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Studies on Aflatoxin-Producing Fungi in Stored Maize (*Zea mays L.*) and the Use of Bentonite Clay in Reducing their Toxin Levels

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ABSTRACT

Aflatoxins are a group of mycotoxins produced by *Aspergillus* fungi that are both toxic and carcinogenic to animals and humans; however, studies on their removal by natural substances have had great success, and bentonite was seen as a possible remedy in this aspect. The study on aflatoxin-producing fungi in stored Maize (*Zea mays L.*) was conducted on stored maize collected from Muna Market, Maiduguri, based on their year of harvest (2015, 2016, 2017, and 2018). Out of which a total of 49 isolates consisting of *Aspergillus* spp, *Talaromyces islandicus,* and *Scopulariopsis candida* were obtained, these include *Aspergillus niger* 38.78%, *A. flavus* 12.24%, *A. oryzae* 12.24%, *A. fumigatus* 10.20%, *A. parvisclerotigenus* 6.12%, *A. aflatoxiformans* 4.08%, *Scopulariopsis candida* 14.29%, and *Talaromyces islandicus* 2.04%. The molecular assay confirms the identity of the isolates amplified using universal ITS primers with 100% query and identity, except sample A (*A. aflatoxiformans* 087-A2) has 99.62% identity compared with NCBI library. The total aflatoxin profile of the stored maize collected for the 2015 harvest year was 100 ppb before treatment and reduced to 2.2 ppb after treatment. In contrast, the 2016 maize sample, which had 1.8 ppb before treatment, reduced to 1.7 ppb; the 2017 sample had 1.9 ppb reduced to 0.9 ppb; and 2018 had 3 ppb before treatment reduced to 0.3 ppb after treatment; the AfB1 profiles depict that 2015 maize sample had 60 ppb before treatment and reduced to 1ppb after treatment. The 2016 maize sample had one ppb reduced to 0.9 ppb, whereas the 2017 sample had 1 ppb and reduced to 0.6 ppb, and 2018 had 2 ppb and detoxified to 0 ppb after treatment. The Bentonite clay used was found to reduce the toxin levels of the stored maize.

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Introduction

Aflatoxins are a group of mycotoxins produced by *Aspergillus* fungi that are both toxic and carcinogenic to animals and humans (Murphy *et al*., 2006), are also chemical compounds produced by Aspergillus species commonly present in a variety of agricultural and livestock

products(Usman *et al*., 2019). Aflatoxin B1 (AfB1) and mixtures of aflatoxin B1, G1, and M1 are proven human carcinogens (IARC1993). Aflatoxin B1 is the most toxic and abundant of the aflatoxins. An estimated 4.5 billion people in developing countries are chronically exposed to uncontrolled amounts of aflatoxins (Williams *et al.,* 2004). Before 1985, the Food and Agriculture Organization (FAO) estimated global food crop contamination with mycotoxins to be 25% (Escola *et al.,* 2020)

The nutritional value of stored maize grains (*Zea mays L.*) could vary significantly due to the interaction between the physical, chemical, and biological factors. (Chulze, 2010).. Aflatoxin causes a serious problem in many foods. Still, it is most abundant in maize and maize products because maize could be infected even in the field under specific environmental conditions (Benkerroum, 2020). The contamination of maize depends on the co-existence of the susceptibility of hybrids and the favorable environmental conditions for the proliferation of aflatoxigenic fungi (Blandino *et al.,* 2009).

Bentonite clay is a naturally occurring material composed predominantly of the clay mineral smectite. Most bentonites were formed by altering volcanic ash in marine environments and occur as layers sandwiched between other types of rocks. The smectite in most bentonites is the mineral montmorillonite (Hillier and Lumsden, 2008). Using clay-based adsorbents has effectively reduced the toxic effects of aflatoxin contamination in animal feeds (Philips, 1999). Specifically, bentonite clays are adsorbent aluminum silicates that are capable of adsorbing aflatoxin within the clay Smectite layer, which allows any adhering aflatoxin to pass through the gastrointestinal tract unabsorbed (McClure *et al.,* 2014)

Methods and Materials

The bentonite clay sample was collected from Monguno, Borno State. Under the aseptic condition, the clay was collected using a clean spade and parked into a sterile polyethylene bag safe for transport to the laboratory; the clay's pH and temperature were determined on-site. The stored Maize grain samples were collected from Muna Market of Maiduguri based on their year of harvest (2015, 2016, 2017, and 2018) and it was transported to Microbiology Laboratory, Department of Science Laboratory Technology of Ramat Polytechnic, Maiduguri, for the analysis.

