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MYCOBIOTA AND AFLATOXIN ASSOCIATED WITH STORED COCOA BEANS IN SOUTH WESTERN NIGERIA

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A B S T R A C T

Cocoa bean is contaminated by mycotoxin-producing fungi due to poor agronomical practices during beans fermentation, drying and storage. This study aims to evaluate the associated mycotoxin-producing fungi in stored cocoa beans. Cocoa beans were collected from Oyo, Osun and Ondo states and that of CRIN (Cocoa Research Institute of Nigeria) serves as control and were bulked into 9 composite samples. The beans were cultured using pour plating technique on potato dextrose agar routinely prepared in the laboratory. The cultured isolates were subjected to morphological identification and aflatoxin was assayed from cocoa beans using thin layer chromatography technique. The moisture content ranged from 5.4 to the highest of 8.0. The pH indicated all were acidic, and it ranged from 0.0 to 6.1. Nine mycobiota species were distinctly identified from the cocoa beans: *Aspergillus flavus*, *Syncephalastrum racemosu*, *Penicillium digitatum, P. roquefortii, Geotichum candida*, *Fusarium graminaerum, A. niger, A. carbonarius*and *Rhizopus stolonifer.* High microbial load was recorded in beans of Abiri (0.312 cfu/ml and 62.0×10^{-5}cfu/ml), Aba nla (304×10^{-3}cfu/ml and 106×10^{-5}cfu/ml), Elebesere camp (200×10^{-3} cfu/ml and 92×10^{-3}cfu/ml), Calendar camp (176×10^{-3}cfu/ml and 9.0×10^{-5}cfu/ml), Amonloje (108×10^{-3}cfu/ml and 50×10^{-5}cfu/ml). All the cocoa beans sample from the selected study location do not show the presence of aflatoxin as classified by both Rf (Retention Factors) value and colour under long wavelength UV light. Aflatoxin was not detected though aflatoxingenic fungi that were isolated in the study, suggesting that aflatoxin production may not be as a result of storage.

Keywords: Pathogens, toxins, storage, contamination, cocoa.

INTRODUCTION

Cocoa trees produce to the maximum of its potential under very humid tropical climates. Mostly grown in West Africa with significant production from Asia and Central and South America. The crop is also an important raw material in chocolate industry. The primary processing of cocoa is carried out on the farm, pods are harvested and opened by hand (Dongo, 2005). The beans are heaped in wooden boxes for the natural fermentation process. There are different ways of contamination of beans by microorganisms including infected farmers' equipment, hands of workers, the pod surface exposed to contaminated objects, pests, leaves of plants and various other tools used during production, harvesting, transportation, and storage (Orole, 2016). The needed categories are yeasts, acetic and lactic bacteria, producing enzymes, alcohol, and acetic and lactic acids (Schwan and Wheals, 2004). These chemical molecules caused the death of embryo and the synthesis of vital antecedents of chocolate aroma (Voigt, 2013). There is a reduction in microbial growth and the production of enzymes by first fermenting the beans for some days, then transferring them to fabricated wooden sun-drying stands or sheets or ground or cemented surfaces to reduce the bean moisture content and acids. Once the beans are dried through the above-mentioned processes, they are stored in the rooms

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before bagging them for sale. The period of beans' conservation in storage is dependent on the economic status of the farmers (Adebayo, 2016).

Cocoa beans are prone to fungal infection at different stages of postharvest handlings. The presence of filamentous fungi in cocoa and chocolate becomes a cause for concern due to the possibility of mycotoxin formation. The intrinsic parameters of cocoa beans such as pH, due to water activity and various organic acids produced during fermentation affect microbial growth. Apart from causing deteriorative alteration of sensorial properties (Copetti *et al*., 2016).

In Nigeria, the main crop of cocoa pods harvesting season is during the peak of rainfall. The pods are broken in the fields and the wet seeds are transported from the field to the homesteads for fermentation. The growth of *A. ochraceus, A. flavus*, *Penicillium* spp, and *Fusarium* spp are enhanced by some of the above-mentioned practces (Fapohunda *et al*., 2020, Dongo, 2005). Cocoa beans and their seeds are infected by the main contaminants called filamentous fungis (Adebayo, 2016; Orole, 2016). It has been reported that these mycotoxin-producing fungi that infect cocoa beans and seeds are responsible for stern health hazards to both animals and humans (Mounjouenpou *et al,* 2008; Fapohunda *et al*., 2012). To the best of our knowledge, there is rare evidence on the nature, effects, and occurrence frequency of mycotoxigenic fungi on cocoa produced in Nigeria. Knowing the major fungi in beans and seeds of cocoa is a very vital tool for abating mycotoxin menaces and risks in animal and human health (Copetti *et al*., 2014; Fapohunda *et al,* 2020).

The growth of the mould and the subsequent production of mycotoxins is dependent upon some factors such as relative humidity, and temperature during the growth and development, gathering and harvesting, processing, drying, and storage of cocoa crop produce. (Orole, 2016; Fapohunda *et al,* 2020). Cocoa is processed for both local consumption and exportation to developed countries. It has been recently reported that the production of cocoa is increasing with many farmers coming into the business after it has been neglected for many years (Fapohunda *et al,* 2020). It is of capital importance to properly do the inventory and document the mycobiota associated with cocoa beans in Nigeria to protect the lives and health of Nigerians.

Mycotoxins are produced by fungi as secondary metabolites toxic to humans and animals causing lower performance, sickness, or death when ingested, inhaled, or absorbed through the skin. Agricultural produce when contaminated with mycotoxins-producing fungi present serious problems, for both human and animal health and also reduces the economic value of crops (Adebayo, 2016; Mounjouenpou *et al,* 2008). Naturally mycotoxins are found in various plant products and all the stages of crop production including during crop growth, harvesting, and storage especially when storage facilities and drying technology are absent or can't be afforded by farmers. Many factors including humidity and temperatures significantly contribute to the mould growth and secretion of mycotoxins during crop production and post havest handling (Mounjouenpou *et al,* 2008).

