Chapter

Aflatoxins and Fumonisins Contamination of Maize in Bangladesh: An Emerging Threat for Safe Food and Food Security

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Abstract

Maize (Bhutta) is one of the important growing cereal crops in Bangladesh. Toxigenic fungi such as *Aspergillus* and *Fusarium* infect stored maize grains. Enzyme-linked immusorbent assay (ELISA) was used to determine total aflatoxins and fumonisins contamination in stored maize grains collected from 15 Bangladeshi maize-producing areas. The highest total concentration of aflatoxins (103.07 μg/kg) and fumonisin (9.18 mg/kg) was found in Chuadanga and Gaibandha, whereas the lowest was detected for aflatoxins (1.07 μg/kg) and (0.11 mg/kg) in Dinajpur and Cumilla, respectively. The findings clearly demonstrated that aflatoxin concentrations in samples from six regions and fumonisin concentrations in samples from 10 regions were beyond the regulatory limit of aflatoxin (10 ppb) and fumonisin (1 ppm), respectively, as set by European Union (EU). However, a positive correlation between aflatoxins with toxigenic *A. flavus*, and fumonisins with toxigenic *Fusarium* spp. was observed. The fungi associated with maize grains were identified by sequencing of ITS regions. Moreover, toxigenic *A. flavus* was confirmed using primers specific to *nor*, *apa2*, *omtA* and primer FUM1 for *F. proliferatum* and *F. oxysporum*. Since the Bangladesh Food Safety Authority has not authorized any precise regulation limits for maize mycotoxin contamination, these results will serve as a benchmark for monitoring mycotoxin contamination in maize and also to develop globally practiced biocontrol approach for producing safe food and feed.

Keywords: mycotoxins, maize, threat, food, security

1. Introduction

Maize (Bhutta) or *Zea mays L.* (corn) is one of the supreme vital cereals in the globe which belongs to Poaceae family and it has been ranked as a third position in the last few decades after wheat and rice [1]. A fair number of food and industrial commodities such as maize flour, animal feed, cooking ingredient, corn syrup, grain

alcohol and whiskey are processed from maize [2]. Maize has been known as a significant emerging crop in Bangladesh as well as maize production is familiarized day by day due to its diverse use for feed, food, fish meal and edible oil processing [3]. Bangladesh has achieved 11th position when it comes to average yield which was 8 tons per ha in the year of 2019–2020 [4] and maize production were 40 lakh ton [5]. Anyway, maize plant is quite vulnerable for various fungi as they get favorable environment to infect via fluctuation of humidity and temperature conditions in both of storage and growing phase [6]. In harvesting period less care in drying and storage processing leads to a surge in infection and production of toxin [7]. Dominant pathogens such as *Aspergillus* spp. and *Fusarium* spp. in maize have the capability to destroy seeds, germination procedure in seeds as well as generating vital mycotoxins [8]. Mycotoxins are light molecular weight developed from saprophytic fungi, most significantly *Aspergillus*, *Fusarium* and *Penicillium* as secondary metabolites [9]. Mycotoxins were detected as one of the deadly toxins after the outbreak of ruinous 'Turkey X' in 1960s at England which leads to the death of Turkey poults (100,000) [10]. Mycotoxin comtamination can develop in any stage of food chain especially in the field, during transportation, processing, harvesting and storage [11].

Aflatoxins are mainly hepatocarcinogenic toxins comprising of major three metabolities named Aflatoxin G, M and B under derivative compounds named difurocoumarin [12–14]. The paramount aflatoxin producing fungi globally is *A. flavus* divided into two distinct morphotypes named L and S [15], among them S morphotype was potentially ruinous as it was capable of producing gigantic level of toxins [16, 17]. A significant research has been made by toxigenic communities that innumerable lineages of fungi are belong to S morphotype among them a few were able to engender enormous concentration of both B and G aflatoxins [18]. Several *Aspergillus* spp. is accounted for several toxins such as aflatoxin B is mainly produced from *A. flavus, A. parasiticus* whereas aflatoxins G is developed from *A. nomius.* Moreover, G and B are highly produced inspices, fruits, corn, nuts, peanuts and copra [19, 20]. *A. flavus* is ubiquitous and mostly detected in corn producing toxins, while in peanut *A. parasiticus* is the main culprit of developing toxins [21]. The toxicity level of aflatoxins of different types chronologically are B1 > G1 > B2 > G2 [22]. Basically, aflatoxins levels were found ascendency in the food markets of Bangladesh [23]. Temperature, pH, relative humidity, and the presence of other fungi are predominant factor for developing aflatoxins and substrates [24]. Aflatoxins level surges due to drought, insect damage, and heat during fungal growth [25]. The *AflR* gene regulates the activation of other structural genes including *omt-A*, *ver-1*, and *nor-1*, which are involved in the aflatoxin biosynthesis process [26]. In hot and humid settings, aflatoxins contamination are also thrived [27]. Seasonal variation has been observed in Bangladesh including high humidity, high temperature and seasonal variation in rainfall (http://en.wikipedia.org/wiki/Geography of Bangladesh). Extreme humid conditions significantly triggered the growth of aflatoxins [28], as a result, it is obvious that aflatoxins was reported in maize, cereals and groundnuts and other feed in Bangladesh and exceeding European Union (EU) permissible limit for aflatoxins [29].