Isolation and Characterization

The aflatoxigenic fungi were cultured on Saboroud Dextrose Agar (SDA) at 25 ^{o}C for seven days and stored on 20% glycerol for further analysis (Olivier *et al.,* 2017). Morphological and growth characteristics were studied after subculturing on Sabouraud Dextrose Agar (SDA) for identification purposes. Isolates were identified using cultural and morphological features such as growth patterns, conidial morphology, and pigmentation (Tafinta *et al.,* 2013). Microscopic observation was then carried out by placing a drop of lactophenol cotton blue stain on a glass slide; a portion of the fungal mycelia from a pure culture was added and

covered with a coverslip to avoid air bubbles. Viewing was done using the x10 and x40 objective lens, and organisms were identified (Oyeleke and Manga, 2008).

DNA Isolation / Extraction

The DNA was extracted using a lysis buffer containing hexadecyltrimethylammonium bromide (CTAB). Mycelia mats were pulverized in 5 ml of 3-mm-diameter glass beads in a disposable 50-ml conical centrifuge tube. Ten milliliters of DNA extraction buffer (l.0 M Tris/HCl, pH 7.5; 1% (*w*/*v*) CTAB; 5 M NaCl; 0.5 M EDTA; 1% (*v*/*v*) 2-mercaptoethanol; and proteinase K at 0.3 mg/ml) was added to the powdered mycelia and mixed gently, and the mixture was incubated at 65°C for 30 minutes. The extracts were cooled before adding an equal volume of chloroform. It was then gently mixed and centrifuged at 6000 rpm for 10 minutes. The aqueous supernatant was recovered, and the nucleic acids were precipitated with an equal volume of 2-propanol gently mixed. This resulted in the formation of high molecular-mass DNA, precipitated by centrifugation at 4800 rpm for 5 minutes. The DNA was resuspended in TE buffer solution (Tris-EDTA, pH 8.0) containing RNase A at 10 µg/ml and further purified by phenol-chloroform extraction (A260/A280 ratio of 1.8–2.0). Finally, the DNA was precipitated with 100% ethanol containing 3M sodium acetate, rinsed in 70% (*v*/*v*) ethanol, and resuspended in TE buffer (Wang *et al*., 2012).

Polymerase Chain Reaction (PCR)

PCR was performed in 25µl volume containing 12.5µl master mix, 1µl each of the universal ITS primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was used. 5µl of the DNA template and 70 µl of molecular grade water were added in the preparation. The ITS region was amplified using the following PCR program: initial denaturation at 95°C for 5 minutes, 95°C for 30 seconds, 58°C for 30 seconds, and 75°C for 45seconds (32 cycles) and final elongation at 72°C for 7 minutes; reaction tubes were held at 4°C as final steps of PCR amplification, according to the protocol.

Agarose Gel Electrophoresis

For the agarose gel electrophoresis, 1% agarose gel was prepared by weighing 1g of agarose into 100 ml of (1×) TE buffer and microwaving for 1-2minutes. 2.5µl of ethidium bromide was added, and the cast was set with a comb inserted. The melted agarose was poured onto the cast and allowed to solidify, and then 5µl of the PCR product was mixed with 1µl loading dye. The whole content was loaded into the wells. The gel is placed into the tank with the TE buffer filled to the appropriate mark and run at 100v for 30 minutes; the gel is then dried and viewed using a gel imager (Biorad)(Sambrook and Russel, 2001).

Sequencing and BLAST

The PCR products obtained were sent to Inqaba Biotec Centre, South Africa, for sequencing, and BLAST was performed against the NCBI database.

Detection and Quantification of total aflatoxin and aflatoxin B1

The stored maize samples' total aflatoxin and aflatoxin B1 (AFB1) content Five grams (5g) of the maize samples were weighed and added to 25 ml of methanol (70%). It was allowed to stand for 10 minutes to aid aflatoxin extraction, then filtered using a No. 1 Whatman filter paper. 50 µl of sample filtrate and aflatoxin standard were dispensed in separate dilution wells, each with 100 µl of the conjugate. 100 µl from the filtrate/standard-conjugate mixture was dispensed in the antibody-coated wells. It was then incubated at room temperature for 15 minutes. The content of the wells was discarded, and the wells were washed 3 – 4 times with distilled water. 100 µl of the substrate was added to each well and incubated for 5 minutes to allow for color change (different shades of blue to colorless). 100 µl of stop solution was added, which converts the blue end-point to yellow, and then the mixture was read with an ELISA plate reader at 450nm. The optical densities of standards (0 ppb, 4 ppb, 10 ppb, 20 ppb, and 40 ppb) and those of samples were recorded. A standard curve was generated, which was used to extrapolate the concentrations of total aflatoxin and aflatoxin B1 in the samples. Samples with high levels of aflatoxin were diluted further with 70% methanol to 1/10th to 1/20th of the original concentration to obtain readings within the standard curve range.