Nigeria has a tropical climate with all-year-round high ambient temperature and relative humidity that provide optimal conditions for the growth of mycotoxigenic molds (Dongo, 2005). Thus, the objective of this study was (i) to assess the occurrence frequency of mycotoxin-producing fungi in cocoa beans and (ii) to determine the level of mycotoxins in the stored cocoa beans in the selected States of South Western Nigeria.

MATERIALS AND METHODS

Collection and preparation of cocoa bean: Twenty-seven dry cocoa bean samples, about 1kg from different origins (9 from Oyo; 8 from Osun; 9 from Ondo; store of Cocoa Research Institute of Nigeria (CRIN) as control (1) were obtained during the light cocoa cropping of 2022 and 2023 season in the selected study locations. The beans were collected, bulked, mixed to obtain heterogeneous samples, and kept in sterile and transparent Ziploc bags. Samples were transferred to the laboratory for further studies. The beans were milled using a sterile mechanical grinder, and 1g bean sample was weighed into 9 ml sterile water in a test tube to make a 10^{-1} dilution factor. This was repeated in sets of test tubes to obtain 10^{-3} and 10^{-5} dilution factors. The pH and moisture content of the bean samples were determined using standard laboratory procedures (Copetti, 2018; Barnett and Hunter, 1998).

Table 1. Cocoa beans obtained from selected local government areas and States

S/N	Community	Local Government Area (LGA)	State
1	Laduni	Ido	Oyo
2	Idiya	Ido	
3	Ido	Ido	
4	Oluyole	Oluyole	
5	Gambari	Oluyole	
6	Adeogun	Oluyole	
7	Elesuru	Akinyele	
8	Aba nla	Akinyele	
9	Ijaye	Akinyele	
10	Amonloje	Ife Central	Osun
11	Eleweran	Ife Central	
12	Odan asun	Ife Central	
13	Aba	Ife East	
14	Oyere	Ife East	
15	Kuola	Ife East	
16	Abiri	Ife South	
17	Ara	Ife South	
18	Agewale	Odigbo	Ondo
19	Ewenla	Odigbo	
20	Ajewe	Odigbo	
21	Adebanjo	Ondo West	
22	Bagbe	Ondo West	
23	Ogbbogoro	Ondo West	
24	Owena	Idanre	
25	Calendar camp	Idanre	
26	Elebesere camp	Idanre	
27	Idi-Ayunre (CRIN)	Oluyole	Oyo

Culturing and identification of fungal isolates: The mycobiota was isolated from bean samples using the pour plating method from beans of farmers' stores (treatment) and storage of Cocoa Research Institute of Nigeria (CRIN), One milliliter (1ml) of the third (10^{-3}) and fifth (10^{-5}) dilution factors of the bean pour-plated by inoculating it in a sterile Petriplate. Potato Dextrose Agar (PDA, sterilized in the autoclave at 121°C for 15 minutes was cooled, acidified with 10% lactic acid solution, poured into the inoculated Petri plate, and incubated at ambient temperature (28±2°C) for five to seven days (Dongo, 2005).

Colony growths were observed and recorded after incubation on the 7th day The fungi population and morphological appearance of associated fungi were recorded. Pure cultures of the isolates were observed for purity, and then fungi were identified according to the methods of Samson *et al.,* (2007) and Barnett and Hunter (1998).

Aflatoxin assay using thin layer chromatography technique: The composite samples of the cocoa beans were subjected to aflatoxins analysis using established TLC plate technique and retention factor (Rf) values calculated according to Singh (1991); Copetti (2014).

DATA ANALYSIS

All data collected were analyzed using SAS software package. Correlation analysis of microbial load and water activity of the cocoa bean samples was done. The mean of the microbial population of occurrence of fungi was obtained using Excel.

RESULTS AND DISCUSSION

The cocoa beans were collected through a survey of farmers` stores in the targeted cocoa-growing communities across the study regions which are major cocoa-producing states in Nigeria. The bean samples were obtained from the private stores of farmers and not warehouses. The details of the origin of the stored beans are listed in Table 1. Cocoa beans are a key raw material used in the chocolate industry. Cocoa of the commercial grade should follow and conform to international and standard criteria such as the absence of contaminated pathogenic fungi and the production of mycotoxins

(Aroyeun et al., 2007). The results show that the moisture of the beans ranges from the least moisture of 5.43 in Agewale in Ondo state to the highest water content of 8.00 in Oluyole (Oyo), Amonloje, Ara (Osun) and Ajewe (Ondo). The degree of acidity and alkalinity (pH) of the beans showed that all the cocoa beans are acidic from 0.0 to 6.14 recorded in Ogbogoro (Ondo state) and Adeogun (Oyo state), respectively (Table 2).

Table 2. Microbial population, water content, and pH of cocoa beans in the study areas