Fusarium spp. are among the utmost crucial fungal pathogens of maize, where they cause severe abatement of yield and accumulation of a vast range of harmful mycotoxins in the grain [30]. *Fusarium* spp. also have the ability to infect crucial crops such as potato, wheat, barley, asparagus, mango, oats, rice and other feed and food crops [31]. High moisture conditions triggered the production of *Fusarium* toxins near or at harvesting stage in cereals [32, 33]. Fumonisins toxins can be developed from a numerous species such as *F. moniliforme*, *F. verticillioides*, *F. nygamai*, *F. proliferatum* [34] as well as *A. niger* [35]. Fumonisins comprise of four types of toxins which are A, B, C, and P, among them fumonisin B1 is the most exploited and ruinous one [34]. FB1, FB2, and FB3 were designated as utmost

destructive and highly abundant fumonisin toxins where FB1 is the most ruinous due to its availability of high concentration on host ranging from 70 % to 80 % of all fumonisins [36–38]. Several biotic (temperature, water stress) and abiotic (osmotic stress, pH, and fungicides) factors are resposnsible for *Fusarium* growth and Fumonisin production [39, 40]. At maturity stage damage occurs by insects, during flowering wet warm weather, rain before harvest, humidity, and media composition for both the *Fusarium* spp., all the activites are related to fumonisins production [41, 42]. *FUM1* gene can also expressed by ecological conditions reported by [43, 44]. As *Fusarium* is widespread and ubiquitous in all cereal growing regions of the globe and corresponding mycotoxins are produced which has been influenced by storage methods and crop production [45]. In the midst of milling, storage, processing, cooking of food and feed, *Fusarium* are highly stable due to its structure and humans and animals are exhibited to them to a certain degree [46–48]. In Bangladesh, animal feed samples were detected and found fumonisin contamination mainly maize based feed contamination [49].

An investigation came out that in South Asia has been ranking as the utmost prevalent continent in case of exposing aflatoxins contamination (82 %) in the globe as well as 41 % maize samples were detected higher amount of aflatoxins contamination than the permissible limit of lenient EU criteria [49]. The very first outbreak of mycotoxin (Sterigmatocystin) was found in Bangladesh in rice straw [50], later in maize and poultry birds [51]. Liver cancer and hepatitis B infection promotes carcinogenic potency in specific individuals by aflatoxins [52, 53]. In Japan, in the year of 1991–2009, violation cases were detected exceeded 1500 in foods which were imported at a level of 10–4918 mg/kg [54]. 62 % children with the age of 3 are at a complete risk of infecting with aflatoxins as aflatoxins biomarkers was detected in plasma of their blood [55]. According to WFP (World Food Program), permissible limit of aflatoxins is 10 ppb (10 μg/kg) and for fumonisins it is 1 ppm (1 mg/kg) [56]. Fumonisins toxin may causes esophageal carcinoma in humans [57], as well as contaminated with folate uptake in cellular level [58] and surging the intensity of neural tube defect [59]. 52 % positive rate of fumonisins was found with an overall level of 936 mg/kg in Asia [60]. *Fusarium* mycotoxin can cause leukoencephalomalacia, porcine pulmonary edema and rat hepatocarcinoma in human and livestock as well [55, 61, 62] detected that in Dhaka, Bangladesh 62 % of 3 year old children had aflatoxin biomarkers in their blood plasma revealing chronic aflatoxin exposure as reported earlier that significant amount aflatoxins were found from corn selling in the Bangladeshi market. Probably 1311 cases of liver cancer was detected every year in Bangladesh [63]. In can be deduced from abovementioned fact that determining aflatoxins and fumonisins and all other mycotoxins in food and feed are the prime need for the country like Bangladesh as these mycotoxin substantially subverts our plants yield concurrently human and animal lives as well. Thus, more research needs to be conducted to elicit the specific mycotoxin hampering specific food, feed and plants, besides to find out the plausible management for controlling these mycotoxins. This study highly exhibited the aflatoxins and fumonisins toxin level in Bangladesh from maize samples of different regions as it has been concerned as one of the burning issues for ensuring safety food.

