DETECTION OF AFLATOXIN PRODUCING FUNGI IN MAIZE

A THESIS

BY

MD. RAFIK

REGISTRATION NO. 1705428

SEMESTER: JANUARY-JUNE, 2019

SESSION: 2017

MASTER OF SCIENCE (MS)

IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

JUNE 2019

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JUNE 2019

DEDICATED

TO MY

BELOVED PARENTS

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The Author

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ABSTRACT

The present research work was carried out for the detection of aflatoxin producing fungus from maize samples during the period from July 2018 to July 2019. A total 110 maize samples were collected from 5 different local areas of Dinajpur district. In this study aflatoxin producing fungi was detected by simple traditional identification using macro and micro morphological feature and aflatoxin was detected by using Agra strip total aflatoxin test (20 ppb cutoff). *Aspergillus* spp. was found on 28 out of 50 samples with 56% and 14 out of 60 samples with 23.23% prevalence on basis of area and storing time respectively while aflatoxin producing fungus as well as aflatoxin found on 14 out of 50 samples with 28% and 6 out of 60 samples with 10% prevalence based on area and storing time respectively. In this study; out of 110 maize samples, *Aspergillus* spp was found on 42 maize samples with 38.18% prevalence. But Aflatoxin producing fungus was found on 20 samples with 18.18% prevalence. Their early detection can help to take preventive measures to combat economic and health losses. The present study revealed that area had no significant effect ($p > 0.05$) on the prevalence of *Aspergillus* spp. and aflatoxin producing fungus as well as aflatoxin in maize sample while prevalence of Aspergillus spp. and aflatoxin producing fungus as well as aflatoxin in stored maize was significantly ($p<0.01$) varies highest to lowest according to storing time that means the more storing time, the more aflatoxin producing fungus and the more aflatoxin produce. In this study, aflatoxin level was detected by Agra Strip total Aflatoxin test (20 ppb cutoff). In this study, out of 110 samples 20 aflatoxin producing fungus as well as aflatoxin was found whose toxin level (>20 ppb) resulting earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques.

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CHAPTER I

INTRODUCTION

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* Linkand *Aspergillus parasiticus* speare (Bennett and Goldblatt, 1973; Cleveland and Bhatnagar, 1992; Cotty, 1997; Yu *et al*., 2004). These fungi survive in a wide range of environments and can be found in soil, in plant and animal remains, and in grains and seeds such as maize, peanuts, and tree nuts (Pitt, 2000). These two fungi are responsible for spoilage of stored grains around the world (Paster, 1995). Aflatoxigenic fungi may infect the crop prior to the harvesting period and remain as such while storage and finally appear in corn products. Aflatoxins are probably the most significant mycotoxins worldwide, with an estimated 20000 deaths linked to aflatoxin induced liver cancer per annum in Indonesia (D'Mello J.P.F. *et al*., 1998).

Aflatoxins are polyketide compounds produced by several members of the *Aspergillus* section Flavi (Lee T. *et al*., 2002). Some strains of *Aspergillus flavus* and most strains of *Aspergillus parasiticus* and *Aspergillus nomius* produce aflatoxins with Aspegillus *parasiticus* and Aspergillus *flavus* being the two most agriculturally important species. Infection of maize, peanuts, pistachio nuts, figs, cotton seed meal and other commodities by these species may result in contamination with aflatoxins. Infection may occur in the field, particularly where the plant experiences water, temperature or nutrient stress and accumulation of mycotoxin may increase if harvested material is inappropriately stored (Varga J. *et al., 2003a*). *Aspergillus flavus* produces aflatoxins B1 (AFB1) and B2 (AFB2) while *Aspergillus parasiticus* also produces aflatoxins G1 (AFG1) and G2 (AFG2). Aflatoxin B1 is considered to be the most potent naturally occurring carcinogen known. Aflatoxin M1 is a derivative of AFB1 that is formed and excreted in the milk of humans and animals following consumption of foodstuffs contaminated with AFB1. AFB1 is derived from sterigmatocystin (ST), which is itself carcinogenic (Xu H. X. *et al*., 2000).

Aspergillus flavus is the main fungus that causes pre-harvest aflatoxin contamination in field crops. The Food and Agriculture Organization of the United Nations (FAO) estimated that at least 25% of the world's cereal grains are contaminated by mycotoxins, including aflatoxins (Dowling, 1997). During the years spanning 1977–1994, high

aflatoxin levels (more than 20 ugkg-1) in maize were reported (Moreno and Kang, 1999). Colonization of maize crops prior to harvest in the field (pre-harvest) by aflatoxigenic fungi often resulted in spoilage and aflatoxin accumulation in post-harvest grains during storage (Resnik *et al*., 1996; Nesci *et al*., 2003). Many developing countries like China and Mexico have also set up regulations compatible with those in the United States for human consumption and for trading. Because of the food safety regulations, grains with higher level of aflatoxins are prohibited from trade domestically or internationally (Ellis *et al*., 1991). Economic losses are significant to farmers. Health issues related to aflatoxin contamination of foodstuffs lematic in developing countries where no proper food safety regulation as been established. For both food safety and economic reasons, aflatoxin contamination is therefore a serious concern throughout the world.

Globally, maize (*Zea mays* L.) provides 15% of the proteins and 20% of the calories in diets. Furthermore, in developing countries such as Latin America, Africa and Asia, maize is a staple food and occasionally is the only protein source in their diets (Bhatnagar S. *et al*., 2004). Around 78% of maize samples are contaminated with AF (Jand S.K. *et al*., 2005). Aflatoxins are also known to cause aflatoxicosis as a result of consumption of contaminated agricultural products. All aflatoxin producing fungi may be classified taxonomically to *Aspergillus* section *Flavi. Aspergillus flavus* and *Aspergillus parasiticus* are the two main aflatoxin producers (Frisvad *et al*., 2006). Many agricultural commodities such as maize, peanut are liable to infestation by aflatoxigenic molds and contamination with aflatoxin. Aflatoxin are worldwide important in public health, agriculture and economics. Aflatoxin have been found as contaminant in agricultural and food products especially in cereal and cereals product (Blesa *et al*., 2004; Pietri *et al*., 2004; Abbas *et al*., 2006) and animal feeds (Dalcero *et al*., 1998; Sassahara *et al*., 2005). They are both acutely and chronically toxic to animal including man, causing acute liver damage, liver cirrhosis, onset of tumors and teratogenic effects (Stoloff, 1977).

Corn (*Zea Mays* L.) is one of the major cereals crops of global importance, and has always been an important commodity to be traded overseas as food, feed and an industrial grain crop in several countries including India. Unfortunately, it is also vulnerable to the growth of aflatoxigenic fungi, resulting into subsequent aflatoxin production which causes major yield and economic losses (Oyebangi and Efiuvwevere, 1999). Aflatoxin causes mortality and reduce productivity in farm animal and are also

detrimental to human as high concentration have been associated with liver cancer. A.flavus may parasitically colonizes silks and invade mature corn kernel in the field producing aflatoxins (Payne *et al*., 1988). Aflatoxigenic fungi may infect the crop prior to the harvesting period and remain as such while storage and finally appear in corn products. Aflatoxin contaminated groundnut cake contributed to the death of more than 200,000 broiler chicken in 1994. A poultry farm in Chitradurgh, Karnataka State (India), lost more than 2000 chicken as a result of feeding them with aflatoxin contaminated maize meal. In addition to maize and groundnut, many commodities including spices (Jelinek *et al*., 1989; Vasanthi and Bhat, 1998) are contaminated by aflatoxin. In India, human disease outbreak attributable to consumption of aflatoxin contaminated maize have been described by Krishnamachari *et al*. (1975).

Aim of the study:-

- To detect the presence of aflatoxin producing fungus in maize samples.
- To detect aflatoxin from the maize samples.

CHAPTER II

REVIEW OF LITERATURE

Agbetiameh *et al.* (2018) examined for the first time the prevalence of aflatoxin contamination in maize and groundnut in major producing regions across three agroecological zones (AEZs) in Ghana. Furthermore, the distribution and aflatoxin-producing potential of *Aspergillus* species associated with both crops were studied. Out of 509 samples (326 of maize and 183 of groundnut), 35% had detectable levels of aflatoxins. Over 15% of maize and 11% of groundnut samples exceeded the aflatoxin threshold limits set by the Ghana Standards Authority of 15 and 20 ppb, respectively. The L morphotype of *Aspergillus flavus* dominated communities with 93.3% of the population, followed by *Aspergillus* spp. with S morphotype (6%), *A. tamarii* (0.4%), and *A. parasiticus* (0.3%). Within the L morphotype, the proportion of toxigenic members was significantly ($P< 0.05$) higher than that of atoxigenic members across AEZs.

Camiletti *et al.* (2018) characterized 83 *A. flavus* isolates from two maize regions of Argentina and evaluated for their ability to produce or lack of producing mycotoxins in order to select atoxigenic strains to be used as potential biocontrol agents (BCA). All of the isolates were tested for aflatoxin and cyclopiazonic acid (CPA) production in maize kernels and a liquid culture medium. Eight atoxigenic isolates were compared for their ability to reduce aflatoxin and CPA contamination in maize kernels in coinoculation tests. The *A. flavus* population was composed of 32% aflatoxin and CPA producers and 52% CPA producers, and 16% was determined as atoxigenic. In coinoculation experiments, all of the atoxigenic strains reduced aflatoxin from 54 to 83% and CPA from 60 to 97%.

Falade *et al.* (2018) aimed at investigating metabolites produced during fungal development on maize and their correlation with aflatoxin levels. Maize cobs were harvested at R3 (milk), R4 (dough), and R5 (dent) stages of maturity. Individual kernels were inoculated in petri dishes with four doses of fungal spores. Fungal colonisation, metabolite profile, and aflatoxin levels were examined. Grain colonisation decreased with kernel maturity: milk-, dough-, and dent-stage kernels by approximately 100%, 60%, and 30% respectively. Aflatoxin levels increased with dose at dough and dent

stages. The strongest positive and negative correlations were with arabitol (R = 0.48) and turanose and (R = −0.53), respectively. Several metabolites were interconnected with the TCA; interconnections of the metabolites with the TCA cycle varied depending upon the grain maturity.

Norlia *et al.* (2018) demonstrated the one-step method yields more accurate and precise calculations of the model prediction uncertainty than the two-step method. Four uncertainty propagation methods were assessed. The current work assesses the applicability of these techniques by considering the effect of experimental uncertainty and model input uncertainty. The linear approximation was demonstrated not always to provide reliable results. The Monte Carlo method was computationally very intensive, compared to its competitors. Polynomial chaos expansion was computationally efficient and accurate but is relatively complex to implement. Finally, the sigma point method was preferred as it is (i) computationally efficient, (ii) robust with respect to experimental uncertainty and (iii) easily implemented.

Lagogianni *et al.* (2018) investigated the efficacy of azoxystrobin, boscalid, cyprodinil, fludioxonil and cyprodinil + fludioxonil to reduce *A. flavus* growth, sporulation and aflatoxin production in *in vitro*, and in maize field studies. Based on *in vitro* inhibition of mycelial growth, the most effective fungicides were cyprodinil (EC_{50}< 0.05 μg mL^{-1}) and fludioxonil (EC_{50} <0.11 μg mL^{-1}), while the least effective was boscalid (EC_{50} 4.35-4.50 μg mL^{-1}). Azoxystrobin almost completely inhibited the conidium germination at > 0.5 μg mL^{-1}. Further evaluation of the fungicides on maize seeds infected with *A. flavus* demonstrated that all the fungicides reduced conidium production by 76 to 94%, and reduced aflatoxin contamination. In a 2-year field study, application of cyprodinil + fludioxonil reduced *A. flavus* ear rot severity by 40%, and was the most effective formulation for reducing aflatoxin contamination, by 83%. The other four single ingredient fungicides also decreased aflatoxin production on maize kernels (fludioxonil by 80%, cyprodinil by 75%, boscalid by 74% and azoxystrobin by 67%).

Thathana *et al.* (2017) identified Aspergillus flavus in soil and maize and at determining their aflatoxin-producing potentials. Five hundred and fourteen isolates obtained from maize and soil in Kenya were cultivated on Czapeck Dox Agar, Malt Extract Agar, Sabouraud Dextrose Agar, Potato Dextrose Agar, and Rose-Bengal Chloramphenicol Agar. Forty-three presumptive A. flavus isolates were identified; aflatoxin was detected

in 23% of the isolates by UV fluorescence screening and in 30% by Thin-Layer Chromatography (TLC). The aflatoxins produced were: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), and aflatoxin G_1 (AFG_1); some isolates produced only AFB_1, whereas others produced either AFB_1 and AFB_2 or AFB_1 and AFG_1. The highest incidence of A. flavus (63%) and aflatoxin production (28%) was recorded in samples from Makueni District. Isolates from Uasin Gishu (21%) and Nyeri (5%) were non-aflatoxigenic. Bungoma District recorded 11% positive isolates of which 2% were aflatoxin producers.