Results

From this study, the frequency of the isolates obtained in the stored Maize samples collected from Muna Market Maiduguri, a total of 49 isolates consisting of *Aspergillus* spp, *Talaromyces islandicus,* and *Scopulariopsis candida* were obtained from the maize samples with *Aspergillus niger* has the highest frequency of occurrence 38.78%, followed by *A. flavus* 12.24%, *A. oryzae* 12.24%, *A. fumigatus* 10.20%, *Scopulariopsis candida* 14.29%, *Talaromyces islandicus* 2.04%, *Aspergillus parvisclerotigenus* 6.12%. *A. aflatoxiformans* had the lowest frequency of occurrence, with 4.08%. The distribution of isolates obtained across the year of harvest reveals that 2015 maize sample has a total of 21 isolates (42.86%), 2016 maize sample has 11 isolates (22.45%), 2017 has 10 isolates (20.41%), and 2018 has 7 isolates (14.29%) as shown in Table 1.

Table 1: Frequency of occurrence of fungi isolated from stored maize samples collected from Muna Market, Maiduguri, harvested between 2015-2018.

Isolates/harvest year	2015	2016	2017	2018	Total
Aspergillus niger	8(42%)	3(16%)	5(26%)	3(16%)	19(100%)
Aspergillus flavus	1(17%)	2(33%)	2(33%)	1(17%)	6(100%)
Aspergillus fumigatus	2(40%)	1(20%)	1(20%)	1(20%)	5(100%)
Aspergillus oryzae	1(16.6%)	3(50%)	1(16.6%)	1(16.6%)	6(100%)

Scopulariopsis candida	3(43%)	2(29%)	1(14%)	1(14%)	7(100%)
Talaromyces islandicus	1(100%)	0(0%)	0(0%)	0(0%)	1(100%)
A. parvisclerotigenus	3(100%)	0(0%)	0(0%)	0(0%)	3(100%)
A. Aflatoxiformans	2(100%)	0(0%)	0(0%)	0(0%)	2(100%)
Total	21(42.86)	11(22.45)	10(20.41)	7(14.29)	49(100%)

The total aflatoxin profile of the maize samples depicts that the maize sample of the 2015 year of harvest had 100 ppb before treatment and reduced to 2.2 ppb after treatment with bentonite clay for 15 minutes; 2016 sample had 1.8 ppb before treatment and reduced to 1.7ppb, whereas the maize sample collected for 2017 year of harvest has 1.9 ppb and reduced to 0.9 ppb, and 2018 maize sample has 3 ppb before treatment and reduce to 0.3 ppb after treatment as showed in **Table 2**.

Table 2: Total Aflatoxin profile of untreated and Bentonite-treated maize samples harvested between 2015- 2018

	Total aflatoxin concentration (ppb)	
Maize year of harvest	Untreated sample	Treated sample
2015	100	2.2
2016	1.8	1.7
2017	1.9	0.9
2018	3	0.3

Table 3 below represents the aflatoxin B1 profile of the maize samples; the sample of the 2015 year of harvest had 60 ppb of Aflatoxin B1 before treatment and reduced to 1 ppb after treatment with bentonite clay for 15 minutes, 2016 sample had 1 ppb of the Aflatoxin B1 before treatment and reduced to 0.9 ppb. In contrast, the maize sample collected for the 2017 year of harvest had 1 ppb and reduced to 0.6 ppb, and the 2018 maize sample had 2 ppb before treatment and reduced to 0 ppb after treatment.

Table 3: Aflatoxin B1 profile of untreated and Bentonite-treated maize samples harvested between 2015- 2018

	AfB1 concentrations (ppb)	
Maize year of harvest	Untreated Sample	Treated Sample
2015	60	1
2016	1	0.9
2017	1	0.6
2018	2	0

The Biorad image indicates that the DNA (450bp-500bp) has been amplified in the samples labeled (A, G, A1, B1, and C1) (**Plate 1**).