2. Materials and methods

2.1 Sample collection

Composite stored maize grain samples were collected from 15 maize growing areas of Bangladesh such as Bogura, Kushtia, Meherpur, Chuadanga, Kishoreganj, Manikganj, Cumilla, Rajshahi, Dinajpur, Rangpur, Natore, Thakurgaon,

Panchagarh, Nilphamary and Jashore. Maize samples were collected from stores of traders in local markets of different districts. Ten markets were sampled in each district having at least five traders in each market. At least two quarter of kilogram unique samples were coalesced from each trader for laboratory analysis. Samples were collected after thoroughly mixing maize in the bag to increase chances of getting the fungi. The samples were stored at temperatures below 4° C to await analysis.

3. Detection of aflatoxins and fumonisins by ELISA method

3.1 Procedure of sample preparation

A representative sample was taken and it was grounded with blender so that 75 % of that grounded portion can pass through a 20-mesh sieve, then thoroughly the sub-sample portion was mixed. 50 g of ground sample was weighed out into a clean conical flask that can be tightly sealed. 250 mL of methanol (70 % methanol diluted in water) extraction solution was added to the ground sample and the flask was sealed. Then the conical flask containing the sample was shaken for 3 min. The sample was allowed to settle down, then the top layer of extract was filtered through a Whatman #1 filter paper and the filtrate sample was collected. The prepared extract was diluted at 1:20 with distilled water. Sample was ready for testing without further preparation.

3.2 Assay protocol for aflatoxins

200 μL conjugate solution was pipetted into dilution wells. 100 μL of each standard or sample extract was added into the dilution wells. The mixture was mixed well and 100 μL of the mixture (conjugate and standard or samples) was transferred into antibody-coated wells. The plate was then incubated for 15 min with slow shaking and washed with distilled water for 5 times. The plate was then tap dried. 100 μL of substrate solution was pipetted into antibody coated wells. The plate was incubated with shaking for 5 min. 100 μL of stop solution was pipetted into antibody coated wells. The absorbance of each well was read at 450 nm with a differential filter at 630 nm. As the aflatoxin limit was (0–40) ppb but we found more than that which was diluted by dilution factor in three regions (Bogura, Nilphamari, Rangpur) by four times dilution.

3.3 Assay procedure for fumonisins detection

200 μL conjugate solution was pipetted into dilution wells with 100 μL of each standard and sample extract. The mixture was mixed well and 100 μL of the mixture (conjugate and standard or samples) was transferred into antibody-coated wells. The plate was then incubated for 15 min with slow shaking and then washed with distilled water for 5 times. The plate was then tap dried. 100 μL of substrate solution was pipetted into antibody coated wells. The plate was incubated with shaking for 5 min. 100 μL of stop solution was pipetted into antibody coated wells. The absorbance of each well was read at 450 nm with a differential filter at 630 nm.

3.4 Isolation, purification, identification and preservation of mycotoxigenic fungi

Isolation & purification of *Aspergillus* spp. and *Fusarium* spp. were collected from stored maize grain samples which was conducted by blotter method [64, 65]. In this

method, 400 maize grains were tested for the identification of toxigenic *Aspergillus* spp. and *Fusarium* spp. for each sample collected from different locations and 40 plastic pestridishes were used for each sample. Then 10 maize grains were placed in the sterile plastic petridish containing three layers of wet blotter papers. The petridish was incubated at 25 ± 1° C under 12/12 h light and darkness cycle for 7 days. Each seed was observed under stereo microscope (Stemi 508, Germany) in order to record the presence of fungal colonies and temporary slides were prepared from the fungal colonies for morphological identification under compound microscope (Primo Star, Germany). Or one of the quarter kilo samples from each trader milled into fine floor using a Laboratory Milling machine. Ten grams of the ground sample was mixed with 100 ml sterile water to make a stock solution and serially diluted up to dilution 10^3. The suspension was plated in Potato Dextrose Agar Medium (PDA) [66, 67] by mixing 1 ml suspension in molten PDA cooled to 40° C. Isolation media was prepared by weighing 39 g of PDA into 1 L of water. The mixture was autoclaved for 15 min at 121° C and 15 PSI pressure. The media was allowed to cool to about 50° C and then amended with 25 ng/L of streptomycin and tetracycline [68, 69]. Petri dishes were labeled appropriately and a milliliter of the diluted sample was poured into a sterile petri dish aseptically and then 18 ml of PDA media at 40° C will was poured on the same plate and the mixture swirled gently to mix. The mixture was allowed to cool and solidify in the laminar flow hood and then sealed using parafilm for incubation. The plates were incubated at room temperature for 5–7 days.