Maryam *et al.*(2017) isolated and identified from four cereal grains; wheat, barley, rice and maize collected from three Libyan cities known to grain producers (Al-Zawia, Subratah and Tripoli) on 1% dextrose-Czapkes agar medium at 28 ± 2°C for 7-15 days using seed-plate method. Two species of *Alternaria* (*A. raphani* and *A.tenusinae*); two species of *Aspergillus* (*A. flavus* and *A. Niger*); three *Fusarium* (*F. graminearum*, *F. moniliforme* and *F. solani*); one *Rhizopus* species (*Rhizopus stolonifer*) and two species of *Penicillium* (*P. digitatum* and *P. notatum*) were isolated from the grains. The densities of these fungi and their frequencies of occurrence have been investigated. It can be concluded that for human public health, cereal grains of production chain must be subjected to quality control and microbiological examinations.

Maina *et al.* (2016) evaluated the importance of xenia on *A. flavus* infection and aflatoxin accumulation in seed of eight inbred lines with different levels of resistance to *A. flavus* infection and aflatoxin contamination. Resistant and susceptible maize lines were hand-pollinated following a diallel mating design to produce seed for trials. Grain was plated on agar to determine the extent of *A. flavus* infection and analyzed to measure aflatoxin content. Significant differences were detected among seed parents for both aflatoxin accumulation and *A. flavus* infection in both 2003 and 2004. The effects of pollen source were not significant on aflatoxin contamination or *A. flavus* infection in either 2003 or 2004. These results are consistent with xenia having little or no effect on *A. flavus* infection or aflatoxin accumulation.

Fatih *et al.* (2016) aimed to review fungal mycotoxins in foods, their roles and significance in human nutrition and health. This paper provided comprehensive information on the mycological quality and mycotoxin safety of foods. The review showed that moulds are multicellular fungi that form thin thread like structures called

hyphae. They are widely distributed and found wherever moisture is present with adequate nutrients that can sustain their growth. Fungi are major spoilage of foods and feedstuffs.

Karthikeyan *et al.* (2013) collected 60 pre- and post- harvest maize samples from major maize growing areas in Tamil Nadu, India. Aflatoxin contamination was observed in 40.22% of the samples tested of which, 22.97% of pre-harvest and 53.93% post-harvest maize samples were found to be infected with AFB1 and 12.05% of the total samples exceeded WHO permissible limit of 20 µg/kg. AFB1 contamination ranged from 0 to 149.32 µg/kg. 28 *A. flavus* isolates were isolated and grouped into three sets based on aflatoxin producing potential viz., highly aflatoxin producing isolates, medium producing isolates and no aflatoxin producer or traces of aflatoxin producing isolates.

Tóth *et al.* (2012) investigated the occurrence of these species and their mycotoxins on maize in Hungary after harvest in two consecutive years. In 2010 and 2011, 81.94% and 14.33%, respectively, of the samples were found to be contaminated with potentially toxigenic isolates. Several *Aspergillus flavus* isolates were identified, which are potential aflatoxin producers. Other mycotoxin producing species were also isolated, including black *Aspergilli*, which potentially produce ochratoxins and fumonisins, and *A. clavatus*, which produces patulin. In 2010 a large number of *Penicillium* species occurred in the samples, producing a wide range of mycotoxins. The mycotoxin content of the samples was analysed using the ELISA and HPLC techniques. Aflatoxins were not detected in any of the samples, while ochratoxins and fumonisins were successfully identified in some of the maize seeds.

Okoth *et al.* (2012) selected to compare the distribution in maize of Aspergillus spp. and their toxigenicity. Two hundred and fifty-five households were sampled in Nandi and 258 in Makueni, and Aspergillus was isolated from maize. Aspergillus flavus and A. parasiticus isolates were tested for the presence of aflD and aflQ genes. Positive strains were induced to produce aflatoxins on yeast extract sucrose and quantified using liquid chromatography-tandem mass spectrometry (LCMSMS). Aspergillus flavus was the most common contaminant, and the incidence of occurrence in Nandi and Makueni was not significantly different (82.33% and 73.26%, respectively).

Saleemi *et al*. (2012) conducted a study to isolate and identify toxigenic mycoflora of maize and maize-gluten meal. A total of 82 samples of maize and 8 samples of maize-

gluten meal were collected from Faisalabad district of Pakistan over a period of two years. These samples were inoculated on different culture media. Fungal contamination of maize and maize-gluten was 56% and 75% of samples, respectively. Isolation frequencies of different genera isolated from maize were *Aspergillus* 33%; *Penicillium* 28%; *Fusarium* 10%; and *Alternaria* 1%. Isolation frequency among species was maximum for *P. verrucosum*, followed by *A. niger* aggregates, *A. ochraceous*, *A. flavus*, *P. chrysogenum*, *A. parasiticus*, *A. carbonarius*, *Fusarium* spp. and *Alternaria* spp. Relative density of *Aspergillus* isolates was maximum for *A. niger* aggregates and *A. ochraceous* (30% each) followed by *A. flavus* (26%), *A. parasiticus* (11%) and *A. carbonarius* (3%). Percentage of toxigenic fungi among *Aspergillus* isolates was 52%. Aflatoxigenic isolates of *A. flavus* and *A. parasiticus* were 43 and 67% and ochratoxigenic isolates of *A. carbonarius*, *A. ochraceous* and *A. niger* aggregates were 100, 63 and 38%, respectively.

Marín *et al.* (2012) found the main mycotoxigenic fungi in maize in the Mediterranean basin include *Aspergillus* section *Flavi* and several *Fusarium* species, *Fusarium* section *Liseola* being the more widespread. While *Aspergillus* section *Flavi* species developed in the field or in stored maize in Mediterranean countries, *Fusarium* species colonise maize ears in the field. As a consequence, fumonisins are the major contaminants in Mediterranean maize, together with aflatoxins. The prevalence of *Fusarium* section *Liseola* in the Mediterranean countries is closely linked to the activity of insects such as *Sesamia nonagrioides* and *Ostrinia nubilalis*. The incidence of the different mycotoxigenic fungi and mycotoxins across the Mediterranean countries is extensively reviewed in this work.

Gallo *et al.* (2012) identified as *A. flavus* by sequencing of β-tubulin and calmodulin gene fragments. Furthermore, the strains were analysed for the presence of seven aflatoxin biosynthesis genes in relation to their capability to produce aflatoxin B1, targeting the regulatory genes aflR and aflS, and the structural genes aflD, aflM, aflO, aflP, and aflQ. The strains were placed into four groups based on their patterns of amplification products: group I (40 strains) characterised by presence of all seven amplicons; groups II (two strains) and III (nine strains), showing four (AflM, aflP, aflO, and aflQ) and three (aflO, aflP, aflQ) amplicons, respectively; and group IV (16 strains) characterised by total absence of PCR products. Only group I contained strains able to produce aflatoxin B1 (37 out of 40), whereas the strains belonging to the other groups

and lacking three, four or all seven PCR products were non-producers. The results obtained in this study pointed out that A. flavus was the only species responsible for aflatoxin contamination in Northern Italy in 2003, and that the aflatoxin gene cluster variability existing in populations can be useful for understanding the toxicological risk as well as the selection of biocontrol agents.

Karami-Osboo *et al.* (2012) investigated in 373 samples collected during 2006–2008 at harvest stage, from different agro climatic regions of the major maize production area of Iran, including Ardabil (North West), Khuzestan (South West) and Fars at the South of Iran. AFB1 was determined by CD-ELISA and was detected in 146 samples (43.6%), in which only 22.5% were contaminated to higher than MRL level. The amount of aflatoxin in maize samples varied across the years, the percentage of contamination in 2006, 2007 and 2008 were 86.7%, 71.4% and 100% respectively for Ardabil, 63.4%, 41.3% and 24.5% for Khuzestan, 17%, 14.8% and 27.6% for Fars province. The mean of contamination was 15.63, 57.67 and 154.13 μg/kg for Ardabil; 14.41, 35.93 and 1.61 μg/kg for Khuzestan; 0.9, 2.12 and 36.39 μg/kg for Fars province. This is the first report of AFB1 maize contamination to AFB1 in the major maize producing areas of Iran.

Beccari *et al.* (2011) conducted a study to evaluate the presence of mycotoxigenic fungi and mycotoxins in maize grain sampled at harvest. *Fusarium* spp., were the most abundant species detected in maize kernels, followed by *Aspergillus* species of sections *Flavi* and *Nigri* and by *Penicillium* spp. Among *Fusarium* species, *F. verticillioides* was the most prevalent species, as detected by PCR directly on the kernels and on the fungi isolated from the kernels, followed by *F. proliferatum* and *F. subglutinans*. Fumonisins were the predominant mycotoxins with values, on average, of 4.3 and 5.7 mg kg^{-1}, in 2006 and 2007, respectively, with a maximum of 76.3 mg kg^{-1} in the second year. Deoxynivalenol ranged from 0.2 to 3.98 mg kg^{-1} in 2006 (average 1.04 mg kg^{-1}) and from undetectable levels to 14 mg kg^{-1} in 2007 (average 0.86 mg kg^{-1}). Aflatoxins, analyzed only in 2007, averaged 26.3 μg kg^{-1}, with a maximum of 820 μg kg^{-1}. Zearalenone content was always very low. Results indicate that EU legal limits for these mycotoxins were rarely exceeded with low levels across most of the examined area, suggesting that this region could be considered suitable for the production of healthy maize.

Cary *et al.* (2011) identified the aflatoxin contamination problem in maize and cottonseed has been, so several strategies must be utilized simultaneously to ensure a healthy crop, free of aflatoxins. The most widely explored strategy for the control of aflatoxin contamination is the development of preharvest hostresistance. This is because A. flavus infects and produces aflatoxins in susceptible crops prior to harvest. In maize production, the hostresistance strategy has gained prominence because of advances in the identification of natural resistance traits. However, native resistance in maize to aflatoxin contamination is polygenic and complex and, therefore, markers need to be identified to facilitate the transfer of resistance traits into agronomically viable genetic backgrounds while limiting the transfer of undesirable traits. Unlike maize, there are no known cotton varieties that demonstrate enhanced resistance to A. flavus infection and aflatoxin contamination. For this reason, transgenic approaches are being undertaken in cotton that utilize genes encoding antifungal/anti-aflatoxin factors from maize and other sources to counter fungal infection and toxin production.

Donner *et al*. (2009) examined to determine distributions of aflatoxin-producing fungi and to identify endemic atoxigenic strains of potential value as biological control agents for limiting aflatoxin contamination in West African crops. Over 1000 isolates belonging to *Aspergillus* section Flavi were collected from soil of 55 Nigerian maize fields located in three agroecological zones by dilution plating onto modified Rose Bengal agar. The most common member of *Aspergillus* section Flavi (85% of isolates) was the *A. flavus* L-strain followed by the unnamed taxon known as strain S_{BG} (8%), *A. tamarii* (6%) and *A. parasiticus* (1%). Highest incidence of S_{BG} was in Zaria district, and lowest was in Ogbomosho and Ado-Ekiti districts. Only 44% of 492 *A. flavus* isolates produced aflatoxins in liquid fermentation (limit of detection 5 ng g^{-1}). Thirty-two percent of the *A. flavus* isolates produced >1 μg g^{-1} total aflatoxins but no *A. flavus* isolate produced G aflatoxins. When the agroecological zones were compared, significantly ($P < 0.05$) greater proportions of aflatoxigenic *A. flavus* isolates were found in the Northern Guinea Savannah (61%) than in Southern Guinea Savannah (31%).

Karina *et al.* (2008) reported qualitative as well as Semi quantitative rapid strip tests for screening of T-2 mycotoxin in agricultural commodities were developed. Colloidal gold particles were coated with monoclonal anti-T-2 antibodies and used as detector reagent, indicating the strip test results by formation of up to two colored lines in a competitive assay format. The test line comprises a protein conjugate of the T-2 mycotoxin and the

control line an antispecies-specific antibody to confirm the correct test development. To perform the test, 5 g of sample was extracted in a ratio of 1:5 with methanol/water (70:30) by shaking for 3 min and the extract directly used without further cleanup steps. The T-2 toxin lateral flow device (LFD) presented has a cutoff level around 100 µg/kg for naturally contaminated wheat and oat.

