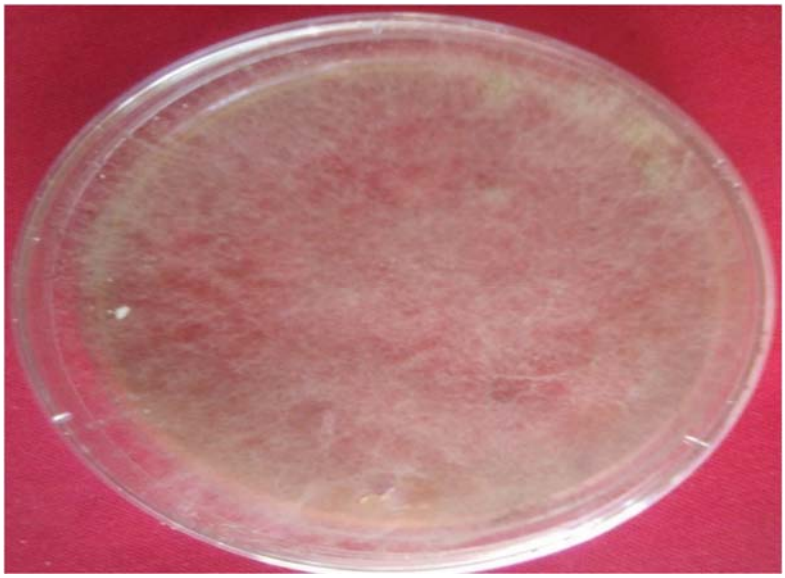

Plate 12(a) Colony of *Mucor racemosum* Fres, growing on Malt Extract Agar after 7 days of incubation at 25^{o}C. Initially white and becoming brownish-grey after one week.

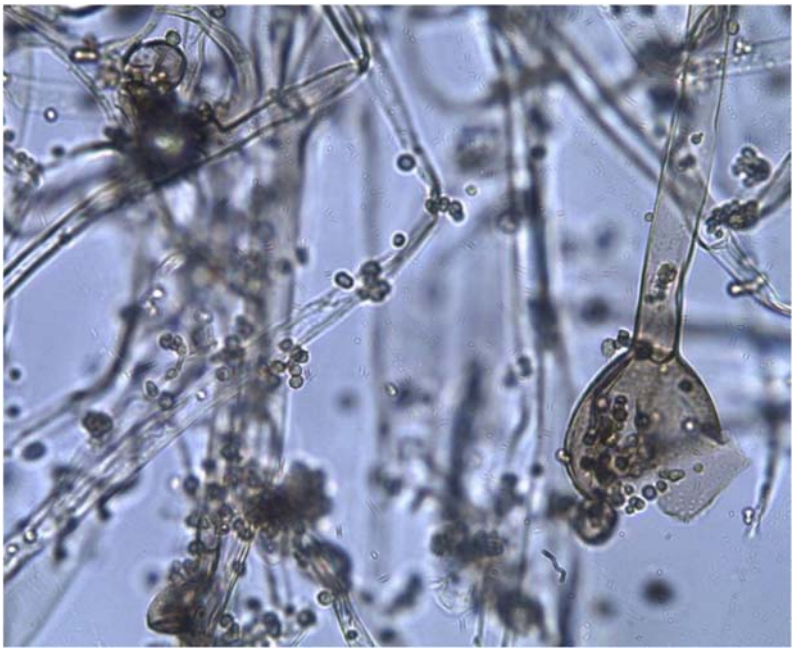

Plate 12(b): High power magnification (x100) of *Mucor racemosum* Fres. Sporangiophore with columella and hyphae, and some sporandiospores.

Plate 13(a) Colony of *Rhizopus stolonifer* (Ehrenb.) Lind, growing on Malt Extract Agar after 7 days of incubation at 25°C. Whitish with brownish sporangiophores and brown-black sporangia. Colony becoming gray-brown

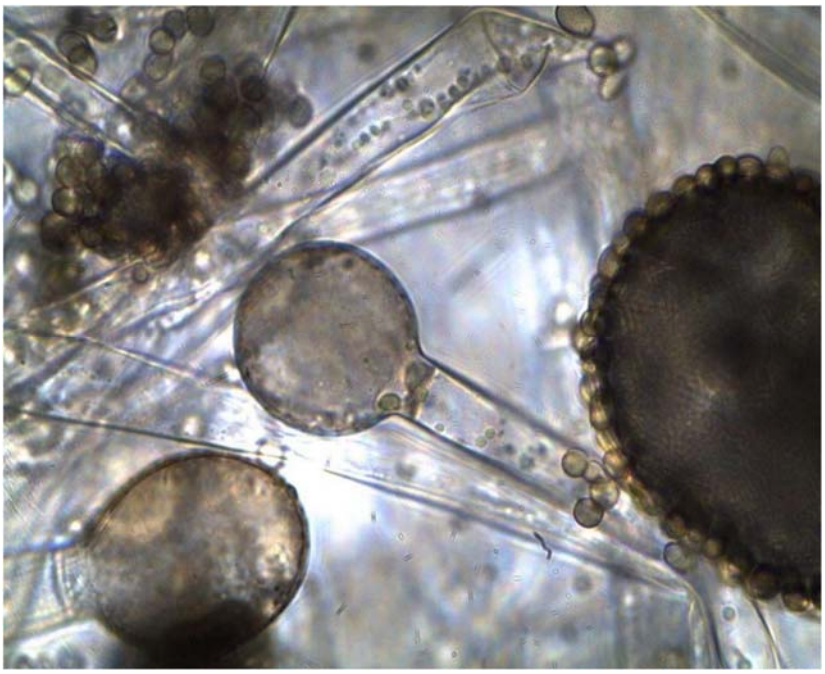

Plate 13(b): High power magnification (x100) of *Rhizopus stolonifer* (Ehrenb.) Lind. Sporangiophore with columella and sporangiospores

Plate 14 (a): Colony of *Syncephalastrum racemosum* Cohn, growing on Malt Extract Agar after a week of incubation at 25^{o}C, white, growing rapidly and covering the petri-dish.

Plate 14b*:* High power magnification (x100) of *Syncephalastrum racemosum* Cohn *Sporangiophore* bearing vesicles forming merosporangia on its surface

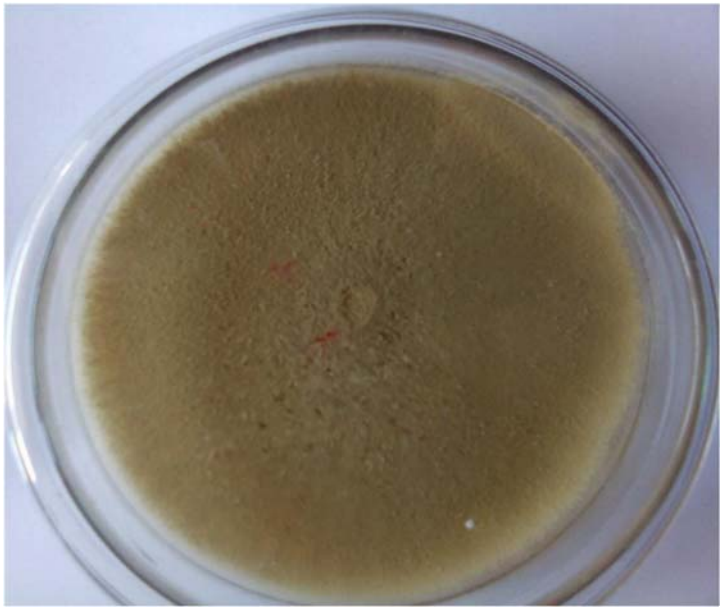

Plate 15(a): Colony of *Talaromyces flavus* (Klocker) Stolk & Samson, growing on Malt Extract Agar after a week of incubation at 25^{o}C, consisting of a basal felt made of numerous ascomata forming a continuous thick yellow layer.

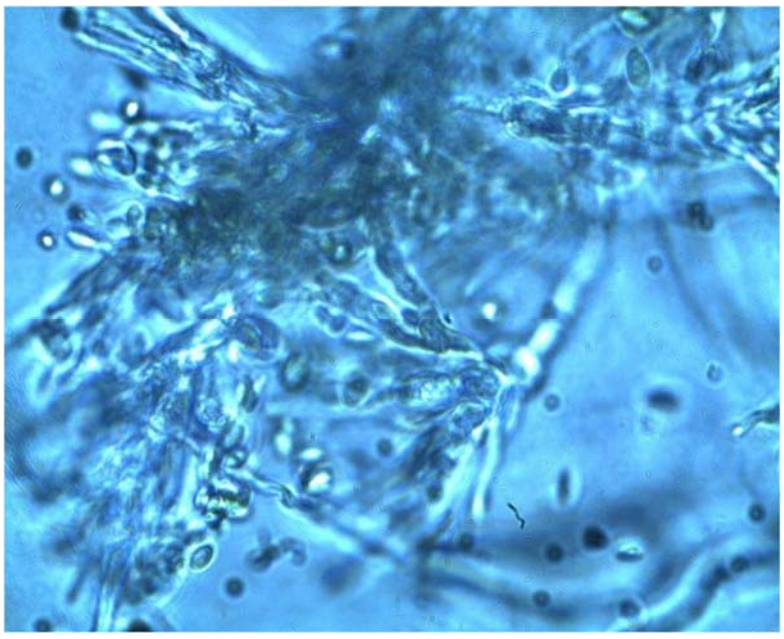

Plate 15(b): Microscopic Photograph (x100 magnification) of Conidiophores and conidia of *Talaromyces flavus* (Klocker) Stolk & Samson.