4. Molecular based identification of fungi

4.1 DNA extraction

Before DNA extraction each purified *Aspergillus* spp. and *Fusarium* spp. was grown on PDA for 7–10 days at 28° C in an incubator. Then a 5 mm culture block was transferred on the conical flask containing PDA broth and the flasks were incubated at 28° C in an incubator for 7–10 days. Mycelium of each isolate was harvested and preserved at −80° C.

Genomic DNA was extracted from the fungal species isolated from maize grains following Wizard Genomic DNA extraction kit (Promega, USA) according to the manufacturer instructions from 100 mg fungal tissue ground with liquid nitrogen. Fungal tissue was processed by freezing with liquid nitrogen and grinding into a fine powder using a microcentrifuge tube pestle or a mortar and it was pestled. 0.04 g of this fungal tissues powder was added to a 1.5 ml microcentrifuge tube. 600 μl of Nuclei Lysis Solution was added and it was vortexed for 1–3 s to wet the tissue. The sample was incubated at 65° C for 15 min. 3 μl of RNase Solution was added to the cell lysate, and the sample was mixed by inverting the tube 2–5 times. The mixture was incubated at 37° C for 15 min. The sample was allowed to cool to room temperature for 5 min before proceeding. 200 μl of Protein Precipitation Solution was added, and it was vortexed vigorously at high speed for 20 s. The sample was centrifuged for 3 min at 13,000–16,000 × *g*. The precipitated proteins were formed into a tight pellet. The supernatant was carefully removed containing the DNA (leaving the protein pellet behind) and it was transferred to a clean 1.5 ml microcentrifuge tube containing 600 μl of room temperature isopropanol. The solution was gently mixed by inversion until thread-like strands of DNA form a visible mass. Then the sample was centrifuged at 13,000–16,000 × *g* for 1 min at room temperature. The supernatant carefully decanted. 600 μl of room temperature 70 % ethanol was added and was inverted gently into the tube several times to wash the DNA. It was centrifuged at 13,000–16,000 × *g* for 1 min at room temperature. The

ethanol was aspirated carefully using either a drawn Pasteur pipette or a sequencing pipette tip. The DNA pellet was very loose at this point and care must be used to avoid aspirating the pellet into the pipette. The tube was inverted onto clean absorbent paper and the pellet was air-dried for 15 min. 100 µl of DNA Rehydration Solution was added and the DNA was rehydrated by incubating at 65° C for 1 h. Periodically the solution was mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4° C. The DNA was stored at 2–8° C.

4.2 Primers, PCR conditions and sequencing of ITS region

The extracted DNA samples were amplified with PCR reaction for ITS regions. The forward primer: ITS1-5.8S (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and the reverse primer rDNA-ITS4 (TCCTCCGCTTATTGATATGC) were used [70]. PCRs were performed in 25 µl reaction volume containing 12.5 µl master mix, 1 µl ITS1, 1 µl ITS4, 9.5 µl Nuclease free water and 1 µl templet DNA (100 ng/µl). PCR products were visualized in 2 % agarose gel, dyed with ethidium bromide and the photograph was taken using a Gel documentation system (Dynamica, GelView Master). The conditions for PCR reaction was: initial denaturation for 5 min at 95° C, followed by 34 cycles at 95° C for 30s, at 55° C for 1 min and at 72° C for 1 min and then final elongation at 72° C for 6 min. The amplified products were stored at −20° C. PCR products were sequenced using ITS1 primer via commercial outsourcing at Macrogen, Korea via Biotech concern. Finaly, Sequence data were imported by Chromas Software version 2. Sequence data were analyzed by BLAST program (Basic Local Alignment Search Tool) and GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