William *et al.* (2008) developed maize germplasm lines with resistance to aflatoxin contamination have been in Mississippi. Four of the aflatoxin-resistant lines and six other lines were used as parents to produce a diallel cross. The diallel cross was evaluated for resistance to aflatoxin contamination in field trials conducted in Mississippi in 2005 and 2006. General combining ability (GCA) and specific combining ability (SCA) were highly significant sources of variation each year. Reciprocal effects were not significant in 2005 or in the combined analysis over years. In the analysis over years, GCA effects for reduced aflatoxin were highly significant for the four lines developed as sources of resistance: Mp313E, Mp494, Mp715, and Mp717. The GCA effect for reduced aflatoxin was also highly significant for Mo18W and NC408. These lines should be useful in developing maize lines and hybrids with resistance to aflatoxin contamination. Breeding methods that maximize the use of GCA should be effective in enhancing resistance to aflatoxin accumulation when using these germplasm lines.

Hawkins *et al.* (2008) evaluated one of the problems in maize hybrids for resistance to kernel infection and aflatoxin contamination is identifying a time period and environmental conditions that are most advantageous. Three maize genotypes (Pioneer Brand 3223, Mo18W × Mp313E, and Mp313E × Mp420) were evaluated from 1998 to 2002 in response to *A. flavus* inoculation and aflatoxin contamination and weather conditions favorable for aflatoxin contamination were identified. The highest aflatoxin levels were observed in 1998 and 2000 (1186 and 901 ng g^{-1}; $P < 0.0001$); while the lowest levels were detected in 1999 (39 ng g^{-1}). Pioneer 3223 had significantly higher levels (1198 ng g^{-1}) than Mp313E × Mp420 (205 ng g^{-1}), and Mo18W ×Mp313E (161 ng g^{-1}; $P < 0.0001$).

Gardner *et al.* (2006) conducted a study to identify germplasm resistant to *A. flavus* infection and aflatoxin accumulation have raised questions regarding the role of xenia, the pollen effect on the embryo and endosperm, in resistance of maize grain to the pathogen. The objective of this study was to evaluate the importance of xenia on *A.*

flavus infection and aflatoxin accumulation in seed of eight inbred lines with different levels of resistance to *A. flavus* infection and aflatoxin contamination. Resistant and susceptible maize lines were hand-pollinated following a diallel mating design to produce seed for trials. The ears were inoculated 14 d after pollination with *A. flavus* spores. Grain was plated on agar to determine the extent of *A. flavus* infection and analyzed to measure aflatoxin content. Significant differences were detected among seed parents for both aflatoxin accumulation and *A. flavus* infection in both 2003 and 2004. The effects of pollen source were not significant on aflatoxin contamination or *A. flavus* infection in either 2003 or 2004. These results are consistent with xenia having little or no effect on *A. flavus* infection or aflatoxin accumulation. The results further suggest that reliable evaluation of *A. flavus* infection and aflatoxin contamination can be gained from open-pollinated field experiments.

Zuoxinliu *et al.* (2006) collected 110 samples from an area of 14.68 million km^2 covering storage length from 1 yr to over 10 yr were determined by high-performance liquid chromatography with fluorescence detection. The results showed that almost all samples collected contained aflatoxins. The average contents in maize, whole grain rice and brown rice were found to be 0.99, 3.87 and 0.88 $\mu g\ kg^{-1}$, respectively. Three-fourths of the total aflatoxins in whole grain rice (3.87 $\mu g\ kg^{-1}$) could be removed by dehusking to as low as 0.88 $\mu g\ kg^{-1}$ in brown

Liu *et al.* (2006) collected 110 samples from an area of 14.68 million km^2 covering storage length from 1 yr to over 10 yr were determined by high-performance liquid chromatography with fluorescence detection. The average contents in maize, whole grain rice and brown rice were found to be 0.99, 3.87 and 0.88 $\mu g\ kg^{-1}$, respectively. Three-fourths of the total aflatoxins in whole grain rice (3.87 $\mu g\ kg^{-1}$) could be removed by dehusking to as low as 0.88 $\mu g\ kg^{-1}$ in brown rice. No significant aflatoxin increase was observed in whole grain rice and brown rice over a 10-yr storage period. In maize, the amount of aflatoxins was significantly higher in 2-yr than 1-yr sample. Aflatoxin G_1 was detected as the major type of aflatoxin in over 40% of all stored grain samples tested and over 92% of rice samples examined. The aflatoxin content in maize and rice is much lower than the regulated maximum amount allowed in foodstuffs in China and other countries. We concluded that these grains are safe for human and livestock consumption and for trading.

Somashekar *et al.* (2004) examined a study in order to isolate aflatoxin-producing fungi as well as to assess aflatoxins in the commodities. Thirty-two samples were contaminated with aflatoxigenic fungi and the total mycoflora and aflatoxigenic fungi in different food and feed commodities were in the range of 0.2–260 and 0–100 cfu×10^3/g, respectively. In total, 136 fungi were isolated, of which 32 were *Aspergillus* flavus strains and 26 of them were found to produce aflatoxins. *A. flavus* group of fungi comprising *A. flavus, A. parasiticus*, A. *oryzae*, *A. sojae* were characterized by using *Aspergillus* differential medium and PCR. The PCR was performed using two different sets of primers specifically targeted to *aflR* and *omt* genes of aflatoxin biosynthesis pathway. Most of the fungi belonging to *A. flavus* group reacted positively with the primers resulting in expected size amplicons of 796 bp for *aflR* and 404 bp for *omt*.

Chen *et al.* (2004) sequenced and identified one of these protein spots as glyoxalase I (GLX-I; EC 4.4.1.5). The full-length cDNA of the glyoxalase I gene (*glx-I*) was cloned. GLX-I constitutive activity was found to be significantly higher in the resistant maize lines compared with susceptible ones. After kernel infection by *A. flavus*, GLX-I activity remained lower in susceptible genotypes than in resistant genotypes. However, fungal infection significantly increased methylglyoxal (MG) levels in two of three susceptible genotypes. Further, MG was found to induce aflatoxin production in *A. flavus* culture at a concentration as low as 5.0 μM. The mode of action of MG may be to stimulate the expression of *aflR*, an aflatoxin biosynthesis regulatory gene, which was found to be significantly upregulated in the presence of 5 to 20 μM MG. These data suggest that GLX-I may play an important role in controlling MG levels inside kernels, thereby contributing to the lower levels of aflatoxins found in resistant maize genotypes.

Fente *et al.* (2001) proposed the use of this compound as an additive for fungal culture media to enhance the natural fluorescence of aflatoxins. The production of aflatoxins coincided with the presence of a bright blue or blue-green fluorescent area surrounding colonies when observed under long-wavelength (365-nm) UV light after 3 days of incubation at 28°C. The presence of aflatoxins was confirmed by extracting the medium with chloroform and examining the extracts by high-pressure liquid chromatography with fluorescence detection.

Wicklow *et al.* (1998) made an evaluation of the genotypic diversity (DNA fingerprinting) of 269 A. flavus strains, including subpopulations isolated from grain

sampled at harvest (91 unique 'fingerprints' or genotypes/128 strains), field soil (26 genotypes/31 strains), maize insects (49 genotypes/52 strains) and air-spora (56 genotypes/58 strains), from a maize field near Kilbourne, Illinois. Eight A. flavus genotypes were isolated from grain samples harvested in different years (1988–1991). Genotype 36, isolated from three maize samples, matched the DNA fingerprint of a K. E. Papa strain NRRL 19997, isolated from maize grown in Georgia. Ninety-eight percent of the A. flavus genotypes produced sclerotia and 53% produced aflatoxin. Contrasts of DNA fingerprints revealed two matches involving subpopulations from grain and soil, one match for grain and maize insects, and no matches for grain and air-spora. The high genotypic diversity recorded for each subpopulation, in addition to a limited sample size, precluded any assessment of the relative importance of these subpopulations as sources of A. flavus infective inoculum. Aspergillus parasiticus was routinely isolated from soil samples.

Angela *et al.* (1995) collected sixty nine samples of maize from pre-harvest standing crops and on-farm storage facilities from 52 smallholder farms located within 4 regions of Honduras during October 1992 and November 1993. Samples were visually assessed for insect damage and fungal spoilage, and the mycoflora quantified on artificial media. The major components of the ear rot complex were *Fusarium moniliforme, F. moniliforme* var. *subglutinans, Penicillium* species, *Stenocarpella maydis, S. macrospora* and *Acremonium* spp.

Kurdikeri *et al.* (1994) reported the percentage seed damage and loss in weight increased with the increase in storage period in all the maize hybrids, while the viability of seeds decreased.

Mahmoud *et al.* (1993) isolated fifty isolates of *Aspergillus flavus* as well as 25 isolates of each of *A. fumigatus, A. ochraceus, A. terreus* and *Fusarium* spp. from poultry feeds were screened for toxin production. Of these isolates, 20% have been detected to exhibited toxigenic activity. The mycotoxin content was determined in samples of grind maize, cotton-seed cake, fish meal, wheat bran and soybean meal. 80% of maize samples were highly contaminated with aflatoxins (480 μ/kg) and zearalenone (40 μ/kg). Aflatoxins B_1 and B_2 were found in 50 and 30% of cotton-seed cake and fish meal, respectively. With wheat bran, 40% of the samples contained aflatoxin B_1 and zearalenone. Soybean meal appeared to be a poor substrate for mycotoxin formation.

Wang*et al*. (1990) stored the seeds maize hybrid Danya (12.1-17% moisture content) at 20-30^{o}C to determine the optimum storage condition. The regration equation between seed moisture contents, storage temp and storage days to achieve an 85% germination rate is presented. Max, and ideal moisture contents or seed storage in summer were 14and <13%, resp. Sealed storage at natural low temperature in spring slowed down the aging rate of maize seeds and offset the effect of high temperature. Hybird and initial germination rates were also factors affecting summer storage of maize.

CHAPTER III

MATERIALS AND METHODS

The present research work was conducted during the period from July, 2018 to July, 2019 in the Bacteriology Laboratory of Microbiology Department, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur.

3.1 Materials

3.1.1 Study Area

Maize samples were collected from 5 different local areas in Dinajpur District of Bangladesh.

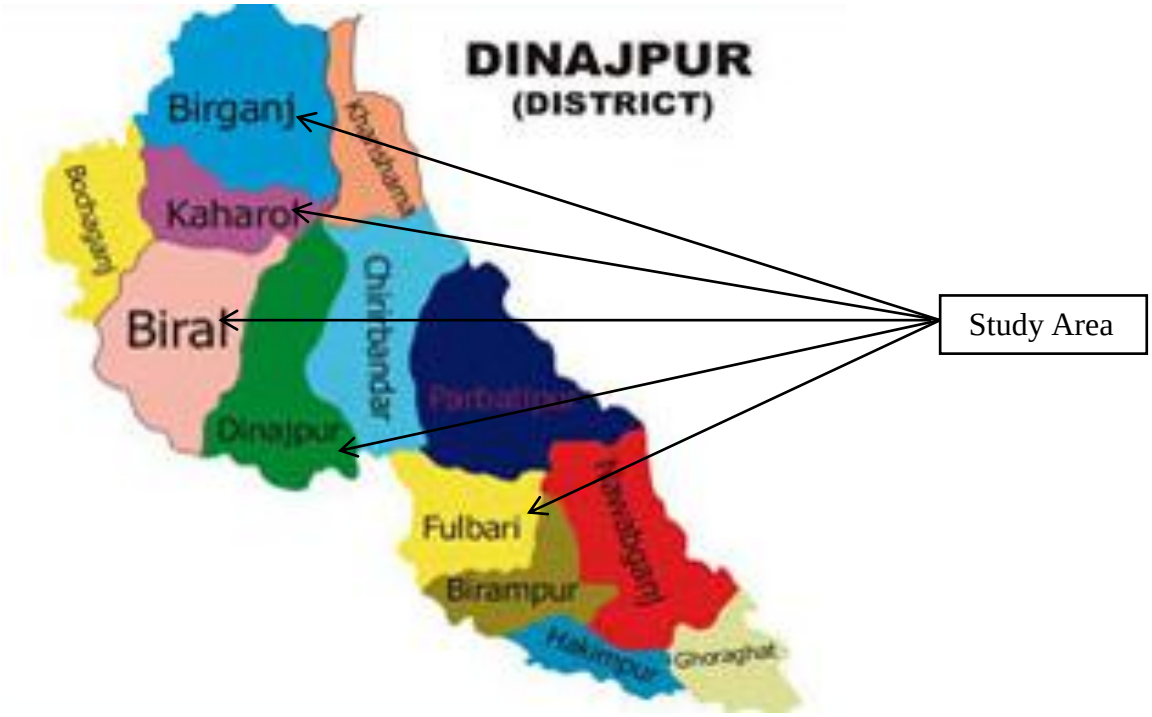


Figure 3.1: Study area map

3.1.2 Study population

From five (5) different local areas of Dinajpur district (Fulbari, Kaharol, Birol, Birganj, Sadar) comprising a total of 110 maize samples were collected aseptically. Among them 50 samples (Table 3.1) were collected from field area and rest 60 samples (Table 3.2) were collected from stored maize based on storing time and brought to the Department of

microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis.