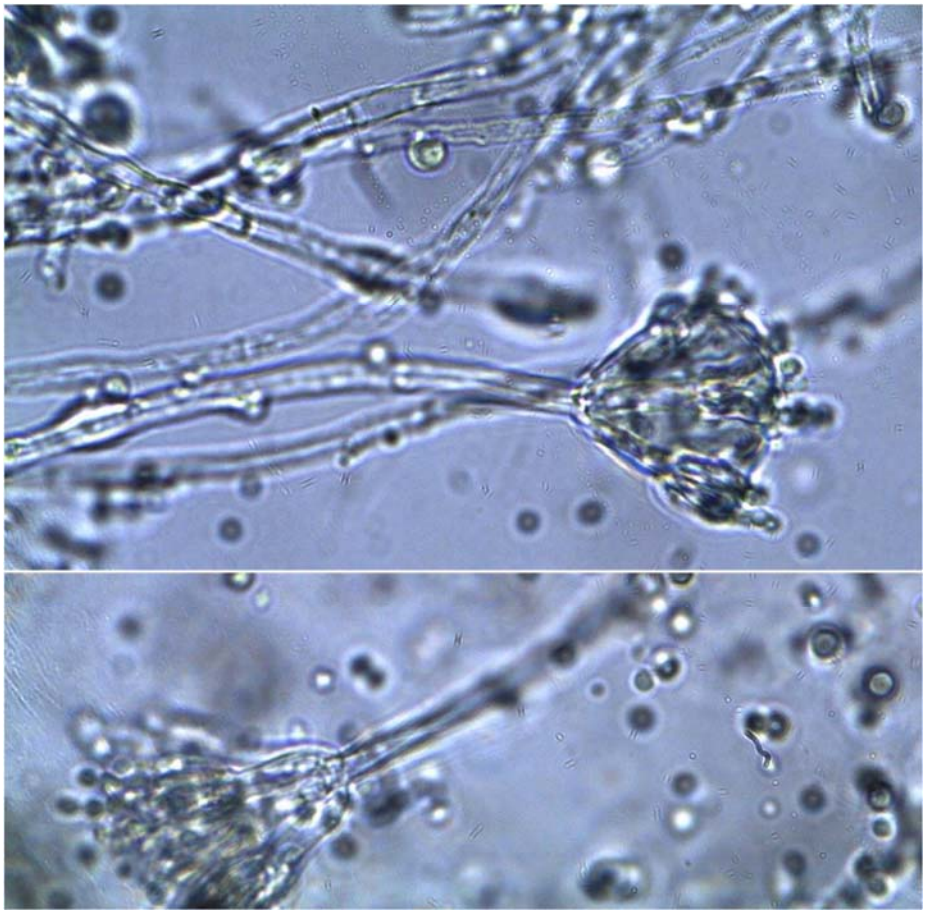

Plate 16: High power magnification (x100) of *Penicillium citrinum* Thom. Conidiophores biverticillate (One stage branched).

4.2 EXPERIMENT 2

Moisture content of cocoa beans obtained from various LBCs in the Agona Swedru, Bremen Asikuman, Assin Bereku and Assin Fosu districts

Table 3 summarises the moisture content of cocoa beans obtained from the various LBCs in the Agona Swedru, Bremen Asikuman, Assin Bereku and Assin Fosu Districts. Beans from Unicom in the Assin Fosu district and Nyonkopa in the Assin Bereku district recorded the lowest (7.05%) and highest (9.86%) moisture contents respectively (Table 3). There were significant differences ($P \leq 0.05$) among the moisture contents recorded for the cocoa beans obtained from the various LBCs (Table 3). The moisture content of cocoa beans obtained from Unicom (7.05%) in the Assin Fosu district, with the exception of those of Fedco (7.37%) and Olam (7.60%) also in the same district was significantly lower ($P \leq 0.05$) than those recorded for the other LBCs (Table 4). The moisture content recorded for cocoa beans obtained from Fedco (7.37%), Unicon (7.05%), Olam (7.60%) and Transroyal (7.89%) in the Assin Fosu district was significantly lower ($P \leq 0.05$). Also, those of Royal Commodities (8.13%) in the Bremen Asikuman district and Royal Commodities (8.18%) in the Agona Swedru district was significantly lower ($P \leq 0.05$) than those of the other LBCs (Table 4). The beans obtained from Olam in the Assin Fosu district also recorded a significantly lower ($P \leq 0.05$) moisture content (7.60%) than those of Olam (8.51%), Atlas (8.71%), Kuapa Kokoo (8.89%) and Nyonkopa (9.86%) in the Assin Bereku district, Transroyal (8.50%), Nyonkopa (8.77%) and Agroecom (8.80%) in the Agona Swedru district, Adikanfo (8.51%) and Produced and Buying Company (8.60%) in the Assin Fosu and Bremen Asikuman districts respectively (Table 3).

The cocoa beans obtained from Nyonkopa (9.86%) in the Assin Bereku district recorded a significantly higher ($P \leq 0.05$) moisture content than those of the other LBCs (Table 3).

Table 3: Moisture content of cocoa beans obtained from various LBC

District	LBC	Moisture content (%)
Swedru	Transroyal	8.50 ± 0.27^{de}
	Agroecom	8.80 ± 0.27^{e}
	Olam	8.24 ± 0.30^{cde}
	Nyonkopa	8.77 ± 0.25^{e}
	Royal Commodities	8.18 ± 0.30^{bcde}
Bremen Asikuman	Produced and Buying Company	8.60 ± 0.30^{de}
	Royal Commodities	8.13 ± 0.22^{bcde}
	Agroecom	8.30 ± 0.30^{cde}
	Olam	8.32 ± 0.25^{cde}
	Nyonkopa	8.22 ± 0.14^{cde}
Assin Breku	Kuapa Kokoo	8.89 ± 0.28^{e}
	Nyonkopa	9.86 ± 0.27^{f}
	Atlas	8.71 ± 0.45^{de}
	Olam	8.51 ± 0.19^{de}
	Agroecom	8.31 ± 0.19^{cde}
Assin Fosu	Adikanfo	8.51 ± 0.26^{de}
	Transroyal	7.89 ± 0.19^{bcd}
	Fedco	7.37 ± 0.20^{ab}
	Unicom	7.05 ± 0.23^{a}
	Olam	7.60 ± 0.22^{abc}
	F (Pr)	**<0.001**

Mean ± standard errors in the same column followed by different letter(s) are significantly different as determined by T's multiple-range test.

4.3 EXPERIMENT 3

Percentage Occurrence of Fungi in Cocoa Samples on PDA from cocoa beans obtained from five Licensed Buying Companies (LBCs) in the Agona Swedru District

Five fungal species viz *Aspergillus flavus, A. fumigatus, A. niger, Cladosporium macrocarpum* and *Rhizopus stolonifer* were resident on dried cocoa beans obtained from the five licensed buying companies (Transroyal, Agroecom, Olam, Nyonkopa and Royal Commodities) in the Agona Swedru District were (Table 4). The incidence of *A. niger* recorded for cocoa beans obtained from the licensed buying companies were Olam

(4.00%), Nyonkopa (5.00%) and Royal Commodities (20.00%). But for Tranroyal and Agroecom (Table 4).

In the Agona Swedru District, the LBC's Transroyal, Agroecom, Olam and Nyonkopa did not record *Cladosporium macrocarpum*, except Royal Commodities, which recorded *C. macrocarpum* incidence of 20.00% (Table 4). *Rhizopus stolonifer* was not recorded on the cocoa beans obtained from Transroyal, Agroecom, and Royal Commodities but on those obtained from Nyonkopa (22.50%) and Olam (42.00%). On the PDA medium, with the exception of the cocoa beans obtained from Royal Commodities, *A. flavus* incidence was recorded for the cocoa beans obtained from Olam (20.00%) and Nyonkopa (40.00%), Transroyal (40.25%) and Agroecom (54.47%) (Table 4). *Aspergillus fumigatus* incidences were recorded for the cocoa beans obtained from Nyonkopa (12.50%), Olam (14.00%), Transroyal (19.75%), Agroecom (25.33%) and Royal Commodities (40.00%) (Table4). The highest fungal incidence (54.67%) was recorded for *A. niger* on cocoa beans obtained from Agroecom (Table 4). There was no significant difference ($P \leq 0.05$) between the incidence of *A. flavus* recorded for Agroecom (54.67%) and the various fungal species recorded for Transroyal, Agroecom, Olam, Nyonkopa and Royal commodities (Table 4).

Table 4: Incidence of fungal species isolated on PDA from cocoa beans obtained from various Licensed Buying Companies in the Agona Swedru District

Licensed Buying Company	Fungi Isolated	% occurrence
Transroyal	*A. flavus*	40.25[ab]
	A. fumigatus	19.75[ab]
Agroecom	*A. flavus*	54.67[b]
	A. fumigatus	25.33[ab]
Olam	*A. flavus*	20.00[ab]
	A. fumigatus	14.00[ab]
	A. niger	4.00[ab]
	Rhizopus stolonifer	42.00[ab]
Nyonkopa	*A. flavus*	40.00[ab]
	A. fumigatus	12.50[ab]
	A. niger	5.00[ab]
	Rhizopus stolonifer	22.50[ab]
Royal Commodities	*A. fumigatus*	40.00[ab]
	A. niger	20.00[ab]
	Cladosporium macrocarpum	20.00[ab]
F (pr)		**0.191**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test.

4.4 EXPERIMENT4

Fungal species isolated on PDA from cocoa beans obtained from Licensed Buying Companies (LBCs) in Breman Asikuma District

The fungal species isolated on PDA medium from cocoa beans obtained from the Breman Asikuma District LBCs: Agroecom, Olam, Nyonkopa, PBC, and Royal Commodities were *Aspergillus flavus, A. fumigatus, A. niger, Absidia corymbifera, Alternaria alternata, Rhizopus stolonifer* and S*yncephalastrum racemosum* (Table 5). With the exception of the cocoa beans obtained from Agroecom and Royal Commodities, the cocoa beans obtained from PBC, Olam and Nyonkopa recorded *A. flavus* incidence of 6.67%, 20.00% and 57.86% respectively (Table 5).