The molecular identity of the isolates that ITS1 and ITS4 primers and sequenced have amplified; all the positive samples had one 100 percent query coverage except sample A

(*Aspergillus aflatoxiformans* 087-A2) with99.62% identity as compared to what was obtained in the NCBI library (**Table 4**)

Table 4, Molecular identity of fungi isolated from aflatoxin-contaminated stored Maize samples.

ID	Best hit for isolate	% Q. coverage	% Identity	Accession No.
A1	*Talaromyces islandicus* strain W.Kh-B.I	100	100	MH727227.1
B1	*Aspergillus parvisclerotigenus* strain Maci262	100	100	MG745384.1
A	*Aspergillus aflatoxiformans* 087-A2	100	99.62	MG662405.1
C1	*Aspergillus flavus* isolate R97190	100	100	MK542007.1
G	*Aspergillus fumigatus* isolate RS_20	100	100	MK267099.1

The phylogenetic tree of the BLAST sequence and the evolutionary relationships reveals that the isolates *Aspergillus flavus, A. aflatoxiformans, and A. parvisclerotigenus* are 100 percent closely related in the Genebank. In contrast, *Talaromyces islandicus* and *Penicillium* are also closely related. Likewise, *Aspergillus fumigatus* had a distant relation with other isolates (Figure 1).

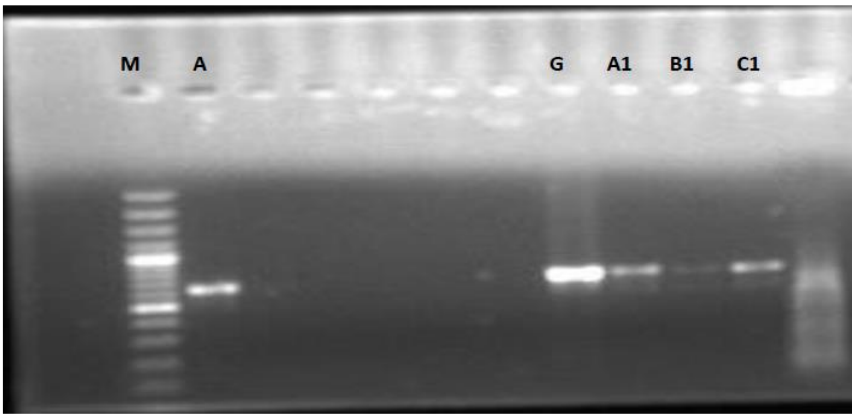

Plate 1: Agarose gel image of the PCR products of the isolates from maize samples collected from Muna Market Maiduguri