Table 3.1: Field area of different location

Location	Number of sample
Fulbari	10
Kaharol	10
Birol	10
Birganj	10
Sadar	10
Total	50

Table 3.2: Different storing time of stored maize

Storing Time (months)	Number of sample
4	14
8	12
12	14
16	10
20	10
Total	60

3.1.3 Glass Wares, Instruments and Appliances

The different kinds of glass wares and appliances used during the course of the experiment were Test tubes (with or without Durham's fermentation tube and stopper),

- Conical flask, Petri dishes
- Pipette, Slides
- Cover slips
- Clean free grease slide
- Stop Watch
- Test tube stand
- Inoculating loop (Straight and coiled)
- Water bath, Detergent power

- Sealed poly bags
- Electronic compact balance
- Aluminum foil roll
- Sterile cotton
- Bacteriological incubator
- Autoclave
- Refrigerator
- Hot air oven
- Compound microscope

3.1.4 Chemicals, Reagents and Solutions

The following reagents were used for conducting the various fungal tests:

- Phosphate buffered saline solution
- Distilled water
- Lactophenol cotton blue stain
- Methanol

3.1.5 Materials required for detection of Aflatoxigenic fungus

- Sabouraud Dextrose Agar (SDA)
- Potato Dextrose Agar (PDA)
- *Aspergillus* differential Agar medium

The media and reagent that have been used for the isolation and identification of aflatoxigenic fungus in maize are mentioned below:

Sabouraud Dextrose Agar (SDA):

Sabouraud Dextrose Agar (SDA) is a selective medium primarily used for the isolation of dermatophytes, other fungi and yeasts but can also grow filamentous bacteria such as Nocardia. The acidic pH of this medium (pH about 5.0) inhibits the growth of bacteria but permits the growth of yeasts and most filamentous fungi. Antibacterial agents can also be added to augment the antibacterial effect. This medium is also employed to determine mycological evaluation of food, contamination in cosmetics and clinically to aid in the diagnosis of yeast and fungal infections. Antibiotics like chloramphenicol,

gentamicin, and tetracycline can be added as selective agents to inhibit bacterial overgrowth of competing microorganisms while permitting the successful isolation of fungi and yeasts. Various other modifications are also reported by using cycloheximide, penicillin, streptomycin, neomycin depending upon the intended use.

Composition of media:

Ingredients	gm/l
Mycological peptone (enzymatic digest of casein and animal tissues)	10 gm
Dextrose	40 gm
Agar	15 gm
pH adjust to 5.6 at 25^0 C	

Directions

1. Suspend 65 g of the medium in one liter of purified water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121° C for 15 minutes.
4. Cool to 45 to 50°C and pour into petri dishes or tubes for slants.
5. For processing of specimen, streak the specimen onto the medium with a sterile inoculating loop in order to obtain isolated colonies.
6. Incubate the plates at 25 – 30°C in an inverted position (agar side up) with increased humidity.
7. Cultures should be examined at least weekly for fungal growth and should be held for 4 – 6 weeks before being reported as negative

Principle and Interpretation:

After sufficient incubation, the plates should show isolated colonies in streaked areas and confluent growth in areas of heavy inoculation. Examine plates for fungal colonies exhibiting typical color and morphology. Additional procedures should be performed to confirm findings.

Yeasts will grow as creamy to white colonies. Molds will grow as filamentous colonies of various colors.

Typical Colony morphology of some fungi in SDA:

Fungi	**Colony morphology**
Aspergillus flavus	Yellow-green, powdery and pale yellowish on reverse
Aspergillus niger	The initial growth is white, becoming black later on giving "salt and pepper appearance" which results from darkly pigmented conidia borne in large numbers on conidiophores and reverse turning pale yellow
Rhodotorula species	pinkish-orangish creamy colonies
Aspergillus fumigatus	Blue – green, powdery and pale yellow on reverse .
Aspergillus nidulans	Greenish-blue with whitish edge , yellow to brownish on reverse
Trichosporon mucoides	White to cream, yellowish, wrinkled
Geotrichum candidum	White to cream colored, flat with aerial mycelium

Potato Dextrose Agar (PDA):

Potato Dextrose Agar (PDA) is used for the cultivation of fungi. Potato Dextrose Agar (PDA) is a general purpose medium for yeasts and molds that can be supplemented with acid or antibiotics to inhibit bacterial growth. It is recommended for plate count methods for foods, dairy products and testing cosmetics. PDA can be used for growing clinically significant yeast and molds. The nutritionally rich base (potato infusion) encourages mold sporulation and pigment production in some dermatophytes.

Composition of Potato Dextrose Agar (PDA)

Ingredients	**gm/l**
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 liter

Directions

Suspend 39 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at15 lbs pressure (121°C) for 15 minutes. Mix well before dispensing. In specific work, when pH 3.5 is required, acidify themedium with

sterile 10% tartaric acid / lactic acid. The amount of acid required for 100 mlof sterile, cooled medium is approximately 1ml. Do not heat the medium after addition of the acid.

Principle and Interpretation

Potato Dextrose Agar is recommended by APHA and F.D.A. for plate counts of yeasts and moulds in the examination of foods and dairy products. Potato Dextrose Agar is also used for stimulating sporulation, for maintaining stock cultures of certain dermatophytes and for differentiation of typical varieties of dermatophytes on the basis of pigment production. It is also recommended by USP, BP, EP and JP for growth of fungi. Potato infusion and dextrose promote luxuriant fungal growth. Adjusting the pH of the medium by tartaric acid to 3.5, inhibitsthe bacterial growth. Heating the medium after acidification should be avoided as it may hydrolyse the agar which can renderthe agar unable to solidify

Aspergillus Differential Agar Base:

Aspergillus Differentiation Medium Base is used for detection of aflatoxin producing Aspergillus species from food samples

Composition of Aspergillus Differential Agar Base:

Ingredients	**gm/l**
Peptone	10.00
Yeast extract	20.00
Ferric Ammonium Citrate	0.50
Dichloran	0.002
Agar	15.00
final ph at (25^0 C) 6.3+$_{-0.2}$	

Directions:

Suspend 22.75 g in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool around 50°C and aseptically add sterile rehydrated contents of 1 vial of Chloramphenicol Selective Supplement (Cat. No. 29231). Mix well.

Principle and Interpretation:

This medium is based on the formulation of Pitt at al. It is a modification of the medium formulated by Bothast and Fennel. Mixture of Chloramphenicol and Dichloran restricts spreading of moulds, inhibits bacterial growth and helps in identification of fungi. Mixture of peptic digest of animal tissue and yeast extract improves growth rate of fungi particularly aflatoxin producing Aspergillus species like Aspergillus parasiticus. Aspergillus flavus develop intense yellow orange colour at the base of the colonies which is a differential characteristic for these species. showed that the orange yellow colouration was due to reaction of ferric ions from ferric citrate with aspergillic acid molecules forming a coloured complex. The number of colonies are reported per gram of food. Cultural Characteristics after 48 – 72 hours at 30°C

Organism (ATCC)	Growth	Colony characteristics
Aspergillus flavus(22547)	+++	Yellowish orange colour on the reverse side of colonies
Aspergillus parasiticus (28285)	+++	yellowish orange colour on the reverse side of colonies
Asperillus niger (9642)	+++	pale yellow colour on the reverseside and black heads on the top of colonies

3.1.6 Materials required for AgraStrip total Aflatoxin Test (20ppb Cutoff)

Materials supplied:

Aflatoxin AgraStrip (20ppb)

Materials Supplied With Kit

1. 1 tube containing 24 aflatoxin test strips
2. 1 tube containing 24 microtiter wells coated with antibody-particle complex
3. 1 bottle of 1.8mL assay diluent

3.1.7 Materials Required But Not Provided With Kit

Extraction Procedure

1. EQMMS2010: Romer Series II Mill or equivalent
2. EQOLE1025: Blender or a tightly sealing jar with lid
3. EQOLE1010: Balance, 400 g
4. EQOLE1050: Graduated cylinder: 100mL
5. 70% methanol or o ACS grade methanol for making 70 % methanol o Distilled or de-ionized water for making 70 % methanol
6. 50% ethanol or o Formula 3A ethanol for making 50 % ethanol o Distilled or de-ionized water for making 50 % ethanol
7. Container with a minimum 50mL capacity
8. COCMY2112: MycoSep 112 clean-up column (for cottonseed only)
9. Whatman#1 filter paper, or equivalent (optional)
10. Filter funnel (optional)

Assay Procedure

1. Single channel pipettor capable of pipetting 50µL with tips
2. EQOLE1300: Timer

3.2 Method

3.2.1 Experimental Design

The experimental work was divided into two (2) steps: the first step was performed for the isolation and identification of aflatoxigenic fungus. In the second step aflatoxin was detected.

Experimental layout-1

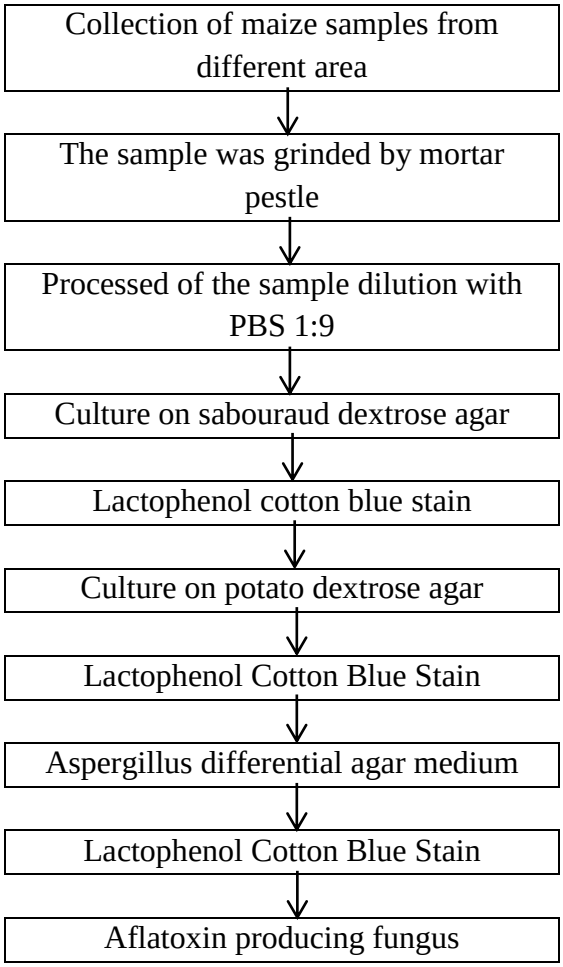


Figure 3.2: The schematic illustration layout of Aflatoxin producing fungus

Experimental layout-2

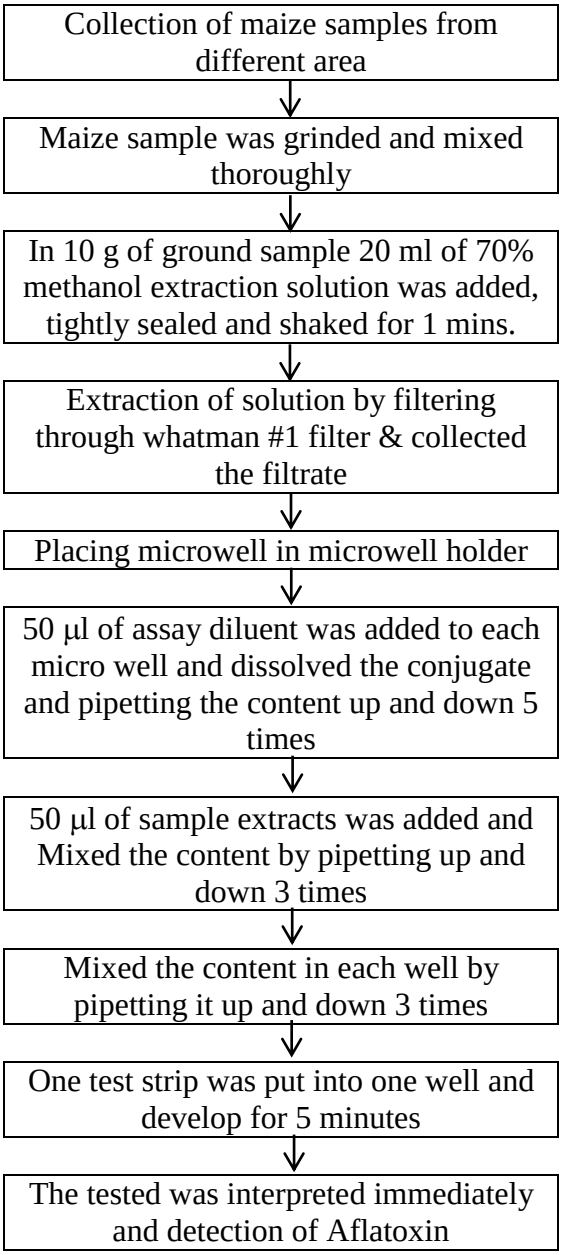


Figure 3.3: The schematic illustration layout of Aflatoxin detection

3.2.2 Samples Collection and transportation

A total of 110 samples were collected for fungal examination. The samples were collected from local area of Dinajpur district, Bangladesh and brought to the microbiology laboratory HSTU. Approximately three hundred (300) g of each maize sample were collected and kept in an ice-box during transportation to the laboratory and stored at 4°C until testing. They were analyzed within 24hours of sampling.