Alternaria alternata was not recorded at all for the cocoa beans obtained from Agroecom, Nyonkopa, PBC, and Royal Commodities but Olam (2.86%). *Rhizopus stolonifer* were recorded for the beans obtained from Royal Commodities (0.80%) and Olam (10.00%) but not Nyonkopa, PBC and Royal Commodities. Amongst the cocoa beans obtained from the various LBCs in the Bremen Asikuma district, only those obtained from Olam recorded a 10.00% incidence of *Syncephalastrum racemosum*. (Table 5). *Absidia corymbifera* was only recorded for the cocoa beans obtained from Agroecom (20.00%) but not those of the following LBCs, Royal Commodities, Olam, and Nyonkopa (Table 5). *Aspergillus niger* was recorded for the cocoa beans obtained from the LBCs Agroecom (20.00%), Olam (31.43%), Royal Commodities (34.84%) and PBC (35.87) but not for those of Nyonkopa (Table 5).

For the various fungal species recorded among the cocoa beans obtained from the LBCs in the Breman Asikuma District, the highest (57.80%) was recorded for *A. flavus* for Nyonkopa cocoa beans which was significantly higher ($P \leq 0.05$) compared to the incidence of *Rhizopus stolonifer* recorded for Royal Commodities (0.80%) and Olam (10.00%), *Syncephalastrum*

racemosum for Olam (10.00%), *Alternaria alternata* for Olam (2.86%), *A. fumigatus* for Olam (5.71%) and *A. flavus* for PBC (6.67%).

Table 5: Incidence of fungi species isolated on PDA from cocoa beans obtained from various Licensed Buying Companies in the Breman Asikuma District

Licensed Buying Company	Fungi	% Occurrence
Agroecom	*A. fumigatus*	40.00^{abcd}
	A. niger	20.00^{abcd}
	Absidia corymbifera	20.00^{abcd}
Olam	*A. flavus*	20.00^{abcd}
	A. fumigatus	5.71^{abc}
	A. niger	31.43^{abcd}
	Alternaria alternata	2.90^{abc}
	Rhizopus stolonifer	10.00^{abc}
	Syncephalastrum racemosum	10.00^{abc}
Nyonkopa	*A. flavus*	57.90^{d}
	A. fumigatus	42.14^{acd}
Produce Buying Company	*A. flavus*	6.70^{abc}
	A. fumigatus	19.30^{abcd}
	A. niger	35.90^{abcd}
Royal Commodities	*A. fumigatus*	24.40^{abcd}
	A. niger	34.84^{abcd}
	Rhizopus stolonifer	0.80^{abc}
F (pr)		**0.020**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.5 EXPERIMENT 5

Fungal species isolated on PDA from cocoa beans from Licensed Buying Companies (LBCs) in the Assin Bereku District

Generally, the cocoa beans from Assin Breku harboured 4 *Aspergillus species* (*A. flavus, A. fumigatus, A. niger, A. parasiticus),* and one species each of *Alternaria alternata, Byssochlamys* (*B. nivea,*) *Pennicillium* (*P. citrinum)* and *Rhizopus stolonifer* (Table 6). None of the cocoa beans obtained from the LBCs Nyonkopa, Atlas, Olam, and Agroecom recorded an incidence of *B. nivea* except Kuapa kokoo which recorded 10.00% incidence.

Among the five LBCs sampled in the Assin Bereku District, only cocoa beans obtained from Kuapa Kokoo recorded an incidence of *P. citrinum* (0.30%). Apart from the cocoa beans obtained from Nyonkopa, Atlas, Agroecom, *Rhizopus stolonifer* was resident on cocoa beans obtained from Kuapa Kokoo (0.15%) and Olam (0.80%). *Aspergillus flavus* were isolated from cocoa beans obtained from Olam (2.40%), Nyonkopa (15.11%) and Kuapa Kokoo (26.91%) but not on Atlas and Agroecom. *Aspergillus fumigatus* were also recorded in samples of cocoa beans obtained from Kuapa Kokoo (12.64%) and Nyonkopa (21.42%) in the Assin Bereku district. None of the cocoa beans sampled from LBCs in Assin Bereku district recorded an incidence of *Alternaria alternata* except those obtained from Nyonkopa (1.54%). *Aspergillus niger* were recorded for cocoa beans obtained from Nyonkopa (13.93%), Atlas (20.00%), Agroecom (20.00%0 and Olam 36.80%) except those of Kuapa Kokoo. *Aspergillus parasiticus* were also resident on cocoa beans obtained from Olam (20.00%), Kuapa Kokoo (30.00%), Agroecom (40.00%), Nyonkopa (48.00%) and Atlas (60.00%). The incidence of *A. parasiticus* recorded for the cocoa beans obtained from Atlas (60.00%).

With the exception of *A. flavus* for Kuapa Kokoo (26.91%), *A. parasticus* for Kuapa Kokoo (30.00%), *A. niger* for Olam (36.80%), *A. parasiticus* for Agroecom (40.00%) and Nyonkopa were significantly higher ($P \leq 0.05$) than the other recorded fungal species. Among the recorded fungi species, *A. parasiticus* incidence on cocoa beans obtained from Nyonkopa was significantly higher ($P \leq 0.05$) than the incidence of *Rhizopus stolonifera* recorded for Kuapa Kokoo (0.15%) and Olam (0.80%), *P. citrinum* for Kuapa Kokoo (0.30%), *A. flavus* for Olam (2.40%) and *B. nivea* for Kuapa Kokoo (10.00%).

Table 6: Incidence of fungal species isolated on PDA from cocoa beans obtained from various Licensed Buying Companies in the Assin Bereku District

Licensed Buying Company	Fungi	% Occurrence
Kuapa Kokoo	*A. flavus*	26.91^{abcd}
	A. fumigatus	12.64^{abc}
	*A. n*iger	30.00^{abcd}
	Byssochlamys nivea	10.00^{ab}
	Penicillium citrinum	0.30^{a}
	Rhizopus stolonifer	0.20^{a}
Nyonkopa	*A. flavus*	15.11^{abc}
	A. fumigatus	21.42^{abc}
	A. niger	13.93^{abc}
	A. parasiticus	48.00^{cd}
	Alternaria alternate	1.50^{a}
Atlas	*A. niger*	20.00^{abc}
	A. parasiticus	60.00^{d}
Olam	*A. flavus*	2.40^{a}
	A. niger	36.80^{abcd}
	A. Parasiticus	20.00^{abc}
Agroecom	*A. niger*	20.00^{abc}
	A. parasiticus	40.00^{bcd}
F (pr)		**0.001**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.6 EXPERIMENT 6

Fungal species isolated on PDA from cocoa beans obtained from Licensed Buying Companies (LBCs) in Assin Fosu District.

The microbial flora of cocoa beans obtained from the five LBCs in the Assin Fosu District were predominated by *Aspergillus* species. Eleven fungal species (*Aspergillus flavus, A. fumigatus, A. niger, A. parasiticus, Absidia corymbifera, Alternaria alternata, Byssochlamys nivea, Cladosporium sphaerospermum, Eurotium herbariorum, Mucor racemosum* and *Penicillium citrinum*) belonging to 8 genera were recorded (Table 7). Among the cocoa beans obtained from the various LBCs in the district, only those of Unicom recorded the incidence of *A. parasiticus* (28.22%). *A. corymbifera* species were recorded only on cocoa beans from Adikanfo (4.00%) and Fedco (30.00%). No species of *A. alternata* were recorded on the cocoa beans obtained from Transroyal and Olam but Adikanfo, Fedco and Unicom recorded 0.39%, 6.67% and 20.00% respectively. In all the samples, only the cocoa beans obtained from Unicom recorded an incidence of *B. nivea* (4.44%). The cocoa beans obtained from Olam and Unicom recorded *C. sphaerospermum* incidence of 0.34 and 0.67% respectively with those of Adikanfo, Transroyal and Fedco recording none. *Eurotium herbariorum* was only recorded for the cocoa beans obtained from Olam (10.00%). The incidence of *A. niger* was recorded for the cocoa beans obtained from Fedco (5.00%) and Olam (18.03%). *Mucor racemosum* incidence was only recorded for the cocoa beans obtained from Fedco (10.00%). Only the cocoa beans obtained from Unicom recorded *P. citrinum* incidence (66.67%). Except for the cocoa beans obtained from Fedco and Unicom, *A. flavus* were recorded on cocoa beans obtained from Olam (6.15%), Adikanfo (39.61%) and Transroyal (66.67%). The percentage incidence of *A. fumigatus* was, Transroyal (13.33%), Adikanfo (36.00%), Unicom (40.00%), Fedco (48.33%) and Olam (65.46%). The incidence of *A. flavus* (66.67%) recorded for the cocoa beans obtained from Transroyal was significantly higher than those of the various

fungal species recorded for the various LBCs except *A. flavus* for Adikanfo (39.61%), *A. fumigatus for* Unicom (40.00%), Fedco (48.33%) and Olam (65.46%).