Sample location	*Colony forming unit (cfu/ml)x 10^{-3}	*Colony forming unit (cfu/ml) 10^{-5}	Moisture content	pH
Laduni (Ido)	38.0	1.5 x	7.10	4.30
Idiya (Ido)	2.0	1.0	7.20	4.95
Ido	2.0	0.0	7.90	5.27
Oluyole	19.5	14.5	8.00	5.78
Gambari (Oluyole)	1.5	0.0	7.70	5.70
Adeogun (Oluyole)	0.0	60.0	7.40	6.14
Elesuru	11.0	9.5	7.20	5.90
Aba nla	304	106.0	7.40	5.15
Ijaye	11.0	2.5	7.50	4.95
Amonloje (Ife Central)	108.0	50.0	8.00	4.84
Eleweran (Ife Central)	68.0	33.0	7.20	4.47
Odanasun (Ife Central)	3.0	1.5	7.30	5.36
Aba (Ife East)	16.0	1.5	7.20	5.61
Oyere (Ife East)	0.5	0.5	7.70	5.35
Kuola (Ife East)	44.5	17.5	7.20	5.72
Abiri (Ife South)	312.0	62.0	7.20	5.09
Ara (Ife South)	1.5	0.5	8.00	4.85
Asewale	0.5	0.5	5.43	5.43
Ewenla	8.0	0.0	5.68	5.68
Ajewe	32.0	7.5	8.00	5.40
Adebanjo	14.5	1.5	7.40	5.58
Bagbe	50.0	4.0	7.90	5.43
Ogbogoro	1.5	0.5	7.50	0.00
Owena	2.0	0.5	7.60	5.38
Calendar camp	176	9.0	7.30	5.87
Elebesere camp	200	92.0	7.50	5.73
Idi-Ayunre (CRIN)	1.5	0.5	7.70	5.70

*Mean of values

The mycobiota analysis of the cocoa beans samples showed high microbial load and population in the beans of Abiri (312.0 x 10^{-3} cfu/ml and 62.0 x 10^{-5}cfu/ml), Aba nla (304 x 10^{-3}cfu/ml and 106 x 10^{-5}cfu/ml), Elebesere camp (200 x 10^{-3} cfu/ml and 92 x 10^{-3}cfu/ml), Calendar camp (176 x 10^{-3}cfu/ml and 9.0 x 10^{-5}cfu/ml), Amonloje (108 x 10^{-3}cfu/ml and 50 x 10^{-5}cfu/ml) sourced from Ife South (Osun state), Akinyele (Oyo state), Idanre (Osun state) and Ife Central (Osun state), respectively.

The microbial population was the same (0.5 x 10^{-5}cfu/ml) in Oyere, Ara both in Osun state an Agewale, Ogbogoro and Elebesere camp all in Ondo state. Similar microbial load records of 1.5 x 10^{-3} were found in the communities above except in Ondo state (Agewale and owena). The 1.5 x 10^{-3} cfu/ml was also recorded in cocoa beans of Gambari origin.

Cocoa beans of Idiya (Oyo state) and Owena (Ondo state) origin had same load of 2.0 x 10^{-3}cfu/ml. The least microbial population in both the third and fifth dilution factors (0.5 x 10^{-3} cfu/ml and 0.5 x 10^{-5}cfu/ml) of the beans samples was recorded in Oyere in Osun state and Agewale in Ondo state (Table 2).

Nine mycobiota isolates from six fungi genera were obtained from the stored cocoa beans obtained from selected growing communities in South Western Nigeria. Figures 1 to 9 show a pictorial representation of the associated above-mentioned fungi on PDA cultured from cocoa bean samples sourced from the areas of study. The figure expressed the picture of the fungi on petri plates and the imaging under a light microscope. The fungi are *Aspergillus flavus* (Figure 1), *Syncephalastrum racemosu* (Figure 2), *Penicillium digitatum* (Figure 3), *Penicillium roquefortii* (Figure 4), *Geotichum*

candida (Figure 5)*, Aspergillus niger* (Figure 6)*, Fusarium graminaerum* (Figure 7), *A. carbonarius* (Figure 8), *and Rhizopus stolonifera* (Figure 9) were cultured through routine laboratory isolation procedures.

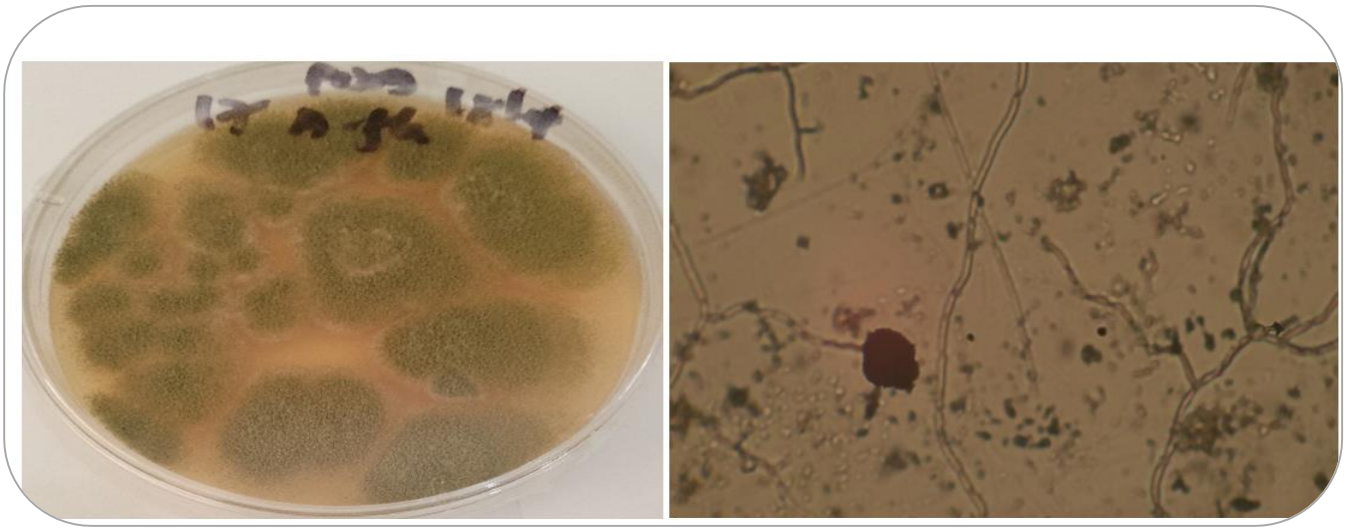

Figure 1. Aspergillus flavus under light miscroscope (x10)