3.2.3 Processing of sample

Three hundred (300) gm maize samples were uniformly homogenized in mortar and pestle using a sterile diluent as per recommendation of ISO (1995). A homogenized suspension was made with the help of mortar and pestle. A quantity of 10 gm homogenate samples transferred carefully into a sterile beaker containing 90 ml of PBS. Thus 1:10 dilution of the samples was obtained.

3.2.4 Techniques for the Isolation and Identification of *Aspergillus* spp.

Culture into different media

Sabouraud Dextrose agar (SDA)

With the help of sterile inoculating loop the processed samples were inoculated into sabroud dextrose agar and incubated at 37^0C for 5-7 days. The incubated media were then examined for the growth of fungi. Colony, greenish colony were found Sabroud dextrose agar.

Potato Dextrose agar (PDA)

With the help of sterile inoculating loop colonies from Sabroud dextrose agar were subculture in potato dextrose agar and incubated at 37^0C for 5-7 days. The incubated media were then examined for the growth of fungi. On PDA the colonies were yellow green with white to cream mycelia and yellow green edges and also in some plates greenish colony were found.

***Aspergillus* differential agar medium**

Presence of Aflatoxigenic fungus in maize is determined by *Aspergillus* differential agar medium. Observed specific colonies from Sabroud dextrose agar and potato dextrose agar were subculture in *Aspergillus* differential agar medium. A bright orange colour on the reverse side of the plates of *Aspergillus* differential agar medium (AFPA) indicated a positive result.

3.2.5 Microscopic study for identification of *Aspergillus* Spp. suspected colonies by lactophenol cotton blue stain (LPCB) method.

Micro morphological characteristics of the pure culture colonies like conidia were observed as wet mount in Lactophenol cotton blue stain for identification by the conidiospore appearance and arrangement (R.Thilagam *et al*. 2016).

1. A drop of 70% ethanol was placed on a clean microscopic glass slide
2. The colony from pure culture was Immersed in the drop of alcohol
3. One or at most two drops of the LPCB were added before the alcohol dries out
4. Holding the coverslip between the index finger and thumb, touch one edge of the drop of mountant with a coverslip edge and lower gently avoiding air bubbles
5. Then this preparation was ready for examination

Make the initial examination using lower power objectives. Switch to higher power (40X) objectives for more detailed examination of spores and other.

3.2.6 Aflatoxin detection by Agra Strip total Aflatoxin Test (20ppb Cut off)

Assay principle

The Agra Strip Aflatoxin Test Method is a one-step lateral flow immuno chromatographic assay with a cutoff level of 20ppb aflatoxin based on an inhibition immunoassay format. Antibody-particle complex is dissolved in assay diluent and mixed with sample extract. The mixed content is then wicked onto a membrane, which contains a test zone and a control zone. The test zone captures free antibody-particle complex, allowing color particles to concentrate and form a visible line. A positive sample with aflatoxin above the cutoff level will result in no visual line in the test zone. Alternatively, a negative sample with aflatoxin below the cutoff level will form a visible

line in the test zone. The line will always be visible in the control zone regardless of the presence of aflatoxin.

Procedure

Extraction Procedure

Representative sample was obtained and it was grinded using a Romer Series II Mill to pass through 75% 20-mesh screen, then mixed thoroughly the subsample portion.

1. Ten (10) g of ground sample was weighed out into a clean jar that was tightly sealed.
2. Twenty (20) mL of 70% methanol extraction solution (i.e. 70/30 (v/v) methanol/water) or 20 mL of 50% ethanol extraction solution (i.e. 50/50 (v/v) ethanol/water) was added and sealed the jar.
3. Then the mixture was vigorously shaken, blended or vortexed for 1minute.
4. The sample was allowed to settle, or filtered the top layer of extract through a Whatman #1 filter and collected the filtrate. Proceeded to the test procedure. (For cottonseed extract, after filtration, placeed 3 mL of extract into a 12 x 75 mm cuvette and pushed the mycosep112 column completely through. Proceeded to the test procedure).

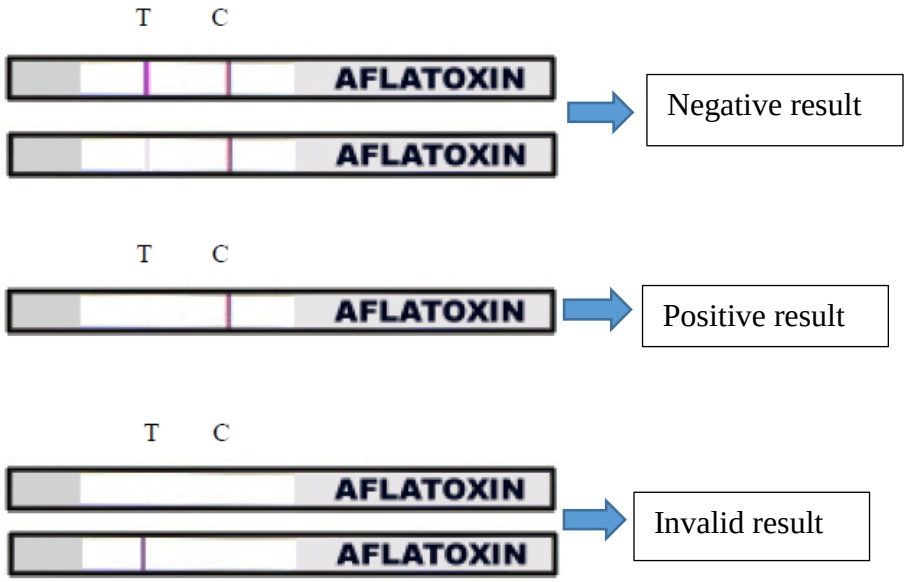
Assay Procedure

1. The microwell sealer was removed, and placed the appropriate number of microwells in a microwell holder. Re-sealed those un-used microwells.
2. Using a single channel pipette, 50µL of assay diluent was added to each microwell. Dissolved the coating conjugate in the microwell by pipetting the content up and down 5 times.
3. 50µL of sample extracts was added to each microwell, mixing the content in each well by pipetting it up and down 3 times.
4. One test strip was put into one well.
5. The test strip was allowed to develop color for 5 minutes.
6. The tested results was interpreted immediately.

Limit of interpretation of the Results

A color line always appears in the upper section of the test strip to indicate that the test strip is working properly. This line is the Control Line (C). A line in the lower section of the test strip indicates the test results. This line is the Test Line (T).

1. **Results less than 20ppb**: 2 lines are visible. This indicates the sample contains total aflatoxin less than 20ppb (negative sample). A negative sample may be determined as soon as 2 lines are visible on the strip (1 min).
2. **Results greater than 20ppb or equal to 20ppb**: 1 line is visible. This indicates the sample contains total aflatoxin great than or equal to 20ppb (positive sample).
3. **Invalid results:** If there is no line in control zone, the test is invalid and the sample should be re-tested by using a valid test strip.



CHAPTER IV

RESULTS

4.1 Prevalence of *Aspergillus* spp. according to area

Area wise Prevalence of *Aspergillus* spp. according to area is presented in table 4.1. The present study revealed that area had no significant ($p>0.05$) effect on the prevalence of *Aspergillus* spp. according to area.

Table 4.1: Prevalence of *Aspergillus* spp. according to area

Name of the area	No. of sample	Positive Case of *Aspergillus* spp.	Prevalence Positive Case of *Aspergillus* spp.	Chi-square test	P- value
Fulbari	10	4	40%	4.55	0.34
Kaharol	10	6	60%		
Birol	10	8	80%		
Birganj	10	6	60%		
Sadar	10	4	40%		
Total	50	28	56%		

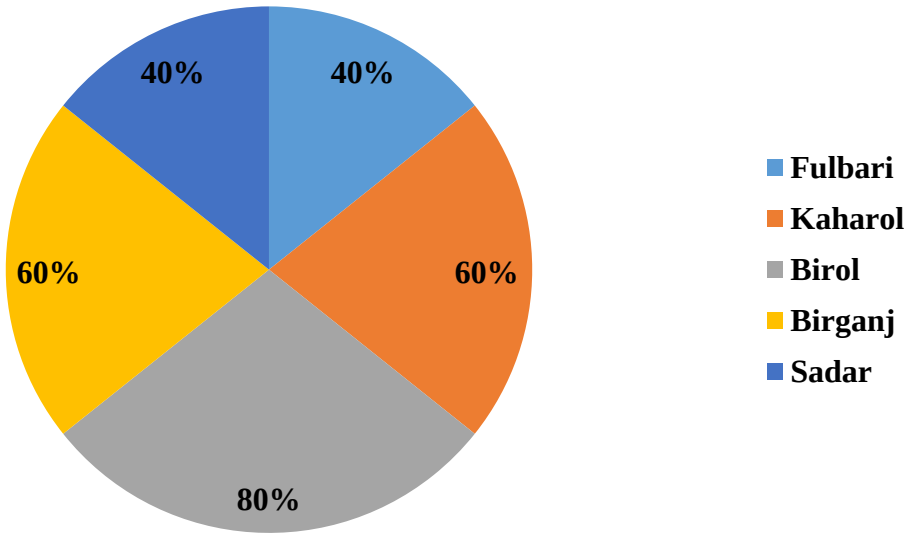


Figure 4.1: Prevalence of *Aspergillus* spp. according to area

4.2 Prevalence of *Aspergillus* spp. according to storing time

Prevalence of *Aspergillus* spp. according to storing time of maize samples are shown in table 4.2. The present findings showed that the prevalence of *Aspergillus* spp. was significantly ($p<0.01$) varied highest to lowest according to storing time of maize sample.