Table 7: Incidence of fungi species isolated on PDA from cocoa beans obtained from various Licensed Buying Companies in the Assin Fosu District

Licensed Buying Company	Fungi	% Occurrence
Adinkafo	*A. flavus*	39.61^{cde}
	A. fumigatus	36.00^{bcd}
	Absidia corymbifera	4.00^{ab}
	Alternaria alternate	0.40^{a}
Transroyal	*A. flavus*	66.70^{e}
	A. fumigatus	13.33^{abc}
Fedco	*A. fumigatus*	48.33^{de}
	A. niger	5.00^{ab}
	Absidia corymbifera	30.00^{abcd}
	Alternaria alternate	6.70^{ab}
	Mucor racemosum	10.00^{abc}
Unicom	*A. fumigatus*	40.00^{cde}
	A. parasiticus	28.22^{abcd}
	Alternaria alternate	20.00^{abcd}
	Byssochlamys nivea	4.44^{ab}
	C. sphaerospernum	0.70^{a}
	Penicillium citrinum	6.70^{ab}
	A. flavus	6.20^{ab}
Olam	*A. fumigatus*	65.50^{e}
	A. niger	18.02^{abcd}
	Cladosporium sphaerospernum	0.40^{a}
	Eurotium herbariorum	10.00^{abc}
F (pr)		**<0.001**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.7 EXPERIMENT 7

Fungal species isolated on Malt Extract Agar (MEA) from cocoa beans obtained from Licensed Buying Companies (LBCs) in the Agona Swedru District

Experiments 7-10 were similar to experiments 3-6, except that Malt Extract Agar (MEA) was used to isolate the fugal sepecies resident on cocoa beans from the 4 districts. In the Agona Swedru district, the fungal species isolated on MEA from the cocoa beans obtained from the LBCs Transroyal, Agroecom, Olam, Nyonkopa and Royal commodities were *Aspergillus flavus, A. fumigatus, A. niger, Absidia corymbifera, Cladosporium sphaerospermum, Eurotium herbariorum, Penicillium citrinum, Rhizopus stolonifer*and *Syncephalastrum racemosum* (Table 8). Among the various LBCs in the district, only the cocoa beans obtained from Agroecom recorded an incidence of *Cladosporium sphaerospermum* (4.00%). *Eurotium herbariorum* incidencewere only recorded for the cocoa beans obtained from Nyonkopa (18.42%) and Agroecom (23.33%). Only the cocoa beans obtained from Nyonkopa in the Agona Swedru district recorded an incidence of *P. citrinum* (1.05%). *Syncephalastrum racemosum* incidencewere only recorded for the cocoa beans obtained from Nyonkopa (2.86%) and Royal Commodities (8.00%). With the exception of the cocoa beans obtained from Olam, *A. flavus* incidence was recorded for the cocoa beans obtained from Nyonkopa (0.67%), Transroyal (6.36%), Agroecom (8.57%), and Royal Commodities (46.00%). The incidenceof *A. niger* were recorded for cocoa beans obtained from Transroyal (5.45%), Nyonkopa (5.71%), Agroecom (6.67%) and Royal Commodities (24.00%). *Aspergillus fumigatus* incidence were recorded for the cocoa beans obtained from Agroecom (19.43%), Nyonkopa (43.24%), Olam (51.00%) and Transroyal (65.45%), except those of Royal Commodities. Aside from the cocoa beans obtained from Royal Commodities, the incidence of *Absidia corymbifera* were Nyonkopa (3.52%), Olam (5.00%), Agroecom (8.00%) and Transroyal (22.73%).

The highest fungal incidence of 65.45% for *A. fumigatus*was recorded for the cocoa beans obtained from Transroyal in the Agona Swedru district which was significantly higher ($P \leq 0.05$) than those of the other fungal species isolated, except those of *Rhizopus stoloniferf*or Olam (44.00%), *A. flavus* for Royal Commodities (46.00%) and *A. fumigatus* for Nyonkopa (43.24%) and Olam (51.00%).

Table 8: Incidence of fungi species isolated on MEA from cocoa beans obtained from various Licensed Buying Companies in the Agona Swedru District

Licensed Buying Company	Fungi	%Occurrence
Transroyal	*A. Flavus*	6.40[a]
	A. fumigatus	65.50[c]
	A. niger	5.50[a]
	Absidia corymbifera	22.73[ab]
Agroecom	*A. flavus*	8.60[a]
	A. fumigatus	19.43[ab]
	A. niger	6.70[a]
	Absidia corymbifera	8.00[a]
	C. sphaerospermum	4.00[a]
	Eurotium herbariorum	23.33[ab]
	Rhizopus stolonifer	10.00[a]
Olam	*A. fumigatus*	51.00[bc]
	Absidia corymbifera	5.00[a]
	Rhizopus stolonifera	44.00[bc]
Nyonkopa	*A. flavus*	0.70[a]
	A. fumigatus	43.24[bc]
	A. niger	5.70[a]
	Absidia corymbifera	3.50[a]
	Eurotium herbariorum	18.42[ab]
	Penicillium citrinum	1.10[a]
	Rhizopus spp.	24.52[ab]
	S. racemosum	2.90[a]
Royal Commodities	*A. flavus*	46.00[bc]
	A. niger	24.00[ab]
	Rhizopus stolonifer	22.00[ab]
	S. racemosum	8.00[a]
F (pr)		**<0.001**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.8 EXPERIMENT 8

Fungal species isolated on MEA from cocoa beans obtained from Licensed Buying Companies (LBCs) in Breman Asikuma District

For the Bremen Asikuma district, the fungal species resident on the cocoa beans were *Aspergillus flavus, A. fumigatus, A. niger, Absidia corymbifera, Cladosporium macrocarpum, Eurotium herbariorum, Penicillium citrinum, Rhizopus stolonifer* and *Syncephalastrum racemosum.* (Table 9). The incidence of *A. flavus* on cocoa beans obtained from the various Produce Buying Company were 5.24% (PBC), 20.00% (Nyonkopa), 38.95% (Olam) 63.47% (Royal Commodities) and 0.00% (Agroecom). *Absidia corymbifera* were only recorded for cocoa beans obtained from Royal Commodities (11.25%). Among the various LBCs in the district, *S. racemosum* was only recorded for the cocoa beans obtained from Royal Commodities (3.75%), Agroecom (13.33%), Nyonkopa (36.84%) and PBC (42.86%).

Incidence of *Cladosporium macrocarpum* was only recorded for the cocoa beans obtained from Agroecom (25.00%) in the district. *Eurotium herbariorum* incidence was only recorded for the cocoa beans obtained from Agroecom (18.18%). *Rhizopus stolonifer* were only recorded for the cocoa beans obtained from Olam (3.55%) and Agroecom (6.67%). *A. fumigatus* were recorded for cocoa beans obtained from Royal Commodities (17.08%), Nyonkopa (23.16%), Agroecom (36.82%), Olam (37.50%) and PBC (41.91%).

There were significant differences ($P \leq 0.05$) among the incidence of the various fungal species (Table 9). The highest fungal species of *A. flavus* (63.47%) was recorded for the cocoa beans obtained from Royal Commodities and with the exception of those of *A. fumigatus* for Agroecom (36.82%) Olam (37.50%) and PBC (41.91%), *A. niger* for Nyonkopa (36.84%) and PBC (42.86%), and *A. flavus* for Olam (38.95%), was significantly higher ($P \leq 0.05$) than those of the other fungi species isolated.

Table 9: Incidence of fungal species isolated on MEA from cocoa beans obtained from various Licensed Buying Companies in the Bremen Asikuma District

Licensed Buying Company	Fungi	% Occurrence
Agroecom		
	A. fumigatus	36.80^{abcd}
	A. niger	13.33^{abc}
	Cladosporium macrocarpum	25.00^{abc}
	Eurotium herbariorum	18.20^{abc}
	Rhizopus stolonifer	6.70^{abc}
Olam	*A. flavus*	38.95^{abcd}
	A. fumigatus	37.50^{abcd}
	Rhizopus stolonifera	3.60^{abc}
	Syncephalastrum racemosum	20.00^{abc}
Nyonkopa	*A. flavus*	20.00^{abc}
	A. fumigatus	23.20^{abc}
	A. niger	36.82^{abcd}
	Absidia corymbifera	20.00^{abc}
Produce Buying Company	*A. flavus*	15.24^{abc}
	A. fumigatus	41.91^{acd}
	A. niger	42.90^{cd}
Royal Commodities	*A. flavus*	63.50^{d}
	A. fumigatus	17.10^{abc}
	A. niger	3.75^{abc}
	Absidia corymbifera	4.44^{abc}
	Penicillium citrinum	11.25^{abc}
F (pr)		**0.003**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.9 EXPERIMENT 9

Fungal species isolated on MEA from cocoa beans obtained from Licensed Buying Companies (LBCs) in the Assin Bereku District

The fungal species resident on cocoa beans on MEA from the LBCs (Kuapa Kokoo, Nyonkopa, Atlas, Olam and Agroecom) in the Assin Bereku district were *Aspergillus flavus, A. fumigatus A. niger, A. parasiticus, Alternaria alternata, Byssochlamys nivea, Cladosporium macrocarpum, Cladosporium sphaerospermum, Eurotium herbariorum, Penicillium citrinum* and *Rhizopus stolonifer* (Table 10). Cocoa beans obtained from only Nyonkopa and Olam recorded *A. alternate,* with incidence of 0.96% 1.00% respectively. *Cladosporium macrocarpum* incidence were only recorded for the cocoa beans obtained from Agroecom (1.43%) and Atlas (1.54%). Among the various LBCs in the district, incidence of *E. herbariorum* were only recorded for those of Olam (5.00%) and agroecom (8.69%). *A. parasiticus* incidence was only recorded for the cocoa beans obtained from Kuapa Kokoo (33.82%). *B. nivea* was only recorded for the cocoa beans obtained from Kuapa Kokoo (0.10%). The incidence of *A. flavus* was recorded for beans obtained from Agroecom (3.83%), Nyonkopa (10.00%), Kuapa Kokoo (19.38%) and Atlas (21.00%). *Cladosporium sphaerospermum* incidences were only recorded for the cocoa beans obtained from Kuapa Kokoo (0.86%) and Atlas (2.22%).