Key

M= Ladder

A= *Aspergillus aflatoxiformans* 087-A2

G= *Aspergillus fumigatus* isolate RS_20

A1= *Talaromyces islandicus* strain W. Kh-B. I

B1= *Aspergillus parvisclerotigenus* strain Maci262

C1= *Aspergillus flavus* isolate R97190

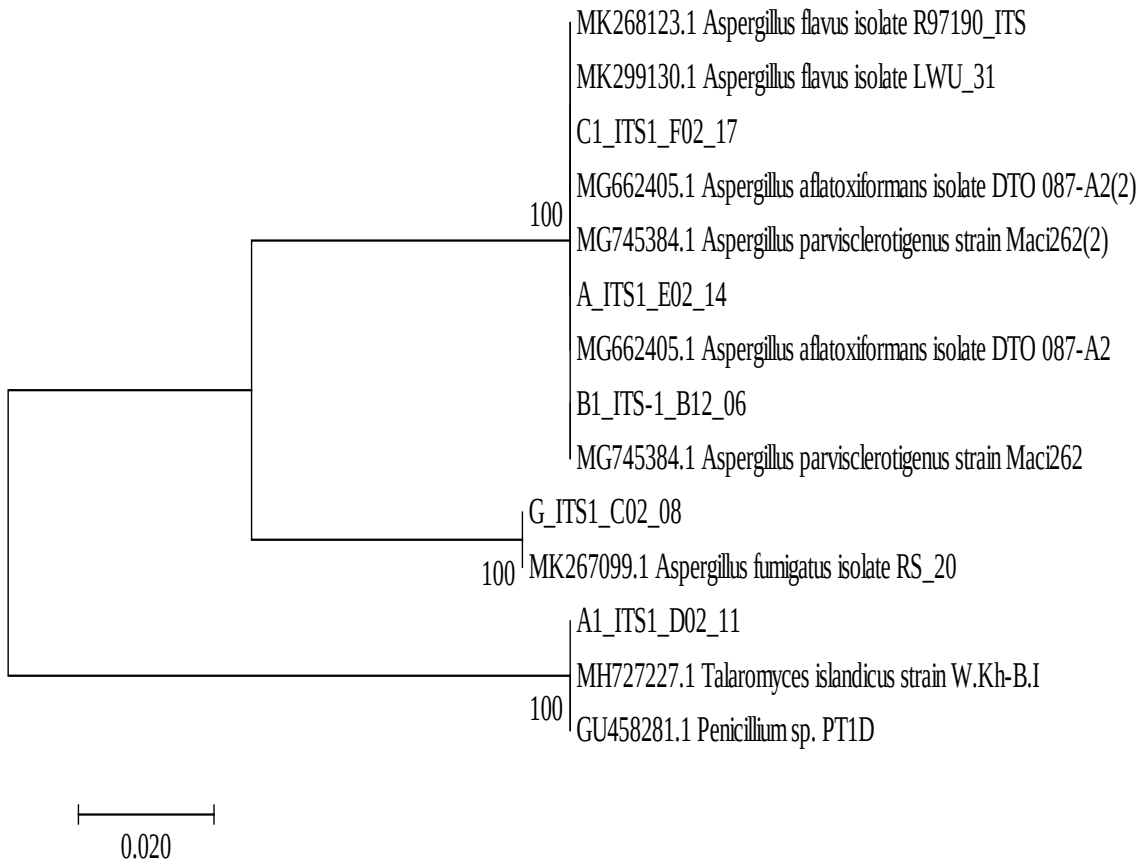

Figure 1: *Neighbor-joining phylogenetic tree of fungi associated with aflatoxin-contaminated maize together with their closest representative available in Genebank*

Discussion

The study on aflatoxins in stored Maize (*Zea mays L.*) was conducted on maize samples collected from Muna Market, Maiduguri, based on their year of harvest (2015, 2016, 2017, and 2018).

A total of 49 fungi isolates consisting of *Aspergillus* spp, *Talaromyces islandicus,* and *Scopulariopsis candida* were obtained from stored Maize samples. These include *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. oryzae*, *A. parvisclerotigenus*, *A. aflatoxiformans*, *Scopulariopsis candida,* and *Talaromyces islandicus.*

The fungal genera predominantly isolated from the stored maize samples were *Aspergillus* spp (83.67%), *Scopulariopsis candida* (14.29%), and *Talaromyces islandicus* (2.04%). *Aspergillus* species had the highest occurrence in all the samples collected from different harvest years, indicating possible contamination of maize samples with total aflatoxin and aflatoxin B1.

In this study, the presence of potentially toxigenic fungi of the genus *Aspergillus* species, *Scopulariopsis candida,* and *Talaromyces islandicus* on the maize grain samples is in contrast with the previous investigation reported by Hell (2003), Krnjaja *et al.* (2007) Amadi and Adeniyi (2009) and Bii *et al*.(2012). In their work, Bii *et al.*(2012) revealed that mycological testing of 86 samples of stored maize, originating from different farms in two locations in Kenya, was found to contain *Aspergillus* (35.8%) and *Fusarium*(15.5%) followed by *Penicillium* (9.2%), *Rhizopus* (5.3%) and others (34.4%).

The co-occurrence of toxigenic fungi is not an uncommon feature. This study included the genera *Aspergillus, Scopulariopsis candida,* and *Talaromyces islandicus,* especially on the maize sample collected for the 2015 harvest year. Also, Remesora *et al.* (2007) isolated the same genera of fungi in healthy and damaged maize but with different frequencies. In a study by Miller (1993), there was frequent joint occurrence of *A. flavus* and *F. verticillioides* on maize. It has already been reported that damage to maize germ has the greatest impact on the proliferation of molds (Tuite *et al.,* 1985).

In addition, the results revealed the presence of *Aspergillus* species similar to those reported by Temu (2016). The selected *Aspergillus* strains showing all five aflatoxins may be produced but not detected because of the inherent detection limits of the analytical Systems (Viegas *et al.,* 2015).