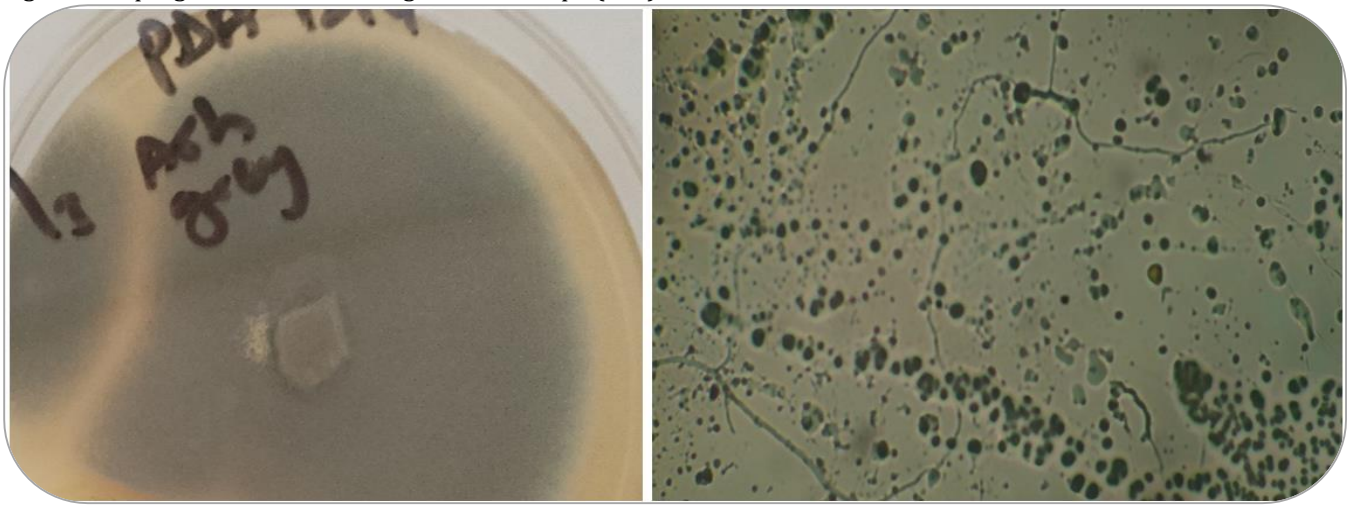

Figure 2. *Syncephalastrum racemosu* under light miscroscope (x10)

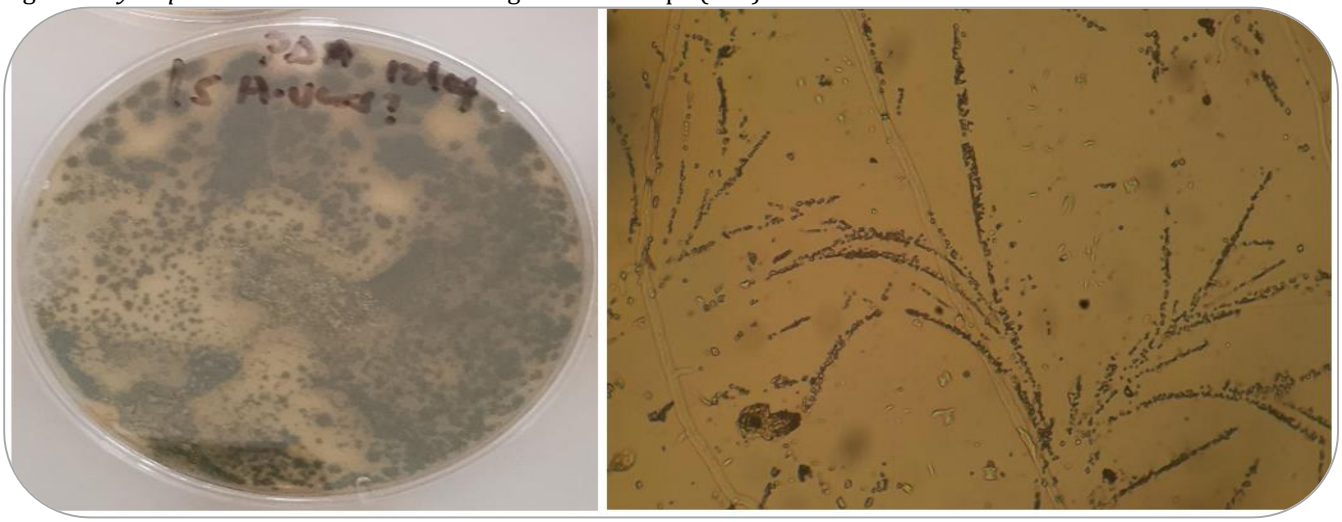

Figure 3. *Penicillium digitatum* under light miscroscope (x10)