Rhizopus stolonifer were recorded on the cocoa beans obtained from Kuapa Kokoo (0.17%), Agroecom (1.44%), Nyonkopa (1.88%), Atlas (2.22%) and Olam (20.00%). *Aspergillus fumigatus* was recorded on cocoa beans obtained from all the LBCs, that is, Olam (2.50%), Atlas (11.61%), Kuapa Kokoo (17.31%), Nyonkopa (19.48%) and Agroecom (39.14%). The incidence of *A. niger* recorded on the cocoa beans were Kuapa Kokoo (4.10%), Agroecom (12.70%), Nyonkopa (26.93%), Olam (34.00%) and Atlas (35.22%). *P. citrinum* was recorded on cocoa beans obtained from Olam (17.50%), Nyonkopa (20.77%), Kuapa Kokoo (24.23%), Atlas

(26.19%) and Agroecom (32.76%). The highest fungal species of *A. fumigatus* (39.14%) was recorded for cocoa beans obtained from Agroecom, which was significantly higher ($P \leq 0.05$) than those of *B. nivea* for Kuapa Kokoo (0.13%), *Rhizopus stolonifer* for Kuapa Kokoo (0.17%), Agroecom (1.44%), Nyonkopa (1.36%) and Atlas (2.22%), *C. sphaerospermum* for Kuapa Kokoo (0.86%) and Atlas (2.22%), *A. alternata* for Nyonkopa (0.96%) and Olam (1.00%), *C. macrocarpum* for Agroecom (1.43%) and Atlas (1.54%), *A. fumigatus* for Olam (2.50%), *A. flavus* for Agroecom (3.83%), *A. niger* for Kuapa Kokoo (4.10%) and *E. herbariorum* for Olam (5.00%) and Agroecom (8.69%).

Table 10: Incidence of fungi species isolated on MEA from cocoa beans obtained from various Licensed Buying Companies in the Assin Bereku District

Licensed Buying Company	Fungi	% Occurrence
Kuapa Kokoo	*A. flavus*	19.40^{abcd}
	A. fumigatus	17.31^{abcd}
	A. niger	4.10^{ab}
	A. parasiticus	33.82^{bcd}
	Byssochlamys nivea	0.10^{a}
	Cladosporium sphaerospermum	0.90^{a}
	Penicillium citrinum	24.20^{abcd}
	Rhizopus stolonifer	0.20^{a}
Nyonkopa	*A. Flavus*	10.00^{abcd}
	A. fumigatus	19.50^{abcd}
	A. niger	26.93^{abcd}
	Alternaria alternate	1.00^{a}
	Penicillium citrinum	20.80^{abcd}
	Rhizopus stolonifer	1.90^{a}
Atlas	*A. Flavus*	21.00^{abcd}
	A. fumigatus	11.60^{abcd}
	A. niger	35.20^{cd}
	Cladosporium macrocarpum	1.54^{a}
	Cladosporium sphaerospermum	2.22^{a}
	Penicillium citrinum	26.20^{abcd}
	Rhizopus stolonifer	2.22^{a}
Olam		
	A. fumigatus	2.50^{a}
	A. niger	34.00^{bcd}
	Alternaria alternate	1.00^{a}
	Eurotium herbariorum	5.00^{ab}
	Penicillium citrinum	17.50^{abcd}
	Rhizopus stolonifera	20.00^{abcd}
Agroecom	*A. Flavus*	3.83^{ab}
	A. fumigatus	39.14^{d}
	A. niger	12.70^{abcd}
	Cladosporium macrocarpum	1.43^{a}
	Eurotium herbariorum	8.70^{abc}
	Penicillium citrinum	32.80^{bcd}
	Rhizopus stolonifer	1.44^{a}
F (pr)		**0.001**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.10 EXPERIMENT 10

Fungal species isolated on MEA from cocoa beans obtained from Licensed Buying Companies (LBCs) in Assin Fosu District

The fungal species isolated from the LBCs (Adikanfo, Transroyal, Fedco, Unicom and Olam) in the Assin Fosu district were *Aspergillus flavus, A. fumigatus A. niger, A. parasiticus, Absidia corymbifera, Alternaria alternata, Byssochlamys nivea, Cladosporium sphaerospermum, Eurotium herbariorum, Penicillium citrinum, Syncephalastrum racemosum* and *Talaromyces flavus* (Table 11). *Aspergillus niger* was resident on only cocoa beans obtained from Unicom (0.84%) and Fedco (12.00%). In the district, only cocoa beans obtained from Unicom recorded an incidence of *A. alternata* (5.71%). *Syncephalastrum racemosum* incidence (4.80%) was only recorded for the cocoa beans obtained from Fedco, whilst cocoa beans obtained from Transroyal recorded an incidence of *P. citrinum* (20.00%). *Talaromyces flavus* was also recorded (5.92%) on cocoa beans obtained from Unicom.

A. parasiticus (2.67%). and *B. nivea* (19.75%) were the only fungal species isolated from cocoa beans obtained from Adikanfo. With the exception of the cocoa beans obtained from Adikanfo and Fedco, *C. sphaerospermum* was resident on all the beans obtained from the remaining LBCs (Transroyal (0.19%), Olam (5.00%), Unicom (8.57%). The incidence of *A. flavus* on the beans were Fedco (9.60%), Transroyal (12.98%), Olam (19.70%) and Adikanfo (38.17%). Apart from Unicom, *Absidia corymbifera* was isolated from the cocoa beans obtained from Fedco (4.80%), Transroyal (15.40%) and adinkanfo (23.89%). The Incidence of *A. fumigatus* were recorded for the cocoa beans obtained from Adikanfo (7.50%), Transroyal (12.39%), Olam (17.94%), Fedco (19.98%) and Unicom (40.84%), whereas *Eurotium herbariorum* was recorded on cocoa beans obtained from Adikanfo (8.00%), Fedco 28.82%), Unicom (38.11%), Transroyal (37.05%) and Olam (57.35%).

The highest fungal species incidence in the district, *E. herbariorum* (57.35%), was recorded on cocoa beans obtained from Olam, which was significantly higher ($P \leq 0.05$) than the other fungal species isolated.

Table 11: Incidence of fungi species isolated on MEA from cocoa beans obtained from various Licensed Buying Companies in the Assin fosu District

Licensed Buying Company	Fungi	% Occurrence
Adinkafo	*A. Flavus*	38.20^{bcde}
	A. fumigatus	7.50^{abc}
	A. parasiticus	2.70^{a}
	Absidia corymbifera	23.90^{abcd}
	Byssochlamys nivea	19.80^{abcd}
	Eurotium herbariorum	8.00^{abc}
Transroyal	*A. Flavus*	13.00^{abcd}
	A. fumigatus	12.40^{abcd}
	Absidia corymbifera	15.40^{abcd}
	Cladosporium sphaerospermum	0.20^{a}
	Eurotium herbariorum	39.05^{cde}
	Penicillium citrinum	20.00^{abcd}
Fedco	*A. Flavus*	9.60^{abcd}
	A. fumigatus	20.00^{abcd}
	A. niger	12.00^{abcd}
	Absidia corymbifera	4.80^{a}
	Eurotium herbariorum	28.82^{abcde}
Unicom	*Syncephalastrum racemosum*	4.80^{a}
	A. fumigatus	40.84^{de}
	A. niger	0.84^{a}
	Alternaria alternate	5.71^{ab}
	Cladosporium sphaerospermum	8.60^{abcd}
	Eurotium herbariorum	38.11^{bcde}
	Talaromyces flavus	5.92^{ab}
Olam	*A. Flavus*	19.70^{abcd}
	A. fumigatus	17.94^{abcd}
	Cladosporium sphaerospermum	5.00^{a}
	Eurotium herbariorum	57.40^{e}
F (pr)		**<0.001**

Mean in the same column followed by different letter(s) are significantly different as determined by Duncan's multiple range test

4.11 EXPERIMENT 11

Aspergillus niger, an Ochratoxin A producing fungi, isolated on PDA from the cocoa beans obtained from the Agona Swedru, Bremen Asikuma, Assin Bereku and Assin Fosu Districts

The incidence of *A. niger* on cocoa beans obtained from the four districts (Agona Swedru, Bremen Asikuma, Assin Bereku and Assin Fosu) ranged from 4.60% for those of Assin Fosu to 24.43% for those of Breman Asikuma (Figure 3). There were significant differences ($P \leq 0.05$) among the incidence of *A. niger* recorded on PDA from the cocoa beans obtained from the various districts (Figure 1). The incidence of *A. niger* recorded for the cocoa beans obtained from the Bremen Asikuma (24.43%) and Assin Bereku (24.15%) was significantly higher ($P \leq 0.05$) than those of Agona Swedru (%.80%) and Assin Fosu (4.60%) cocoa beans (Figure 3).

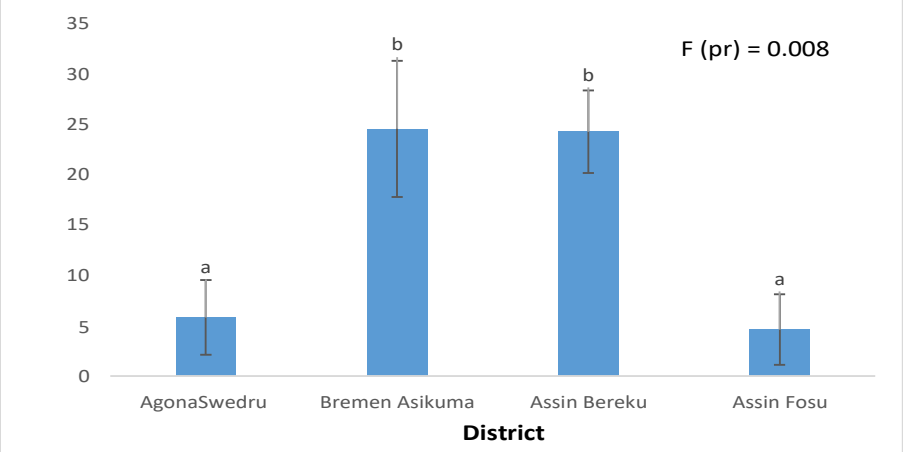

Figure 3: Incidence of *A. niger* isolated on PDA from the cocoa beans obtained from various Districts. Error bars indicate standard errors of means.