5. PCR based detection of aflatoxin producing *Aspergillus* spp

5.1 PCR primers and amplification

Primers *nor-1* FP (5′-ACCGCTACGCCGGCACTC TCGGCAC-3′) and *nor-1* RP (5′-GTTGGCCGCCAG CTTCGACACTCCG-3′) were set to amplify an amplicon of 400 bp of norsolorinic acid reductase; *omtA* FP (5′-GGCCCGGTTCCTTG GCTCCTAAGC3′) and *omtA* RP (5′-CGCCCCAGTGAGACCCTTCC TCG-3′) to amplify a 1024 bp fragment of sterigmatocystin O-methyltransferase; and *aflR* FP (5′-TATCT CCCCCCGGGCATCTCCCGG-3′) and *aflR* RP (5′-CCGTCAGACAGCCACTGGACACGG-3′) to amplify a 1032 bp fragment of regulatory protein (*aflR*), involved in aflatoxin biosynthesis. The nucleotide sequence of all these genes from *A. parasiticus* are available at NCBI, GenBank at aceession numbers L27801 (*nor-1*), SRRC 2043 (*aflR*) and SRRC 143 (*omt-1*). PCR was performed in 15 µL of reaction volume containing 7.5 µl master mix, 1 µl forward primer, 1 µl of reverse primer and 4.5 µl nuclease free water and 1 µl of extracted DNA as template (with a total concentration of 100 ng of genomic DNA per reaction). PCR condition for *nor 1* primer initial denaturation for 5 min at 94° C, followed by 35 cycles at 94° C for 30 s, at 67° C for 30 s and at 72° C for 30 s and then final elongation at 72° C for 10 min [71]. PCR condition for *omtA* and *aflR* primer initial denaturation for 10 min at 95° C, followed by 30 cycles at 94° C for 1 min, at 65° C for 2 min and at 72° C for 2 min and then final elongation at 72° C for 5 min [71]. PCR products were separated by electrophoresis on a 1 % agarose gel with 0.5 % ethidium bromide in 1× TBE buffer and visualized under a Gel documentation system (Dynamica, GelView Master). 1 kb plus DNA Ladder (BioLabs, New England) was used as molecular size marker for the analysis of fragment size.

6. PCR based identification of mycotoxigenic *Fusarium* spp

6.1 Primers for PCR amplification

Primers specific for fumonisins producing *Fusarium* spp. (*FUM1* Forward-CCATCAC AGTGGGACACAGT, *FUM1* Reverse-CGTATC GTCAGCATGATGTAGC) were used previously [72]. PCR were performed in mixture 15 μl volume containing 1 μl of DNA sample, 7.5 μl of master mix, 1 μl *FUM1* forward primer, 1 μl *FUM1* reverse primer, 4.5 μl nuclease free water. PCR was performed using T100 Thermocycler (BioRad, Hercules, USA). The PCR condition for *FUM1* regions include 94° C for 4 min for initial denaturation, followed by 35 cycles of denaturation at 94° C for 1 min, primer annealing at 58° C for 1 min, primer extension at 72° C for 1 min. The final extension was set at 72° C for 10 min. 4 μl of the PCR product was electrophoresed on 1.5 % agarose gel, stained with ethidium bromide, illuminated and documented using Gel documentation system (Dynamica, GelView Master).

7. Statistical analyses

The collected data were analyzed statistically by using Minitab software version 17 (www.minitab.com). The mean of all the treatments were compared by critical difference value at 5 % level of significance.

8. Results

8.1 Determination of total Aflatoxins contamination in stored maize grain samples collected from some selected growing areas of Bangladesh

The study was performed at the Laboratory of Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh. Composite stored maize grain samples were collected from 15 maize growing areas of Bangladesh including Panchagarh, Thakurgaon, Dinajpur, Nilphamari, Rangpur, Lalmonirhat, Gaibandha, Bogura, Natore, Kushtia, Jashore, Chuadanga, Kishoreganj, Manikganj and Cumilla.

In terms of total aflatoxins concentration in μg/kg, the highest and lowest amount of aflatoxins concentration was recorded in Chuadanga (101.57 μg/kg) and Dinajpur (1.08 μg/kg) which exposed no significant relationship to each other. The moderate amount of afalatoxin level was detected in Gaibandha (68.73 μg/kg), Kushtia (31.48 μg/kg), Kishoreganj (30.86 μg/kg), Rangpur (20.56 μg/kg) and Cumilla (11.91 μg/kg) revealing more aflatoxins contamination than the regulatory limit (10 μg/kg) in which only aflatoxins concentration from Kushtia and Kishoreganj revealed statistically significant data, besides, rest of the location exhibited below level of aflatoxins contamination of regulatory limit showing more or less statistically significant data.