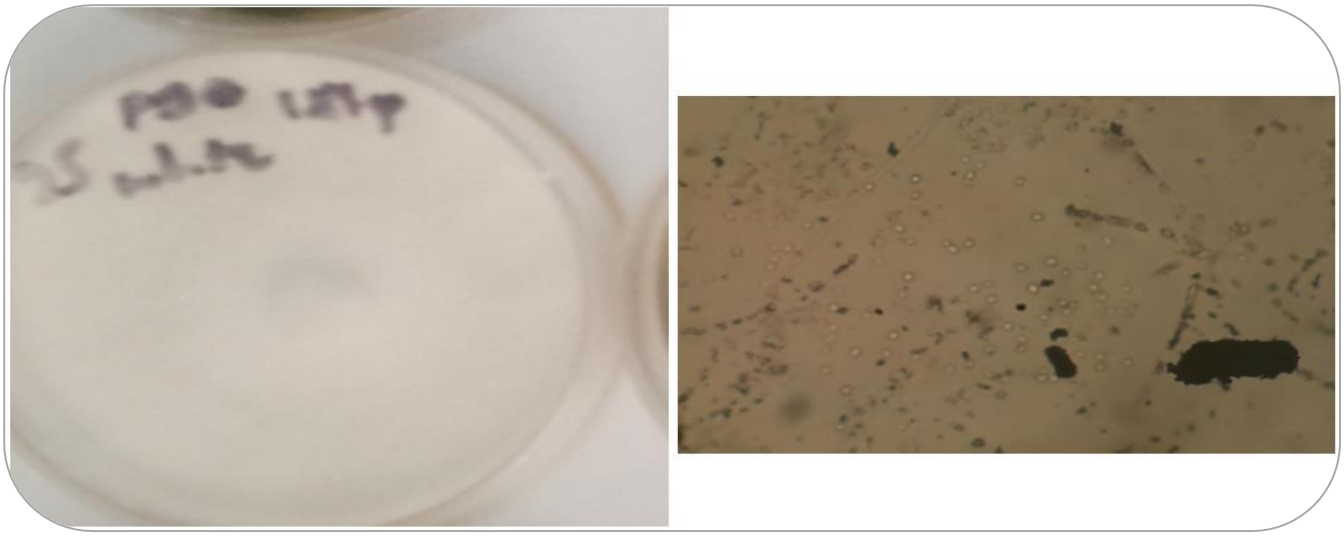

Figure 4. *Penicillium roquefortii* under light miscroscope (x10)

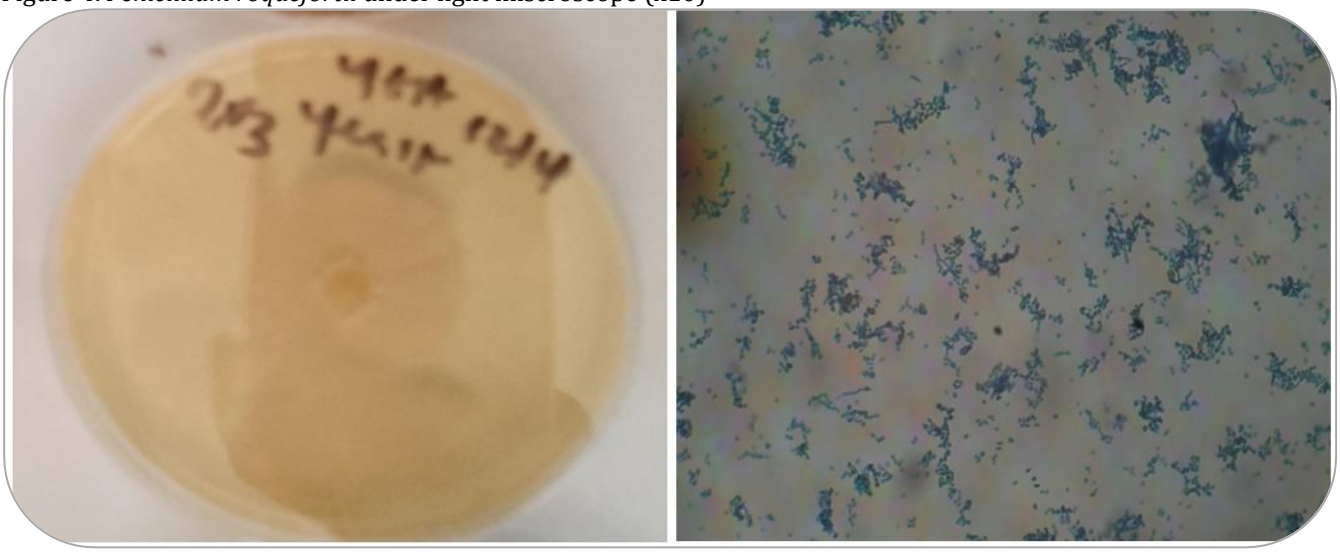

Figure 5. *Geotichum candida* under light miscroscope (x10)

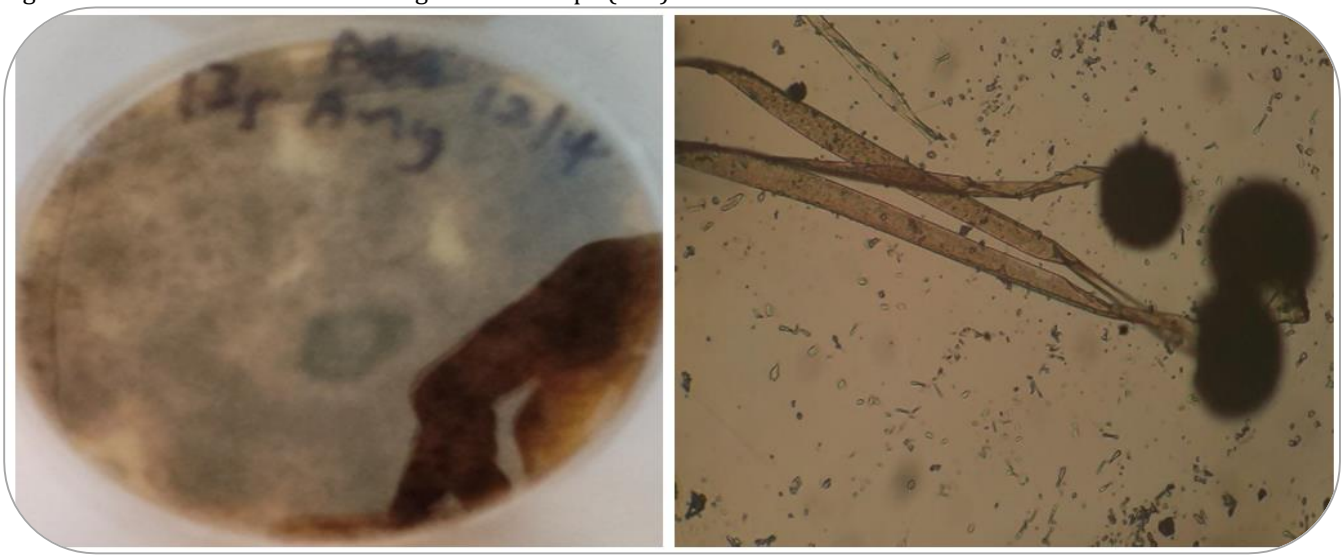

Figure 6. *Aspergillus niger* under light miscroscope (x10)