4.12 EXPERIMENT 12

***Aspergillus niger* (ochratoxin A producing fungi) isolated on MEA from the cocoa beans obtained from the Agona Swedru, Bremen Asikuma, Assin Bereku and Assin Fosu Districts**

*Aspergillus niger*was resident on cocoa beans obtained from the Agona Swedru, Bremen Asikuma, Assin Bereku and Assin Fosu Districts (Figure4) The cocoa beans obtained from Assin Bereku district recorded the highest incidence of *A. niger* (22.59%) and the lowest was recorded on that of Assin Fosu (2.57%) (Figure 4). The incidence of *A. niger* recorded for the cocoa beans obtained from the Agona Swedru (7.28%) and Bremen Asikuma (19.36%) districts each was significantly ($P \leq 0.05$) not different from those of Assin Fosu (2.57%) and Assin Bereku (22.59%) (Figure 4). The incidence of *A. niger* recorded on the MEA medium for the cocoa beans obtained from the Assin Bereku District (22.59%) was significantly higher ($P \leq 0.05$) than that of Assin Fosu (2.57%) (Figure4).

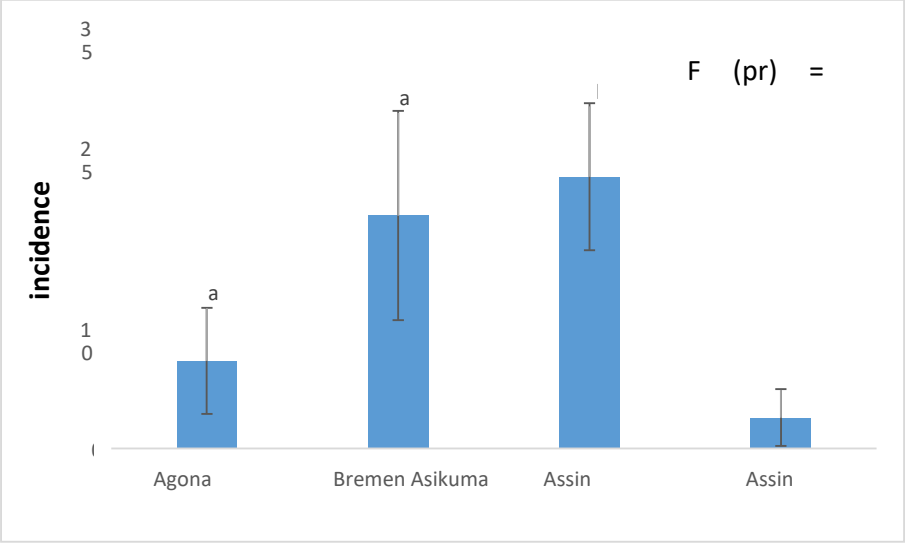

Figure 4: Incidence of *A. niger* isolated on MEA from the cocoa beans obtained from various Districts. Error bars indicate standard errors of means.

4.13 EXPERIMENT 13

Ochratoxin A Analysis

The HPLC-FLD Ochratoxin A Concentration (ppb) in cocoa beans obtained from various Licensed Buying Companies in the four districts in the central region, Agona Swedru (Transroyal, Agroecom, Olam, Nyonkopa and royal commodities), Breman Asikuma (Agroecom, Olam, Nyonkopa, PBC, and Royal commodities), Assin Bereku (Kuopa Kokoo, Nyonkopa, Atlas, Olam, Agroecom) and Assin Fosu (Adinkanfo, Transroyal, Fedco, Unicom and Olam) showed that OTA was not detected in all the samples (Table 13).

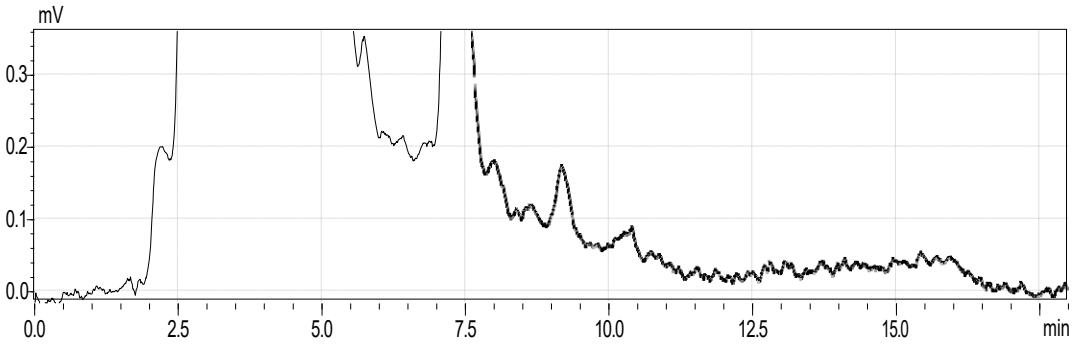

Figure 5: HPLC-FLD Chromatogram of Cocoa bean sample with no OTA detection

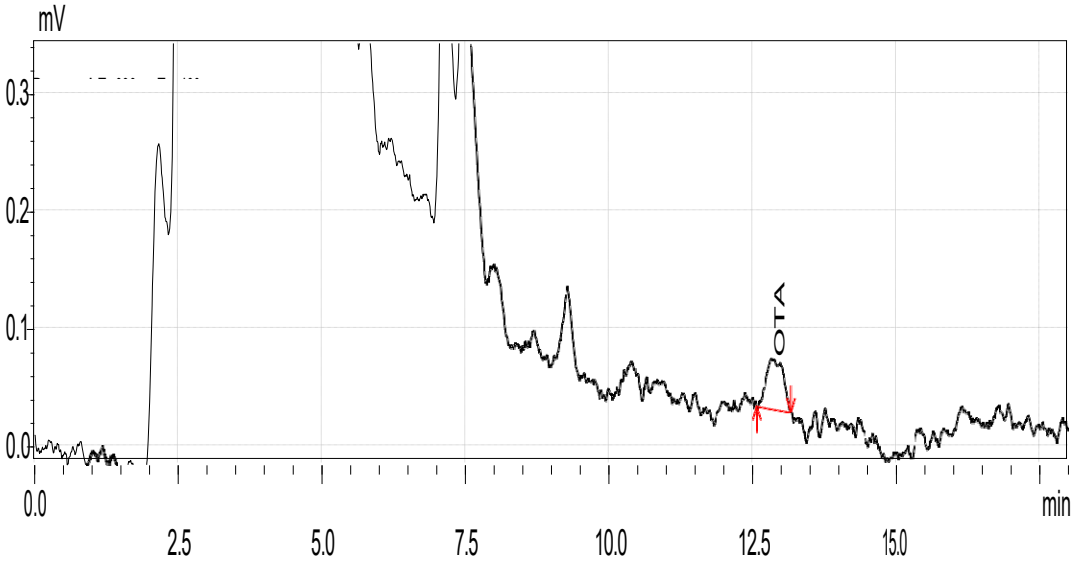

Figure 6: HPLC-FLD Chromatogram of Spiked Cocoa beans samples

Table 12: HPLC-FLD Ochratoxin A Concentration (ppb) in cocoa beans obtained from various Licensed Buying Companies in the four districts in the central region

Sample ID	District	LBC	Concentration (ppb)
A	Agona Swedru	Transroyal	ND
B	Agona Swedru	Agroecom	ND
C	Agona Swedru	Olam	ND
D	Agona Swedru	Nyonkopa	ND
E	Agona Swedru	Royal Commodities	ND
G	Bremen Asikuma	Agroecom	ND
H	Bremen Asikuma	Olam	ND
J	Bremen Asikuma	Nyonkopa	ND
K	Bremen Asikuma	PBC	ND
W	Bremen Asikuma	Royal Commodities	ND
L	Assin Bereku	Kuapa Kokoo	ND
M	Assin Bereku	Nyonkopa	ND
N	Assin Bereku	Atlas	ND
P	Assin Bereku	Olam	ND
Q	Assin Bereku	Agroecom	ND
R	Assin Fosu	Adinkafo	ND
T	Assin Fosu	Transroyal	ND
T	Assin Fosu	Fedco	ND
U	Assin Fosu	Unicom	ND
V	Assin Fosu	Olam	ND

ND= Not Detected

CHAPTER FIVE

DISCUSSION

Cocoa is an import cash crop in Ghana and a major source of foreign exchange earnings for the country's economy.

In 2013, agriculture employed 53.6% of Ghanas total labour force (FAPDA 2016), with the crop sector leading in the agricultural industry ("Ghana" Natureduca (in Spanish). Retrieved April 2013).

In 2019, Cocoa provided the second largest source of total export earnings of 2.11 billion GHS, equivalent to 366 million U.S. dolars from 850,000 tons, representing 30% of the gross domestic product (GDP) of Ghana's economy (Statista 2021).

Ghana produces about 20% of the world's cocoa and the second largest producer in the world. Ghana is also noted to produce the highest premium cocoa worldwide. Ghana's economic growth and development process. (Agriculture Sector in Ghana Review itrade.gov.il/ghana/files/2020/05/Agriculture-Sector-Review.pdf).