Table 4.2: Prevalence of *Aspergillus* spp. according to storing time

Storing time (months)	No. of Sample	Positive Case of *Aspergillus* spp.	Prevalence Positive Case of *Aspergillus* spp.	Chi-square test	P- value
4	14	0	0%	23.59	0.00***
8	12	0	0%		
12	14	2	14.28%		
16	10	6	60%		
20	10	6	60%		
Total	60	14	23.23%		

***Correlation is highly significant at the 0.01 level (2-tailed)

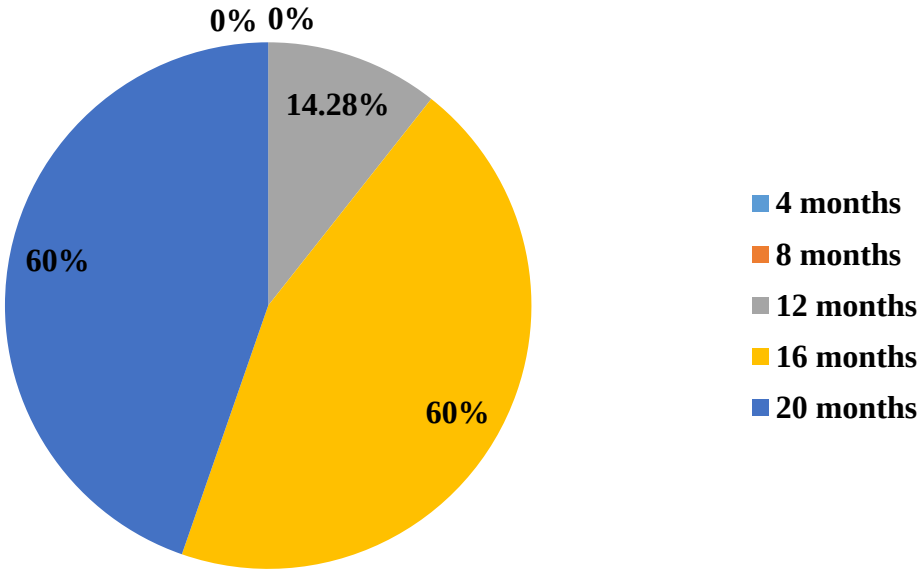


Figure 4.2 Prevalence of *Aspergillus* spp. according to storing time

4.3 Prevalence of aflatoxin producing fungus in maize according to area

Area wise prevalence of aflatoxin producing fungus in maize is presented in table 4.3. The present study revealed that area had no significant ($p>0.05$) effect on the prevalence of aflatoxin producing fungus in maize sample.

Table 4.3: Prevalence of aflatoxin producing fungus in maize according to area

Name of the area	No. of sample	aflatoxin producing fungus	Prevalence of aflatoxin producing fungus	Chi-square test	P- value
Fulbari	10	2	20%	2.38	0.67
Kaharol	10	2	20%		
Birol	10	4	40%		
Birganj	10	4	40%		
Sadar	10	2	20%		
Total	50	14	28%		

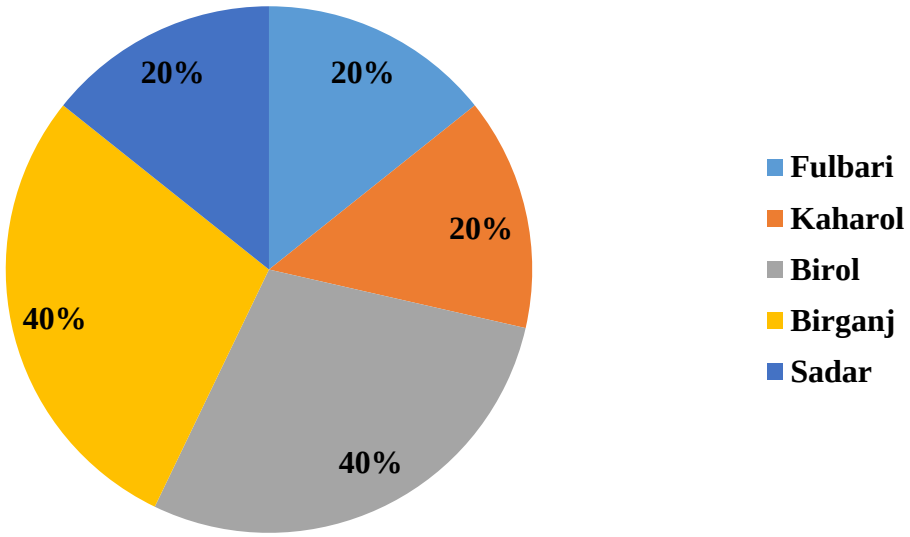


Figure 4.3: Prevalence of aflatoxin producing fungus in maize according to area

4.4 Prevalence of aflatoxin producing fungus in maize according to storing time

Prevalence of aflatoxin producing fungus in maize according to storing time of maize samples are shown in table 4.4. The present findings showed that the prevalence of aflatoxin producing fungus in maize sample was significantly ($p<0.01$) varied highest to lowest according to storing time of maize sample.

Table 4.4: Prevalence of aflatoxin producing fungus in maize according to storing time

Storing time(months)	No. of Sample	aflatoxin producing fungus	Prevalence of aflatoxin producing fungus	Chi-square test	P- value
4	14	0	0%	15.56	0.004**
8	12	0	0%		
12	14	0	0%		
16	10	2	20%		
20	10	4	40%		
Total	60	6	10%		

***Correlation is significant at the 0.01 level (2-tailed)

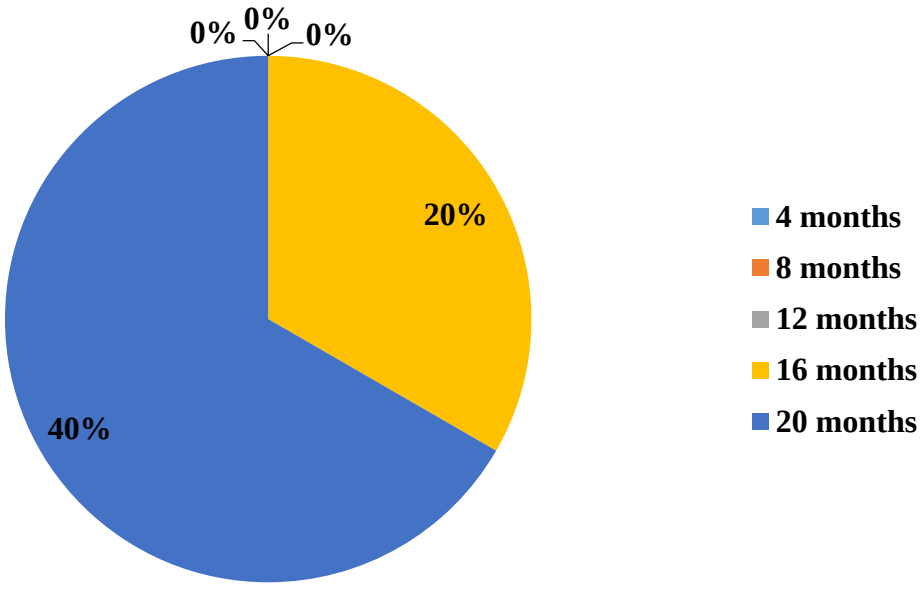


Figure 4.4: Prevalence of aflatoxin producing fungus in maize according to storing time

4.5 Screening of Aflatoxin by Agra Strip Total Aflatoxin Test (20ppb Cutoff)

Level of Aflatoxigenic fungus was determined by Agra Strip Total Aflatoxin Test (20ppb Cutoff)

Table 4.5: Screening of Aflatoxin by AgraStrip Total Aflatoxin Test (20ppb Cutoff)

Sample No.	Positive case for *Aspergillus* spp.	Toxin level	
		positive (>20 ppb)	Negative (<20 ppb)
1-10	3	2	8
11-20	4	2	8
21-30	3	2	8
31-40	5	3	7
41-50	4	2	8
51-60	2	1	9
61-70	6	3	7
71-80	3	1	9
81-90	5	2	8
91-100	4	1	9
101-110	3	1	9
Total=110	42	20	90
Prevalence (%)	38.18%	18.18%	81.82%

4.6 Isoaion and Identification of Aflatoxigenic *Aspergillus* spp

Culture into different media

Sabroud Dextrose agar (SDA)

Greenish colonies were found on Sabroud dextrose agar (Plate 4.2, 4.3).

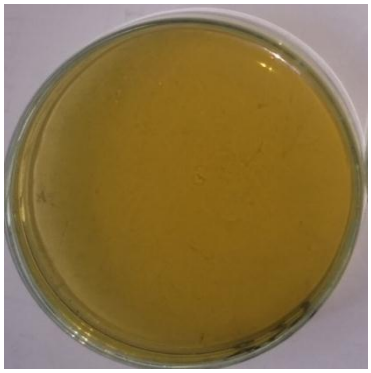


Plate 4.1: Control SDA agar

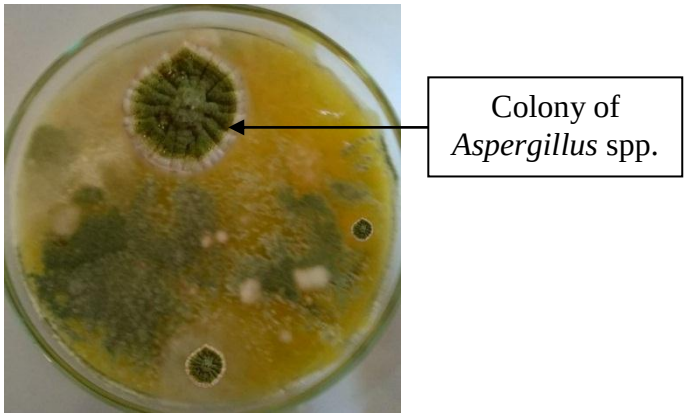


Plate 4.2: Greenish colonies in SDA agar

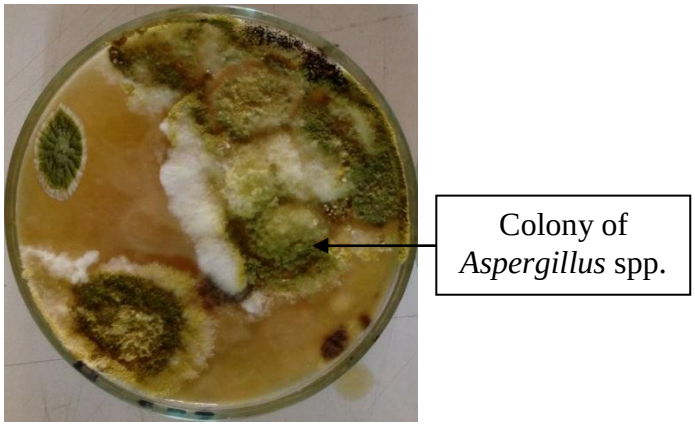


Plate 4.3: Greenish colonies in SDA agar

Potato Dextrose agar (PDA)

On PDA the colonies were yellow green with white to cream mycelia and yellow green edges and also in some plates greenish colony were found (Plate 4.5, 4.6).

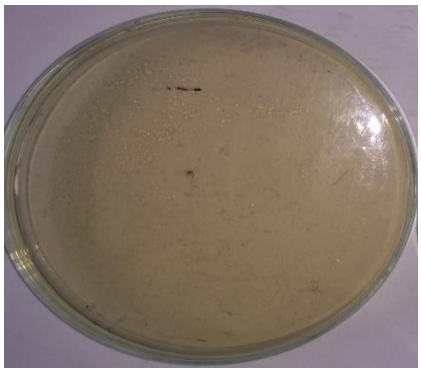


Plate 4.4: Control PDA

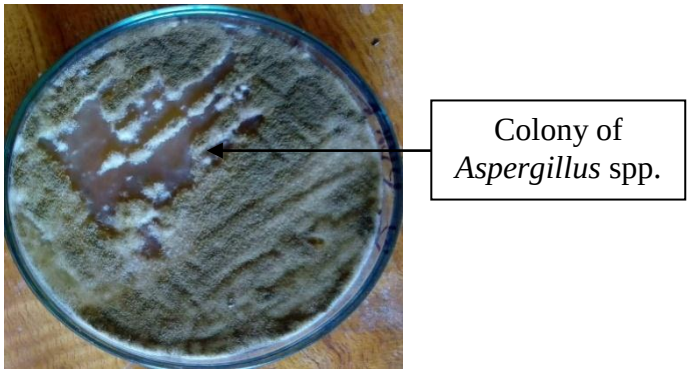


Plate 4.5: Cream mycelia and Yellow green edges

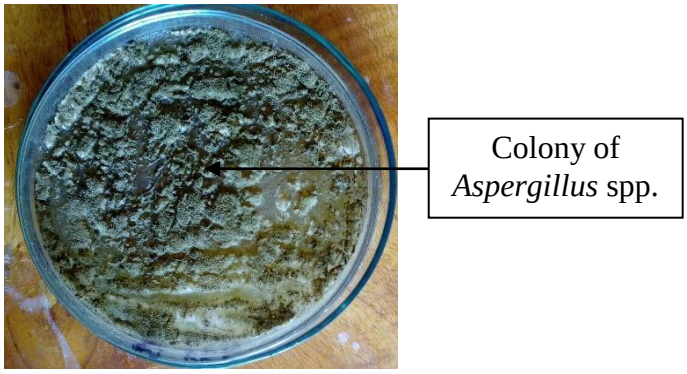


Plate 4.6: Cream mycelia and Yellow green edges

Aspergillus Differential Agar Medium (ADA)

In the present study, Aspergillus differential agar base medium was used to detect aflatoxin producing ability of the fungal isolates. A bright orange colour on the reverse side of the plates of Aspergillus flavus parasiticus agar medium (AFPA) indicated a positive result (Plate 4.7, 4.8).