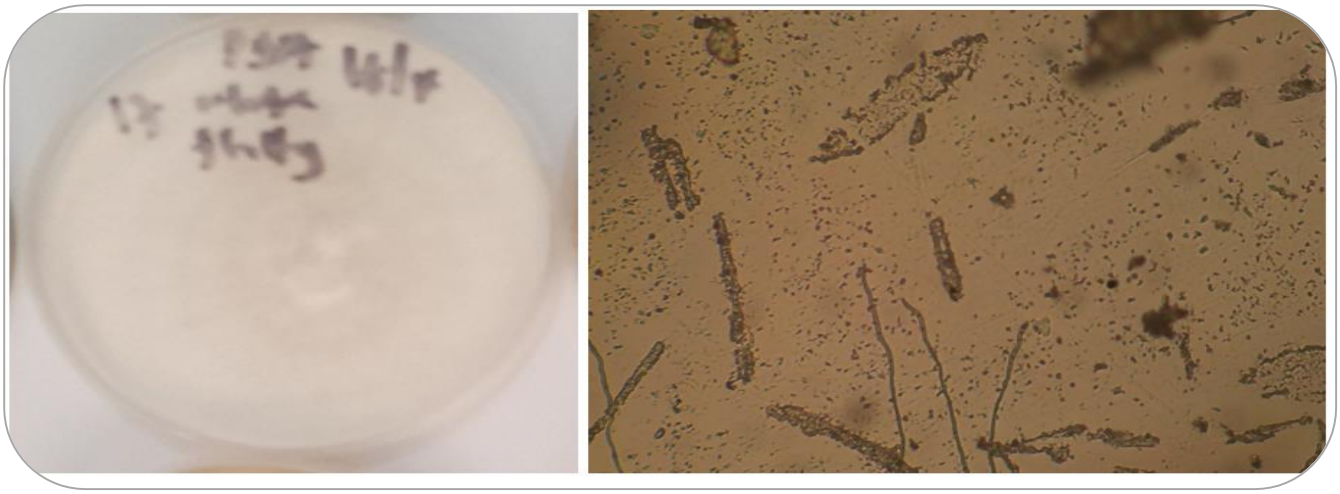

Figure 7. *Fusarium graminaerum* under light miscroscope (x10)

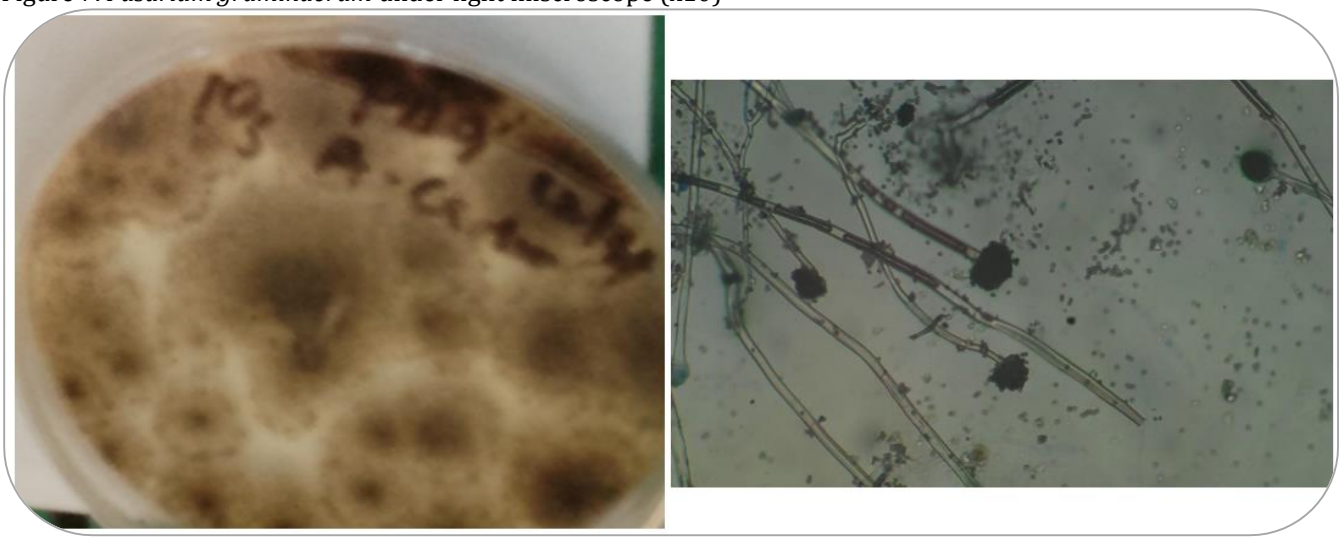

Figure 8. *Aspergillus carbonarius* under light miscroscope (x10)