Cocoa production is mostly carried out by smallholding farms or families and it involves over 800,000 families or households. Over the years, through the interventions of the Ghana Cocoa Board (COCOBOD), cocoa production has increased in Ghana, resulting in improvement of the socioeconomic lives of cocoa farmers and their families.

The production of quality and premium cocoa is of paramount interest to all producing countries since cocoa is traded on the international market under very high and strict regulatory standard requirement on the physical and chemical properties of the cocoa beans (Adeyeye et al. 2010; Bateman, 2015).

To maintain the quality and reduce the incidence of Ochratoxigenic fungi and Ochratoxin A contamination of cocoa beans produced from Ghana and the emerging health issues associated, calls for continuous monitoring of cocoa beans from farm gate to departure at the harbour/port.

This work was carried out to determine the moisture content of cocoa beans sold to the various LBCs by the farmers, fungal loads, the ochratoxigenic fungi present and subsequently the Ochratoxin loads in cocoa from the four districts in the central Region of Ghana.

The method of extraction as described in document No. SANTE/11945/2015 for OTA in cocoa was partially modified and validated. The LOD was found to be 1ng/Kg and the LOQ was 2ng/Kg. A linear range from 1.0 -20 ng/mL and a correlation coefficient (R^2) of 0.9997obtained for the entire range of studied concentrations.

Repeatability (RSD_r) and reproducibility (RSD_R) expressed as RSD were 5.5% and 9.8% respectively with mean recovery of OTA spiked at 2.5 and 5ng/kg in 10 replicates were consistent and more than 90%.

Dryness level (Moisture content) of cocoa beans is an important quality control measure both locally and internationally. The allowed moisture content of cocoa beans is between 7 and 8%.

In addition, cocoa beans with moisture content greater than 8% weight by weight (w/w) under storage leads to proliferation of moulds and the development of musty odours (Hamid and Lopez 2000, Hii et al. 2009) whilst too dried beans below moisture content of 6.5% leads to increase levels of broken beans.

In recent times, it has been established that not all water available in a food product (moisture content) is available for the development of microbes which causes deterioration in food, some water molecules are bounded to the matrix of the food substance. Water activity (aw), which is the amount of water which is not bounded to the matrix of the food substance and available for the growth and development of microbes is preferably measured instead of the moisture content (Mabbett 2013).

The result of the moisture content determination (Table 3) reveals that cocoa beans from Unicom in the Assin Fosu district had the lowest moisture content value of 7.05% and the highest moisture content of 9.86% for the cocoa beans obtained from Nyonkopa in the Assin Bereku district.

There was a significant difference ($P \leq 0.05$) among the moisture content recorded for the cocoa beans obtained from the various LBCs (Table 3).

During sampling of cocoa beans from the LBC, it was revealed that no LBC had a sciencetific means of measuring the mosture content of the cocoa beans purcheased from the farmers/societies.

The moisture content of the of the cocoa beans were determined by crude method largely based on the experience of the quality control officer. This was done by passing the hands through the beans severally to determine whether the cocoa beans are properly dried and ready to be bagged and sold to the Cocoa Marketing Company (CMC)of Ghana COCOBOD.

This therefore explains the varying moisture content of the cocoa beans sold to the LBCs and the alleged and widely circulated adjustment of scales by some LBCs to accommodate the losses they might incur due to further drying before bulking and handing over to QCC.

Henderson (1984) and Hamid and Lopez (2000), reported that cocoa beans are hygroscopic and therefore increase in moisture content equilibrium with increase in relative humidity and temperature (ie. above 70% and 25^{o}C respectively),

Although the rate at which dried cocoa bean absorbs moisture with varing temperature and relative humidity (RH) and the holding time of dreid cocoa beans by the LBC's was not determined by this work, it was gathered during sampling that LBC's after redrying, bagging and weighing of the cocoa beans, it is transported to CMC of COCOBOD. To prevent the re-absorption of moisture at the warehouse, during transportation to CMC and to sustain the exportation of high-quality cocoa bean, it is important that cocoa is well dreid and the moisture content of dried cocoa beans kept at levels within 6% and 7.9% in storage. This implies that the initial storage moisture content is very important.

The farmers did not properly dry cocoa beans samples obtained from LBCs with moisture content above 8%. This is because cocoa beans are not stored for a longer period before selling to the LBCs and therefore the possibility of cocoa beans absorbing moisture under long storage is ruled out. It is also possible these cocoa beans are product of past cocoa seasons, which were not stored under the right conditions of temperature and humidity and have absorbed water from the environment, resulting in high moisture content.

The isolation of the various fungal species on the cocoa beans obtained from the various LBCs in the districts was not surprising since the handling of cocoa beans from the farm gate to the point of storage involved several processes that could result in mould infestation of the cocoa beans.

Apart from diseased and rotten pods, which are known to be contaminated with fungal spores and/ or mycelia, dry cocoa beans produced from healthy pods is likely to be infested with fungal spores and mycelia, which will germinate when conditions are favourable.

The methods through which dried cocoa beans are produced, (i.e., from pod breaking to obtain the cocoa bean through fermentation and drying of the fermented beans in open space) are all likely means of introducing fungal spores into the final dried cocoa bean.

This processes if properly done according to the recommendation for processing of cocoa beans by CAOBISCO/ECA/FCC 2015, reduces the fungal loads to the minimal or acceptable levels.

A similar observation was made by (Ribeiro et al. 1986; Ardhana and Fleet, 2003; Mounjouenpou et al. 2008; Sanchez-Hervas et al. 2008) revealing the presence of mould at the fermentation, drying and storage stage of cocoa beans production.

Therefore, dried cocoa beans obtained from the farm gates can be possibly contaminated with fungal spores and or mycelia and when not properly dried and stored before selling to the LBCs. With the right amount of moisture, temperature, humidity, and other conditions, these spores or mycelia are likely to germinate, posing quality and health issues to the final product and the consumers.

The presence of fungi in cocoa beans reduces the quality of the beans. For instance, the presence of fungi reduces the aesthetic value of the cocoa beans, making it unattractive. It also renders the cocoa beans unsafe for consumption.

It has been established that cocoa bean with more than 3% internally contaminated fungi (Determined by cut test) are unsafe for consumption and results in cocoa bean rejection (CAOBISCO/ECA/FCC, 2015).

The result of the fungal isolation and identification revealed the presence of 15 fungal species belonging to 12 genera isolated on Malt Extract Agar and or Potato Dextrose Agar from cocoa beans obtained from the four districts sampled. The following species were encountered.

Aspergillus (*A. flavus*, *A. fumigatus*, *A. niger* and *A. parasiticus*), *Cladosporium* (*C. macrocarpum* and *C. sphaerospermum*), *Absidia corymbifera*, *Alternaria alternata*, *Byssochlamys nivea*, *Eurotium herbariorum*, *Mucor racemosus*, *Penicillium citrinum*, *Rhizopus stolonifera, Syncephalastrum racemosum* and *Talaromyces flavus.* (Table 2 and Plates 1-15).

More species were isolated on Malt Extract Agar compared to Potato Dextrose Agar. *Byssochlamys nivea* and *Talaromyces flavus* were not isolated on PDA whilst *Mucor racemosus* was also not isolated on MEA. (Table 2) although Samson at el. (1988) reported the isolation these three fungi by MEA.

Several researchers (Hansen and Welty, (1970); Ogundero, (1983); Ardhana and Fleet, (2003); Schwan and Wheals, (2004); Cam et al. (2007) have documented the contamination of cocoa beans by fungi (*Aspergillus* and *Pennicilliun*).

Four species of *Aspergillus* (*A. flavus*, *A. fumigatus*, *A. niger*, *A. parasiticus*), were isolated in this study and this agrees with Pitt and Hocking (1997) that *Aspergillus* species dominated deteriorating fungi in the tropics.

Among the list of fungi isolated (Table 2)*, A. flavus*, *A. fumigatus*, *A. niger*, *A. parasiticus*, *Cladosporium sp*., *Absidia corymbifera Eurotium sp., Mucor sp.*, *Penicillium citrinum*, *Rhizopus sp. And Syncephalastrum racemosum* were part of fungi isolated from cocoa beans from Brazil (Copetti et al. (2011).

Rahmadi and Graham, 2008 also reported the presence of *A. flavus, A. fumigatus, A niger, Cladosporium sp*. and *Penicillium citrinum*, among other fungi isolated from cocoa beans obtained from Indonesia and Queensland, Australia.

Aspergillus niger, a known Ochratoxin producing fungi, also has the ability to attack the kernel of the beans during fruiting stage (ICMSF, 2005).

With the exception of *A. niger* which is known to be an ochratoxigenic fungus (Frisvad *et al*., 2004, Perrone et al. 2007), all the remaining fungi have not been associated with OTA production although some are known to produce other mycotoxins e.g., *A. flavus, A. parasiticus* (Aflatoxins). (Frisvad et al. 2005; Kostarelou et al. 2014).

Another common fungus, which was isolated in almost all the cocoa beans samples, *Aspergillus fumigatus*, is known to be associated with cocoa beans spoilage as it has the ability to deteriorate the tissues of the beans and allowing other fungi (*A. niger, A flavus, Penicillium, Eurotium sp.,* and *Mucor sp*.) to penetrate and cause internal mouldiness in cocoa.

The variation in the type of fungal species and occurrence of the fungal species isolated on PDA and MEA from the cocoa beans obtained from some LBCs could be due to the differences in the nutritional component of the two media (PDA and MEA).

PDA is noted for the cultivation of fungi in the presence of acid or antibiotics to retard the growth of bacteria.

It has the nutritional component of dextrose and potato infusion and the nutritional component of MEA are (High concentration of maltose, Dextrin and glycerol as carbon sources and gelatin peptone as source of nitrogen) and helps to provide an acidic environment and nutrient helpful for the growth and metabolism of fungi.