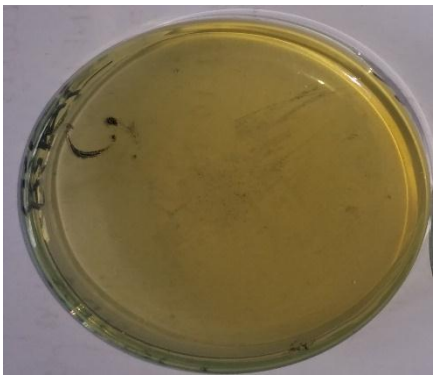


Plate 4.7: Control ADA agar

Plate 4.8: Orange color colony formation in ADA agar

Microscopic study for identification of *Aspergillus* Spp. suspected colonies by lactophenol cotton blue stain (LPCB) method

Characteristics conidia were found under microscope by lactophenol cotton blue stain (LPCB) method (Plate 4.9, 4.10).

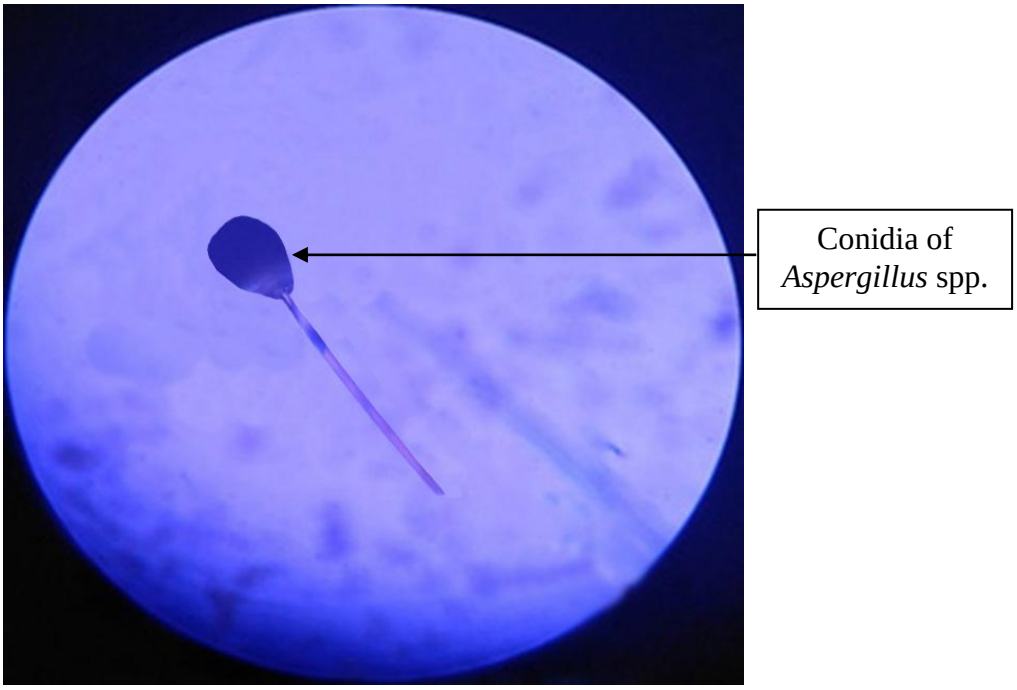


Plate 4.9: Lactophenol cotton blue stain (LPCB) from SDA agar colony

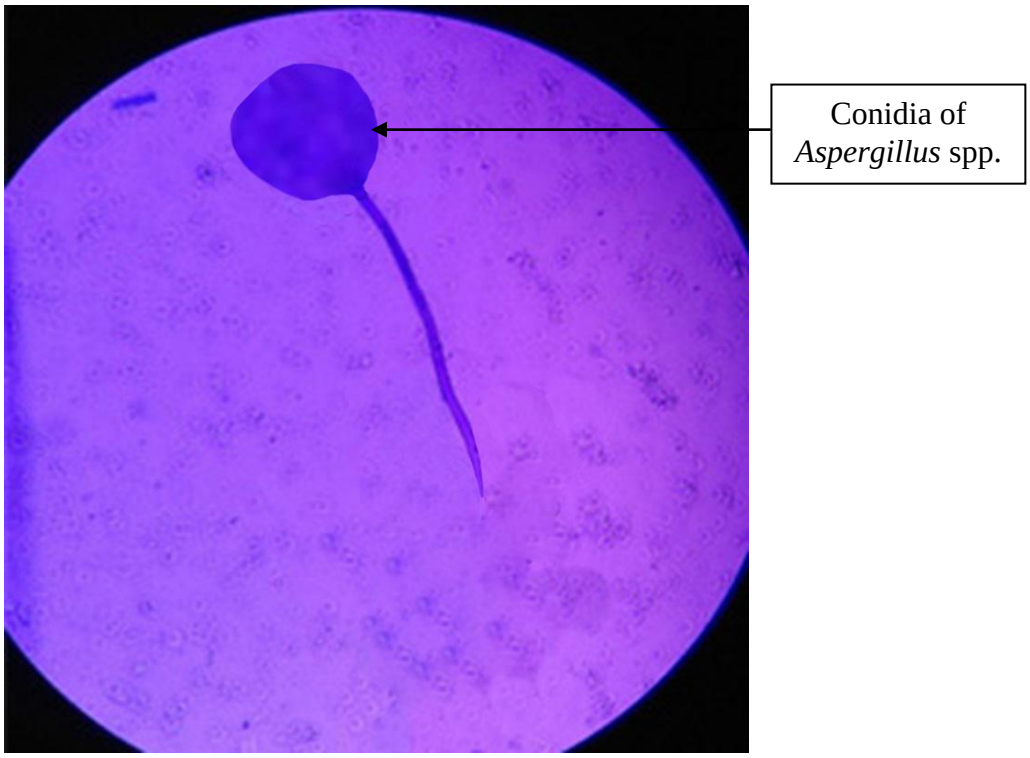


Plate 4.10: Lactophenol cotton blue stain (LPCB) from PDA agar colony

CHAPTER V

DISCUSSION

The experiment was carried out for detection of potential aflatoxin producing fungus from maize samples. The five (5) locations Fulbari, Kaharol, Birol, Birganj and Sadar were selected for sample collection. For this study a total of 110 maize samples were collected. A series of test were conducted for detection, frequency distribution and toxicity level of aflatoxin producing fungus from maize samples.

In culture media, greenish colonies were found on Sabroud dextrose agar, yellow green with white to cream mycelia and yellow green edges and also in some plates greenish colony were found on potato dextrose agar, a bright orange colour on the reverse side of the plates on Aspergillus differential agar medium which indicated positive result, characteristics conidia were found under microscopic examination by lactophenol cotton blue stain (LPCB) method.

Aspergillus spp. was found on 28 out of 50 samples with 56% and 14 out of 60 samples with 23.23% prevalence on basis of area and storing time respectively while aflatoxin producing fungus as well as aflatoxin found on 14 out of 50 samples with 28% and 6 out of 60 samples with 10% prevalence based on area and storing time respectively. In this study; out of 110 maize samples, *Aspergillus* spp was found on 42 maize samples with 38.18% prevalence. But Aflatoxin producing fungus was found on 20 samples with **18.18%** prevalence which is more or less Rouhollah *et al.* (2012) and Hindai *et al*. (2019).

The present study revealed that area had no significant effect ($p > 0.05$) on the prevalence of *Aspergillus* spp. and aflatoxin producing fungus as well as aflatoxin in maize sample while prevalence of Aspergillus spp. and aflatoxin producing fungus as well as aflatoxin in stored maize was significantly ($p<0.01$) varies highest to lowest according to storing time that means the more storing time, the more aflatoxin producing fungus (Liu *et al*., 2006) and the more aflatoxin produce. In this study, aflatoxin level was detected by Agra Strip total Aflatoxin test (20 ppb cutoff). In this study, out of 110 samples 20 aflatoxin producing fungus as well as aflatoxin was found whose toxin level (>20 ppb).

Several intensive research works on aflatoxin contamination in agriculture products have been done worldwide, but report on occurrence and identity of toxigenic fungi and level of mycotoxin in different products of Bangladesh is very little. On this purpose, this study is an effort to investigate distribution of toxigenic fungi in maize. Permitted level of aflatoxin in Bangladesh is 4μg/kg, and previous studies conducted in Bangladesh reports that agricultural commodities such as maize, rice etc. are contaminated with 33 to 480 parts per billion (ppb) of aflatoxin (Dawlatana *et al.* 2002). Present study as well as previous reports (Begum *et al*. 1994, Dawlatana *et al*.2002, Bakr. M. A. 1991 and Mohammad *et al*. 2019) indicate that grains and feeds of Bangladesh are contaminated with toxigenic fungi and aflatoxin. Grains and feeds are consumed in large quantity in Bangladesh and aflatoxin contamination of these products pose threat to the consumers of Bangladesh. *Aspergillus flavus* was found to be most predominant species in grains and feed samples which is in accordance with Reddy *et al*. (2004).

The Food and Drug Administration (FDA) has established action levels of 20 parts per billion (ppb) for grain and feed products. Mixing aflatoxin contaminated grains with sound grains for sale is illegal but less than 20 ppb aflatoxin can be sold as normal grain.

According to this study, in our maize samples the aflatoxins are found to be as 20ppb or more. So it was a great concern for human health because aflatoxin contamination in food and feed product is a serious risk for public health having long-term health effects. Because it has a potent liver toxin known to cause cancer in animals. Their early detection can help to take preventive measures to combat economic and health losses. That earlier detections can be made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques. Aflatoxin contaminated maize used in poultry feed is an alarming issue due to its bio-accumulation in poultry that can be subsequently be deleterious for humans. Therefore, the management of storage is very important and improvement in storage conditions for preventing the spoilage and reducing the toxicity contamination is recommended.

CHAPTER VI

SUMMARY AND CONCLUSION

The recent study was conducted for detection of potential aflatoxin producing fungus from maize samples. A total of 110 maize samples were collected from different areas of Dinajpur district in Bangladesh for this study. Out of 110 maize samples, *Aspergillus* spp. was found on 42 maize samples with 38.18% prevalence. But Aflatoxin producing fungus as well as aflatoxin was found on 20 samples with 18.18% prevalence. The present study revealed that area had no significant effect ($p > 0.05$) on the prevalence of *Aspergillus* spp. and aflatoxin producing fungus as well as aflatoxin in maize sample while prevalence of *Aspergillus* spp. and aflatoxin producing fungus as well as aflatoxin in stored maize was significantly ($p<0.01$) varies highest to lowest according to storing time that means the more storing time, the more aflatoxin producing fungus produce and the more aflatoxin produce. In this study, aflatoxin level was detected by Agra Strip total Aflatoxin test (20 ppb cutoff). In this study, out of 110 samples 20 aflatoxin producing fungus as well as aflatoxin was found whose toxin level (>20 ppb).

Grains are stored for long time before distribution which make them vulnerable to aflatoxin contamination crops and contamination level in different food products. Continuous surveillance should be conducted to detect aflatoxin contaminated. Early detection can help to take preventive measures to combat economic and health losses. The study showed that earlier detections made by simple traditional identifications using macro and micro morphological fungal features rather than adopting the time and cost consuming molecular identification techniques. Good agricultural management practice should be employed to reduce contamination risk of aflatoxin producing fungus in agricultural commodities.

REFERENCES

Agbetiameh D, Ortega-Beltran A, Awuah RT, Atehnkeng J, Cotty PJ, Bandyopadhyay R. Prevalence of aflatoxin contamination in maize and groundnut in Ghana: population structure, distribution, and toxigenicity of the causal agents. Plant disease. 2018 Apr 16; 102(4):764-72.

Angela M. Julian Peter W. Wareing Sarah I. phillipsvictor F. P. Medl Ockmary V. Macdonaldluis E. Delrio. Fungal contamination and selected mycotoxins in pre- and post-harvest maize in Honduras. Mycopathologia. 1995, 129(1): 5–16

Bakr MA. Status of research on detection and control of mycotoxins in food grains in Bangladesh.1991.

Bennett JW, Goldblatt LA. The isolation of mutants of Aspergillus flavus and A. parasiticus with altered aflatoxin producing ability. Sabouraudia. 1973; 11(3): 235-41.

Bhatnagar S, Betrán FJ, Rooney LW. Combining abilities of quality protein maize inbreds, Crop Sci. 2004; 44: 1997-2005.