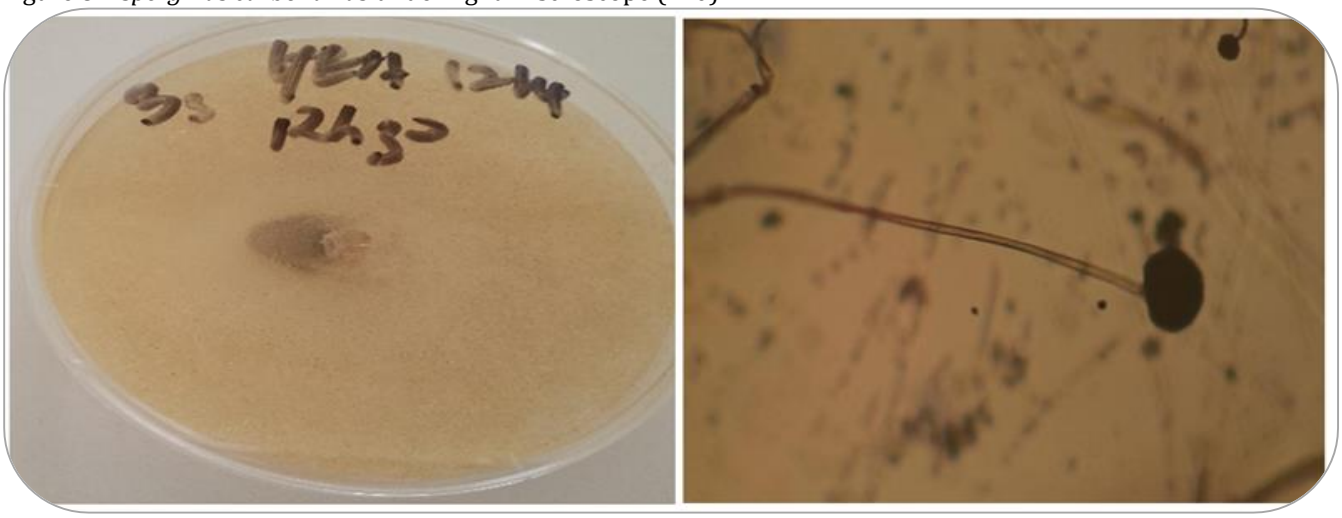

Figure 9. *Rhizopus stolonifer* under light miscroscope (x10)

The occurrences of the fungi associated with store cocoa beans in the study varies across study locations, although the variation margin was not very distant. Percentage association of the fungal isolates was the highest

(38.60%) in Osun State and similarity of 29.50 % and 27.25% occurrences were recorded in Oyo and Ondo States respectively while the control recorded4.45 %.

Out of the total of nine fungal isolates cultured from cocoa bean from study locations, five fungi were reportedly associated with the beans sourced from Oyo state, all fungi except one were cultured from Osun State, and only four in Ondo State (Table 3). *Syncephalastrum racemosu* was recorded only in Osun State l while *P. digitatum, P. roquefortii were recorded* in Osun and Oyo States respectively.

The record of the microbial analyses also showed that each of *A. niger* and *F. graminaerum* was associated with cocoa beans in the three study States except Ondo and Oyo States respectively.

However, *G. candida, A. flavus* and *R. stolonifera* were all commonly recorded in the study states. In Oyo State, *G. candida and A. flavus* were the most (9.08%) associated fungi while *P. roquefortii* occurrence was 2.27% in the same state (i.e. in Oyo State).

Rhizopus stolonifera and *A. flavus* (9.08%) were the highest occurrences in stored cocoa beans sourced from Osun and Ondo States respectively but the least common fungi: *A. carbonarius, P. digitatum* and *S. racemosu* occurrences were 2.27% in Osun while 4.54% were recorded for *F. graminaerum* in Ondo State.

Table 3. Percentage (%) of fungal isolates associated with cocoa beans in study locations

S/N	Fungal isolates	Oyo state(%)	Osun state(%)	Ondo state(%)	Control (%)	Overall(%)
1	*Geotichum candida*	9.08	6.82	9.08	2.27	27.25
2	*Aspergillus flavus*	9.08	4.54	9.08	2.27	24.98
3	*Rhizopus stolonifer*	4.54	9.08	4.54	0	18.16
4	*Aspergillus niger*	4.54	4.54	0	0	9.08
5	*Fusarium graminaerum*	0	4.54	0	0	9.08
6	*Aspergillus carbonarius*	0	2.27	0	0	2.27
7	*Penicillium roquefortii*	2.27	0	0	0	2.27
8	*Penicillium digitatum*	0	2.27	0	0	2.27
9	*Syncephalastrum racemosu*	0	2.27	0	0	2.27
	Total(%)	29.52	38.6	27.25	4.54	99.9

Dongo (2005) in his work has reported *Aspergillus* as the most frequently isolated genus in cocoa beans, with occurrence of 46.3% of total genera isolated. *Aspergillus flavus,* and *A. tamarii*were the most prevalent species followed by *A. oryzae. Aspergillus parasiticus, A. niger,* and *A. sojae* were less frequently isolated. *Fusarium* ranked next in the number of cases of isolation while *Rhizopus* and *Penicillium* are included (Dongo, 2005).

Earlier works have reported similar fungal incidences on different crops, for example, Hasan and Abdel-Sater (1993) studied Mycoflora and Aflatoxin in regular and decaffeinated black tea and indicated that *Aspergillus* was isolated in all samples comprising 92.3% of total fungi.

Their results equally showed that *A. flavus* was the most commonly encountered. On the fungal contamination of fruits and vegetables, (Peter *et al.,* 1990) reported *Aspergillus* as the most frequently encountered genera in 95% of the samples.

The microbial load in the third and fifth dilution factors were correlated with the moisture and the pH of the cocoa bean samples from the study areas. The results showed a weak negative correlation between the microbial load of the beans at the third dilution factor and the moisture content and pH of the beans. Any increase in moisture and pH of the beans results in a reduction in the microbial population in the third dilution, thus a higher dilution factor is required to have a better expression of the microbial population.

However, there exists a positive correlation between the fifth dilution factor and the moisture content of the beans, although very weak. Any increase in moisture brings about an increase in the population of the mycobiota expressed in the fifth dilution factor. However, both the fifth dilution factor and moisture content were weakly and negatively correlated with the pH of the cocoa beans (Table 4).