These differences in the nutritional component of PDA and MEA might have influenced the type and occurrence of the various fungi recorded. Since the nutritional requirements vary among fungi. This observation is supported by Gao and Liu, (2010), that the nutritional requirement varies from one fungus to another.

The differences in the type and occurrences of fungal species isolated among the cocoa beans obtained from the LBCs in the districts Agona Swedru, Breman Asikuma, Assin Fosu and Assin Bereku can be attributed to several factors.

For instance, the conditions of storage facilities for the cocoa beans may vary among the LBCs. and also, among the district from which the cocoa beans were obtained.

The influence of storage facility on the occurrence of fungi species on cocoa beans have been reported (Hamid and Lopez (2000).

The variations in the type and occurrence of fungal species isolated on the cocoa beans obtained from the various LBC and districts could also be attributed to the sources from which the various cocoa beans were obtained.

Schwan and Wheals (2004) made a similar report that cocoa pulp inside an un-injured cocoa pod is microbiologically sterile.

The hygienic conditions under which the cocoa beans were handled from the time of pod breaking to obtain the seeds, through fermentation and drying of the seeds to storage of the dried cocoa beans certainly vary from one cocoa farmer to another.

There is therefore the likelihood of each cocoa farmer from whom the cocoa beans were obtained by the LBCs contaminated their cocoa beans with different fungi during the post harvest processes of the cocoa. This is supported by the report of Schwan and Wheals (2004) that the poor handling of cocoa beans by farmers could result in the contamination (pod surface, knives, hands of workers, containers used in conveying pulp and beans, insects and work environment) of the beans by moulds.

The Ochratoxin producing fungus *A. niger* which was isolated on the cocoa beans obtained from the various districts is a major source of concern for cocoa producers, consumers and the trade in general.

Copetti et al. (2010) reported the presence of *A. niger* at all the processing stages and at different isolation frequencies (IF). 51 sample with IF of 3.92% at fermentation, 81 samples with IF of 14.8% at drying stage and 65 samples with IF of 26.15% at storage.

Although all the cocoa beans obtained from the various LBCs in the Agona Swedru, Breman Asikuma, Assin Fosu and Assin Bereku districts each recorded the presence of *A. niger*.

Ochratoxin A was not detected in any of the cocoa beans obtained from these sources. Investigations by Astoreca et al. (2007), Amezqueta et al. (2009) and Kokkonen et al. (2005) have proved that the presence of ochratoxigenic fungi in food samples does not unavoidably result in OTA synthesis.

It is Also possible that some required environmental conditions needed for the production of this Ochratoxin might be absent since Aziz and Moussa (1997), and O'Callaghan et al. (2006) stated that varying environmental conditions such as temperature, water activity level, pH, oxygen availability and presence of some ions influence the production of OTA and varies between different strains of *Aspergillus* and *Penicillium*.

Studies by Copetti et al. (2004), indicate that *A niger* is not a major OTA producing fungi compared to *A. carbonarius* and that *A. carbonarius* is the main source of OTA production in cocoa beans. In their work on cocoa beans sampled from Brazil, different strains of *A. carbonarius, A. niger, A. amellens, A. westerdijkiae* and *A. ochraceus* OTA producing fungi were isolated.

Other works (Abarca et al. (2003); Iamanaka et al. (2005); Leong et al. (2006) and in robusta coffee (Joosten et al. (2001) also support this argument that *A. carbonarius* is a main source of OTA production in grape, grape products and robusta coffee.

The non-detection of OTA below the LOD level (1 ng/Kg) in the cocoa beans samples obtained from the various districts could be attributed to fact that the various LBCs in each of the districts (Agona Swedru, Breman Asikuma, Assin Fosu and Assin Bereku) properly fermented, dry and stored their cocoa beans before selling to the LBCs. The proper storage of the beans did not create the conducive conditions and environment for *A. niger* to establish on the cocoa beans to produce the OTA.

In addition, the recorded *A. niger* on cocoa beans from the various LBC's from these Districts and the absence of Ochratoxin A. in the cocoa beans sampled could also imply that the cocoa beans contained the *A. niger* spores which could not germinate or germinating hyphae which could not advance in germination on the cocoa beans due to proper storage condition before selling to the various LBCs.

The *A. niger* spores or germinating hyphae, which could have been on the cocoa beans only, germinated on the PDA and MEA media, which had the required nutrient and incubated in the conducive environment necessary for their establishment.

Also, despite the presence of the *A. niger* on the cocoa beans obtained from the various LBCs, the *A. niger* loads may not be high enough to lead to the production of ochratoxin in the cocoa beans (Amezqueta et al. 2009; Astorreca et al. 2007; Kokkonen et al. 2005).

In addition, Ochratoxin was not detected in the various cocoa beans sample obtained from the various LBCs because the cocoa farms from whom the beans were bought might have been properly fermented, which results in the inhibition of the growth of the detected ochratoxingenic fungi *A. niger*.

Physical damage and poor plant health are other conditions that predisposes plant products to OTA production (Amezqueta et al. 2009). This implies that Ghana Cocoa Board recommendation to farmers not to include damaged and diseased pods with healthy pods during fermentation process (Abrokwah et al. 2013) is strictly adhered to.

In addition, sucrose and glucose promote OTA production (Muhlencoert et al. 2004) and acetic acid, lactic acid and citric acid produced by bacteria inhibits the growth of OTA producing fungi. The breakdown of sucrose, glucose, and the production of acetic acid, lactic acid and citric acid which is achieved by the action of micro-organisms and enzymes on the pulp and cocoa beans respectively (Hansen et al. 1998; Biehl et al. 1990) which is realized during proper fermentation recommended by Ghana COCOBOD might have inhibited its production.

The non-detection of Ochratoxin on the cocoa beans obtained from the various LBCs could also be attributed to the fact that the moisture content range of 7.05 to 8.89% recorded for the cocoa beans with only one sample from Assin Breku (Nyonkopa) recording 9.86% in addition to other interacting risk factors did not support the fungal growth. This is supported by the report of Minife (1999) that in order to prevent fungal growth, the moisture content of stored cocoa beans should not exceed 8.0%.

Another factor could also be that since the farmers do not store the dried beans for a longer period before selling to the LBCs for further drying and bulking, available ochratoxigenic fungal spores or mycelia do not get enough time to grow to the stage of toxin production.

This has been established by (ICMSF, 2005; Pitt, 2006) that longer storage periods could lead to fungal proliferation especially *Aspergillus niger* and *Eurotium*, and OTA production.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study has revealed that various fungal species were associated with the dried cocoa beans obtained from the LBC's in the Agona Swedru district (Transroyal, Agroecom, Olam,

Nyonkopa and Royal commodities), Breman Asikumen district (Agroecom, Olam, Nyonkopa, PBC and Royal commodities), Assin Bereku district (Kuapa Kokoo, Nyonkopa, Atlas, Olam, and Agroecom) and Assin Fosu district (Adinkanfo, Transroyal, Fedco, Unicom and Olam) The various fungal species on the dried cocoa beans were isolated on PDA and MEA.

Fungal species belonging to 12 genera were isolated on both Malt Extract Agar and Potato Dextrose Agar from cocoa beans obtained from the four districts sampled were *Aspergillus* (*A. flavus*, *A. fumigatus* Fres., *A. niger*, *A. parasiticus*), Cladosporium (*C. macrocarpum*, *C. sphaerospermum*), *Absidia corymbifera*, *Alternaria alternata*, *Byssochlamys nivea*, *Eurotium herbariorum*, *Mucor racemosus*, *Penicillium citrinum*, *Rhizopus stolonifer*. *Syncephalastrum racemosum* and *Talaromyces flavus*.

More species were isolated on Malt Extract Agar compared to Potato Dextrous Agar. *Byssochlamys nivea* and *Talaromyces flavus* were not isolated on PDA whist *Mucor racemosus* was also not isolated by MEA.

The moisture content of the cocoa beans obtained from the various districts under study ranged from 7.05% to 9.86% for the LBC's Unicom in the Assin Fosu district and Nyonkopa in the Assin Bereku district respectively.

The Ochratoxin A producing fungus, *A. niger*, was isolated on PDA and MEA for all the dried cocoa beans obtained from the various districts.

On PDA, the highest incidence of *A. niger* (24.43%) and the lowest (4.6%) were recorded for the dried cocoa beans obtained from the Breman Asikuman and Assin Fosu districts respectively.

On MEA, the cocoa beans obtained from Assin Bereku district recorded the highest incidence (22.59%) of the Ochratoxin producing fungi *A. niger* and the lowest (2.57%) recorded for the Assin Fosu district.

Despite the isolation of the Ochratoxin producing fungus *A. niger* in the sampled dried cocoa beans, Ochratoxin A was not detected in any of the sampled cocoa beans.

6.2 RECOMMENDATION

This study should be replicated in other cocoa growing regions in the country. Future study should include the measurement of all other parameters that are considered when grading and sealing cocoa beans.

Cocoa Farmers or societies should be given Field Moisture content meters to enable them to monitor the drying of their cocoa beans in order to obtain the acceptable limit of 7.5% to 8.0%

Studies needed in tracing cocoa beans quality from farmhouse to the ports of departure to find out the point of contamination if any and the necessary recommendation given.

QCC should insist on LBCs doing further drying during the bulking stage before bagging since very few farmers or societies are able to dry to moisture content of 7.0% and develop similar research or studies targeting farms and societies to produce seasonal data on fungal flora and OTA level.

Similar studies are recommended for all Codex members in other to develop code of standard in controlling ochratoxigenic fungi in cocoa beans thereby reducing OTA in cocoa beans and cocoa products.

CHAPTER SEVEN

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