Contamination of maize by Aflatoxins has been increasing worldwide because of climate change, the growing of high-yielding hybrids susceptible to infection with toxigenic fungi, and the accumulation of Aflatoxins in crop products, especially in maize and other cereals such as wheat, millet, and rice. Some Aflatoxins could be synthesized in maize before harvest, but their concentration may increase during the storage period and further in the food chain after harvest. Preventive measures, such as fast drying of maize for medium and long-term storage in hygiene-maintained warehouses or stores without insects and microorganisms and proper regulation of grains moisture content, could significantly reduce the Aflatoxins contamination of maize grains (Miller, 1993).

The total aflatoxin profile of the stored Maize samples collected for the 2015 year of harvest has a 100ppb aflatoxin level far above the least accepted value of 20ppb in Nigeria and Africa at large, according to the Food and Agricultural Organization (FAO) accepted value and reduced to 2.2 ppb after treatment with bentonite clay for 15 minutes. Bouraima *et al.* (1993) found that aflatoxin levels in stored maize in Benin were 14 ppb for B1 and 58 ppb for G1. Udoh *et al.* (2000) reported that 33% of maize samples from different ecological zones in Nigeria were contaminated with aflatoxin.

Hell *et al.* (2000) also reported that 9.9% to 32.2% of maize samples of different ecozones in the Benin Republic prepared for storage had aflatoxin levels of more than five ppb, and the levels increased to 15% and 32.2% after six months of storage. Meanwhile, Kpodo (1996) reported that maize samples from silos and warehouses in Ghana contained aflatoxin levels in the range of 20 to 355 ppb, while fermented maize dough collected from

major processing sites contained aflatoxin levels of 0.7 to 313 ppb. Hence, Kpodo (1996) got far above what was obtained in this study: 100 ppb total aflatoxin and 60 ppb aflatoxin B1 before treatments with the bentonite clay.

Generally, 20 ppb is the maximum limit of total aflatoxins in food meant for human consumption in most countries, including the USA, Nigeria, and UAE, and it is as low as 4 ppb in the EU nations (Tiffany, 2013). Aspergillus, Fusarium, and Penicillium species have been implicated in mycotoxins contamination of foods and feeds. *Aspergillus* and *Penicillium, which require low water activity to grow, are considered storage fungi, while Fusarium spp., which requires high water content, is considered* field fungi (Whitlow and Hagler, 2013). Were as 2016 maize sample was reduced to 1.7ppb from 1.8ppb before treatment, and that of the 2017 maize sample was 1.9ppb before treatment and was detoxified by the bentonite clay to 0.9ppb after treatment for 15 minutes. Lastly, the sample collected for the 2018 harvest had 3 3ppb before treatment and reduced to 0.3 ppb after treatment with the clay for 15 minutes; this follows the work of Phillip (1999) that the use of clay-based adsorbents has proved effective at reducing the toxic effects of aflatoxin contamination in animal feeds specifically bentonite clays.

Subsequently, the aflatoxin B1 (AfB1) profile of the stored Maize samples before and after treatment with the bentonite clay for 15 minutes each reveals that the maize sample of 2015 year of harvest has the highest aflatoxin B1 level of 60ppb and reduced to 1 ppb after treatment, while 2016 and 2017 maize sample have 1ppb each before treatment and they reduces to 0.9 and 0.6ppb respectively, the maize sample collected for 2018 year of harvest has 2ppb AfB1 level before treatment and detoxify to 0 ppb after treatment. With the exception of 2015 maize samples, the remaining samples are within the limited accepted value and are safe for use as food and feed (Tiffany, 2013).

Conclusion

This study on the aflatoxins in stored Maize (*Zea mays L.*) revealed the presence of *Aspergillus* species (*Aspergillus niger, A. flavus, A. fumigatus, A. oryzae, A. parvisclerotigenus,* and *A. aflatoxiformans*), *Scopulariopsis candida,* and *Talaromyces islandicus* in the Maize sample. Total aflatoxin and aflatoxin B1 were detected in the stored Maize. Bentonite clay was found to reduce the levels of total aflatoxin in the stored Maize sample from 100 ppb before treatment. (Which is far above the total accepted limit of 20 ppb in food and feeds in Nigeria and Africa) to 2.2 ppb after treatment with the bentonite clay. Similarly, the bentonite clay was also found to reduce the AfB1 in the stored Maize sample for the 2015 year of harvest from 60ppb before treatment to1ppb after treatment, which is within the accepted limit for aflatoxin in food and feeds for animals and humans in Nigeria.

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