Table 4. Correlation analysis of microbial load and water activity in cocoa beans

	Logcfu/ml (10-3)	*Logcfu/ml (10-5)*	*Moisture content*	*pH*
Logcfu/ml (10^{-3})	1			
Logcfu/ml (10^{-5})	0.306794118	1		
Moisture content	-0.046340617	0.115312624	1	
pH	-0.037821463	-0.032728999	-0.075340068	1

Table 5 shows the value of retention factors (Rf) of the cocoa bean samples sourced from selected study areas in the extracts of the beans when eluted using chloroform: acetone (96:4 v/v) solvent was obtained and matched with the reference. All the extracts tested far exceed the Rf of the aflatoxin reference.

Table 5. Result of aflatoxin analysis

S/N	Sample location (LGA)	Retention factor (Rf) value
1	Ido	0.96
2	Oluyole	0.96
3	Akinyele	0.89
4	Ife Central	0.94
5	Ife East	0.90
6	Ife South	0.90
7	Odigbo	0.90
8	Ondo west	0.86
9	Idanre	0.93
	Reference	0.58

This confirmed the absence of aflatoxin in the samples tested as shown in Figure 10. This finding corroborates the earlier report of Fapohunda et al. (2020) which stated that none of the extracts eluted from chloroform: acetone solvent has a greenish blue color of aflatoxin when observed in a dark cupboard under a long wavelength UV light.

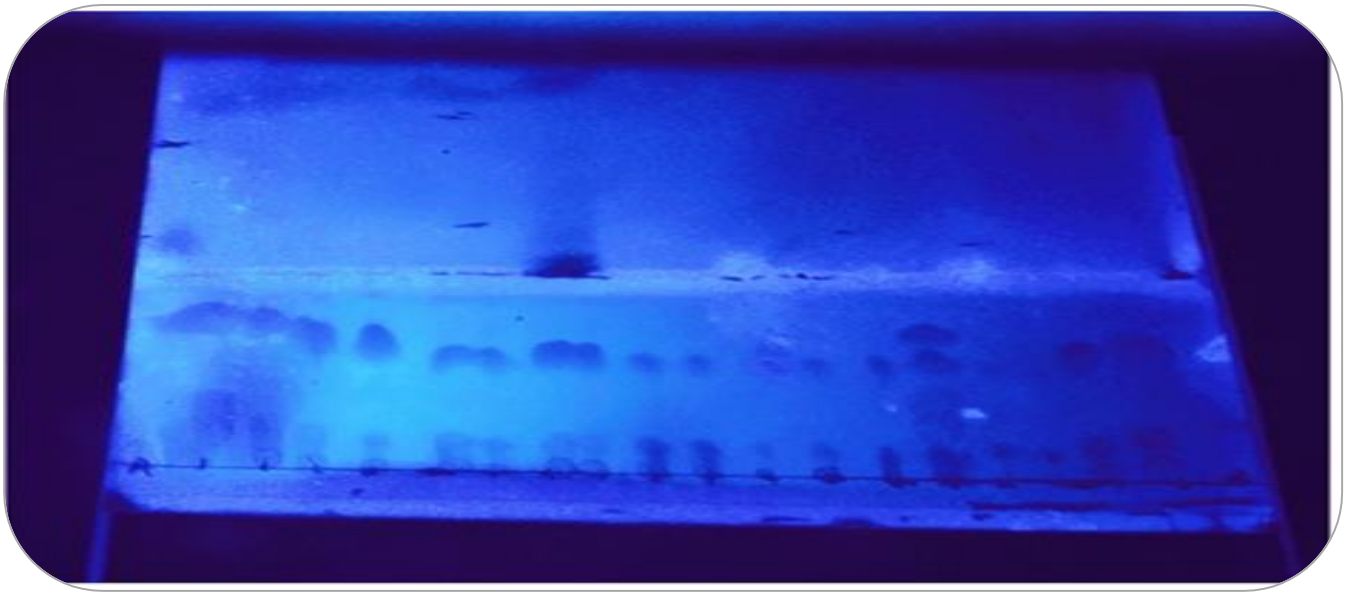

Figure 10. TLC plate of extracts testing for aflatoxin

This study contradicts the findings of (Baby *et al*., 2015) which reported the detection of aflatoxin from *A. flavus* and *A. oryzae,* although other reports had it that *A. niger*, *A. oryzae*, and *A. tamarii* have never produced aflatoxin.

However, none of the other isolates were found to produce aflatoxin. *Aspergillus niger* and *A. violaceofuscus* have the potential to produce ochratoxin A (OTA) but detected only a trace level of OTA from *A. violaceofuscus* (Baby *et al*., 2015).

Syncephalastrum racemosum has been reported to cause various diseases in humans (Khrisna *et al.,* 2012). *Aspergillus niger* has been reported to produce beneficial compounds such as amylase enzyme (Kareem *et al.,* 2010), as well as citric acid (Frisvad *et al.,* 2011). Some strains of *A. niger* have been reported to produce mycotoxins such as fumonisin and ochratoxin (Chinnasamy *et al.,* 2011).

Aspergillus flavus is most known for its ability to produce aflatoxins [Fapohunda *et al*.,; IARC, 2012), toxic metabolites which have been rated as class 1A carcinogen by the International Agency for Research of Cancer (IARC, 2012). It also produces a protease enzyme that may be useful to industry.

CONCLUSION

Aspergillus flavus, A. niger, A. carbonarius were isolated

and none of the cocoa bean extracts contained aflatoxin when subjected to analysis using the TLC. Though these are potential aflatoxin producing fungi present in the stored Cocoa beans from three different states in Nigeria. There was no correlation between the aflatoxin content and aflatoxin producing fungi isolated from stored cocoa beans in the study. These showed that strains of aflatoxingenic fungi isolated in the study may be a non-toxin producer. This might indicate that aflatoxin production may not be as a result of storage, but more likely linked to adverse conditions during harvesting, drying and transportation stage.

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