Camiletti BX, Moral J, Asensio CM, Torrico AK, Lucini EI, Giménez-Pecci MD and Michailides TJ. Characterization of Argentinian endemic Aspergillus flavus isolates and their potential use as biocontrol agents for mycotoxins in maize. Phytopathology. 2018; 108(7): 818-28.

Cary JW, Rajasekaran K, Brown RL, Luo M, Chen ZY, Bhatnagar D. Developing resistance to aflatoxin in maize and cottonseed. Toxins. 201; 3(6): 678-96.

Chen ZY, Brown RL, Damann KE, Cleveland TE. Identification of a maize kernel stress-related protein and its effect on aflatoxin accumulation. Phytopathology. 2004; 94(9): 938-45.

Cleveland TE, Bhatnagar D. Molecular strategies for reducing aflatoxin levels in crops before harvest. InMolecular approaches to improving food quality and safety 1992 (pp. 205-228). Springer, New York, NY.

Cotty PJ. Aflatoxin-producing potential of communities of Aspergillus section Flavi from cotton producing areas in the United States. Mycological Research. 1997 Jun;101(6):698-704.

Covarelli L, Beccari G, Salvi S. Infection by mycotoxigenic fungal species and mycotoxin contamination of maize grain in Umbria, central Italy. Food and Chemical Toxicology. 2011 Sep 1;49(9):2365-9.

D'Mello J.P.F., Macdonald A.M.C., Postel D., Dijksma W.T.P., Dujardin A. and Placinta C.M., Pesticide use and mycotoxin production in Fusarium and Aspergillus phytopathogens, Eur. J. Plant Path., 104, 741–51 (1998).

Dawlatana M, Shahida S, Rahim M, Hassan MT. Investigation on the occurrence of ochratoxin A in maize in Bangladesh. Bangladesh Journal of Scientific and Industrial Research. 2008;43(4):495-500.

Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ. Distribution of Aspergillus section Flavi in soils of maize fields in three agroecological zones of Nigeria. Soil Biology and Biochemistry. 2009 Jan 1;41(1):37-44.

Dowling, F.S., Fumonisin and its toxic effects. Cereal Foods World 42, 1997, 13–15.

Ellis, W.O., Smith, J.P., Simpson, B.K., 1991. Aflatoxins in food: occurrence, biosynthesis, effects onorganisms, detection and methods of control. Critical Reviews in Food Science and Nutrition 30,403–439.

Emanuela Camera, Marzia Scarpari, Marta Punelli, Valeria Scala, Marco Zaccaria, Chiara Nobili, Matteo Ludovici, Anna A. Fabbri, Massimo Reverberi, and Corrado Fanelli (2014) Lipids in *Aspergillus flavus*-maize interaction.Front Microbiol Jan 22 2014.

Falade T, Chrysanthopoulos P, Hodson M, Sultanbawa Y, Fletcher M, Darnell R, Korie S, Fox G. Metabolites identified during varied doses of aspergillus species in zea mays grains, and their correlation with aflatoxin levels. Toxins. 2018 May;10(5):187.

Fatih Yildiz and Samuel A.O. Adeyeye (2016) Fungal mycotoxins in foods.*Food Science and Technology* Published online: 05 Aug 2016.

Fente CA, Ordaz JJ, Vazquez BI, Franco CM, Cepeda A. New additive for culture media for rapid identification of aflatoxin-producingAspergillus strains. Appl. Environ. Microbiol.. 2001 Oct 1;67(10):4858-62.

Gallo A, Gaetano ST, BAttiLAni P, LoGRieCo AF, PeRRone G. Molecular characterization of an Aspergillus flavus population isolated from maize during the first outbreak of aflatoxin contamination in Italy. Phytopathologia Mediterranea. 2012 Mar 9;51(1):198-206.

Gardner HD, Williams WP, Windham GL. Effects of xenia on Aspergillus flavus infection and aflatoxin accumulation in maize inbreds. Crop science. 2006 Sep 1;46(5):2151-4.

Hawkins LK, Windham GL, Williams WP. Occurrence of aflatoxin in three maize (Zea mays L.) hybrids over 5 years in Northern Mississippi. Mycopathologia. 2008 Mar 1;165(3):165-71.

Jand, SK, Kaur P, Sharma NS. Mycoses and mycotoxicosis in poultry: A review, Indian J. Anim. Sci. 75 (2005) 465-476.

Jones B.D. and Toal M.E., Mycotoxins in food: a UK regulatory perspective, Aspects Appl. Biol., 68, 1–9 (2003).

Karami-Osboo R, Mirabolfathy M, Kamran R, Shetab-Boushehri M, Sarkari S. Aflatoxin B1 in maize harvested over 3 years in Iran. Food Control. 2012 Jan 1;23(1):271-4.

Karina Grossalber, Alexandra Molinelli, Manuela Führer, Sabine Baumgartner, Michael Sulyok and Rudolf Krska (2008) Development of Qualitative and Semiquantitative Immunoassay-Based Rapid Strip Tests for the Detection of T-2 Toxin in Wheat and Oat. *J. Agric. Food Chem.*, 2008,volume *56* (8), pp 2589–2594.

Karthikeyan M, Karthikeyan A, Velazhahan R, Madhavan S, Jayaraj T. Occurrence of aflatoxin contamination in maize kernels and molecular characterization of the producing organism, Aspergillus. African Journal of Biotechnology. 2013;12(40). among the *A. flavus* isolates from maize.

Kurdikeri MB, Aswathaiah B, Katagall RD, Vasudevan S, Deshpande VK. Extent of seed damage, loss in weight and loss of viability due to infestation of the rice weevil Sitophilus oryzae Linn.(Coleoptera: Curculionidae) in maize hybrids. Karnataka Journal of Agricultural Sciences. 1994;7(3):296-9.

Lagogianni CS, Tsitsigiannis DI. Effective chemical management for prevention of aflatoxins in maize. Phytopathologia Mediterranea. 2018 Apr 1;57(1):186-97.

Lee T., Han Y.K., Kim K.H., Yun S.H. and Lee Y.W., Tri13 and Tri7 determine deoxynivalenol- and nivalenolproducing chemotypes of Gibberella zeae, Appl. Env. Micro., 68, 2148–54 (2002).

Liu Z, Gao J, Yu J. Aflatoxins in stored maize and rice grains in Liaoning Province, China. Journal of Stored Products Research. 2006 Jan 1;42(4):468-79.

Liu Z, Gao J, Yu J. Aflatoxins in stored maize and rice grains in Liaoning Province, China. Journal of Stored Products Research. 2006 Jan 1;42(4):468-79.

Logrieco A., Mulé G., Moretti A and Bottalico., Toxigenic Fusarium species and mycotoxins associated with maize ear rot in Europe, Eur. J. Plant Path., 108, 597–609 (2002).

Mahmoud AL. Toxigenic fungi and mycotoxin content in poultry feedstuff ingredients. Journal of basic microbiology. 1993;33(2):101-4.

Maina AW, Wagacha JM, Mwaura FB, Muthomi JW, Woloshuk CP. Postharvest practices of maize farmers in Kaiti District, Kenya and the impact of hermetic storage on populations of Aspergillus spp. and aflatoxin contamination. Journal of Food Research. 2016;5(6):53.

Marín S, Ramos AJ, Germán CS, Sanchis V. Reduction of mycotoxins and toxigenic fungi in the Mediterranean basin maize chain. Phytopathologia Mediterranea. 2012 Jan 1;51(1):93-118.

Maryam A. S. Abubakr. Isolation and Identification of Fungi from Cereal Grains in Libya. *International Journal of Photochemistry and Photobiology*.2017, 2(1): 9-12.

Mohamed HA, Salauddin M, Hossain MK, Afroz F. Detection of Potential Bacterial Pathogens and Aflatoxigenic Fungi from Grain Samples. Turkish Journal of Agriculture-Food Science and Technology. 2019 May 21;7(5):731-6.

Moreno OJ, Kang KS. 1649801. Aflatoxins in maize: the problem and genetic solutions. Plant Breeding. 1999;118(1):1-6.

Norlia M, Jinap S, Nor-Khaizura MA, Son R, Chin CK. Polyphasic approach to the identification and characterization of aflatoxigenic strains of Aspergillus section Flavi isolated from peanuts and peanut-based products marketed in Malaysia. International journal of food microbiology. 2018 Oct 3;282:9-15.

Okoth S, Nyongesa B, Ayugi V, Kang'ethe E, Korhonen H, Joutsjoki V. Toxigenic potential of Aspergillus species occurring on maize kernels from two agro-ecological zones in Kenya. Toxins. 2012 Nov;4 (11):991-100.

Paster N. Fungi in Stored Grains and Animal Feeds: Their Occurrence and Harm Caused to Animals. Israel Journal of Veterinary Medicine. 1995;50:49-54.

Pitt JI. Toxigenic fungi and mycotoxins. British medical bulletin. 2000 Jan 1;56(1):184-92.

Reddy KR, Saritha P, Reddy CS, Muralidharan K. Aflatoxin B 1 producing potential of Aspergillus flavus strains isolated from stored rice grains. African Journal of Biotechnology. 2009;8(14).

Resnik S, Neira S, Pacin A, Martinez E, Apro N, Latreite S. A survey of the natural occurrence of aflatoxins and zearalenone in Argentine field maize: 1983–1994. Food additives & contaminants. 1996 Jan 1;13(1):115-20.

Rouhollah K. O., Mansoureh M¨ Reza K., Mahdi S. B., Samad S. Aflatoxin B1 in maize harvested over 3 years in Iran. Food Control. 2012 Jan. Vol. 23(1): 271-274.

Saleemi MK, Khan MZ, Ahrar KH, Javed I, HAsAN ZU, Hameed MR, Hameed S, MEHMOOD MA. Occurrence of toxigenic fungi in maize and maize-gluten meal from Pakistan. Phytopathologia Mediterranea. 2012 Mar 9;51(1):219-24.

Somashekar D, Rati ER, Anand S, Chandrashekar A. Isolation, enumeration and PCR characterization of aflatoxigenic fungi from food and feed samples in India. Food microbiology. 2004 Dec 1;21(6):809-13.

Thathana M, Murage H, Abia A, Pillay M. Morphological Characterization and Determination of Aflatoxin-Production Potentials of Aspergillus flavus Isolated from Maize and Soil in Kenya. Agriculture. 2017 Sep 21;7(10):80.

Tóth B, Török O, Kótai É, Varga M, Toldiné Tóth É, Pálfi X, Háfra E, Varga J, Téren J, Mesterházy Á. Role of Aspergilli and Penicillia in mycotoxin contamination of maize in Hungary. Acta Agronomica Hungarica. 2012 Jun 1;60(2):143-9.

Varga J., Rigó K., Tóth B., Téren J. and Kozakiewicz Z., Evolutionary relationships among Aspergillus species producing economically important mycotoxins, Food Tech. Biotech., 41, 29–36 (2003a).

Wang JS, Liu YX, Wang X. Study on summer storage of maize seeds. Journal of Shenyang Agricultural University. 1990;21(3):201-6.

Wicklow ND, McAlpin CE, Platis CE. Characterization of the Aspergillus flavus population within an Illinois maize field. Mycological Research. 1998 Mar;102 (3):263-8.

Williams WP, Windham GL, Buckley PM. Diallel analysis of aflatoxin accumulation in maize. Crop science. 2008 Jan 1;48(1):134-8.

Xu H. X., Annis S., Linz J. and Trail F., Infection and colonization of peanut pods by Aspergillus parasiticus and the expression of the aflatoxin biosynthetic gene, nor-1, in infection hyphae, Physiol. Mol. Plant Path., 56(5), 185–96 (2000).

Yu, J., Bhatnagar, D., Cleveland, T.E., 2004a. Genetics and biochemistry of aflatoxin formation and genomicsapproach for eliminating aflatoxin contamination. In:Romeo, J.T. (Ed.), Recent Advances in Phytochemistry:Secondary Metabolism in Model Systems, vol. 38. Elsevier, Amsterdam, pp. 224–242.

APPENDICES

Composition of media:

Ingredients	gm/l
Mycological peptone (enzymatic digest of casein and animal tissues)	10 gm
Dextrose	40 gm
Agar	15 gm
pH adjust to 5.6 at 25^0 C	

Composition of Potato Dextrose Agar (PDA)

Ingredients	gm/l
Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 liter

Composition of Aspergillus Differential Agar Base:

Ingredients	gm/l
Peptone	10.00
Yeast extract	20.00
Ferric Ammonium Citrate	0.50
Dichloran	0.002
Agar	15.00
final ph at (25^0 C) $6.3+_{-0.2}$	