Total aflatoxins associated with maize grains were detected in 2020, with the supreme value was detected in Chuadanga (30.5 %) followed by Kushtia (29.5 %), Nilphamari (22.5 %), Panchagarh (19.25 %) and the minimal aflatoxins was detected in Manikganj (3.2 %), rest of the samples from other districts revealed lower to moderate level of aflatoxins, moreover, data from Chuadanga and Kushtia, Cumilla, Jashore and Natore, Thakurgaon and Rangpur, Lalmonirhat and Kishoreganj regions revealed ststistically similar data while data from other regions exhibited statistically dissimilar data.

In case of infection rate, toxgenic maize samples were obtained from Panchagarh, Thakurgoan, Gaibandha, Chuadanga, Kishoreganj exhibiting 100 % infection by

A. flavus and no atoxigenic samples were found in those area. Moderate amount of toxigenic *A. flavus* was detected in Jashore followed by Cumilla, Natore, Lalmonirhat, Nilphamari which were 78 %, 75 %, 66 %, 50 % respectively and atoxigenic fungi was detected 22 %, 25 %, 34 %, 50 %, 50 % were detected respectively. Rest of the locations (Dinajpur, Rangpur, Bogura, Kushtia, Manikganj) exhibited higher amount of atoxigenic *A. flavus* compared to toxigenic *A. flavus* (**Table 1**).

The outmost percent aflatoxins concentration over standard limit was found in Chuadanga (915.7 %) followed by Gaibandha (587.3 %), Kushtia (214.8 %), Kishoreganj (208.85 %), Rangpur (105.6 %), Cumilla (19.5 %) revealing that the aflatoxins contamination from those area were beyond the regulatory limit set by EU for aflatoxins (10 ppb), conversely, aflatoxin concentration from other nine locations were below the regulatory limit of aflatoxins (**Table 1**).

8.2 Relationship between aflatoxins producing *A. flavus* and mean aflatoxins concentrations

The regression analysis between toxigenic *A. flavus* percentage and mean aflatoxin concentrations which was positively correlated by observing regression equation where the slope was = 0.55 and y-intercept was = 50.14, coefficient of

Location	Total aflatoxins concentrations (μg/kg)	% *A. flavus* associated with maize grains			Percent total aflatoxins concentration over standard limit
		Total	Toxigenic	Atoxigenic	
Panchagarh	4.96 ± 0.19^{f}	19.25 ± 3.53^{c}	100	0	—
Thakurgoan	1.28 ± 0.10^{g}	18.25 ± 0.43^{cd}	100	0	—
Dinajpur	1.08 ± 0.122^{g}	16 ± 1.73^{de}	25	75	—
Nilphamari	3.04 ± 0.56^{fg}	22.5 ± 3.28^{b}	50	50	—
Rangpur	20.56 ± 0.42^{d}	18.5 ± 2.18^{cd}	44	56	105.6
Lalmonirhat	3.37 ± 0.19^{fg}	9.75 ± 1.00^{g}	50	50	_
Gaibandha	68.73 ± 4.02^{b}	3.75 ± 1.00^{h}	100	0	587.3
Bogura	3.33 ± 0.41^{fg}	11.25 ± 0.66^{fg}	40	60	—
Natore	2.39 ± 1.29^{fg}	13.5 ± 1.80^{ef}	66	34	—
Kushtia	31.48 ± 1.14^{c}	29.5 ± 1.32^{a}	33	67	214.8
Jashore	1.67 ± 0.57^{g}	13.75 ± 1.64^{ef}	78	22	—
Chuadanga	101.57 ± 5.09^{a}	30.5 ± 0.50^{a}	100	0	915.7
Kishorerganj	30.89 ± 0.22^{c}	10.25 ± 1.09^{g}	100	0	208.85
Manikganj	2.57 ± 0.01^{fg}	3.25 ± 0.43^{h}	33.33	66.67	—
Cumilla	11.91 ± 0.30^{e}	14 ± 2.00^{ef}	75	25	19.5
Level of significance	**	**			
LSD	2.07	2.95			
CV	5.07	9.66			

**Significant at 5 % level of significance. Least significant difference (LSD) at P = 0.05 was used for comparing means and the P values were 0.00.*
***Significant at 1 % level of significance. Least significant difference (LSD) at P = 0.05 was used for comparing means and the P values wee 0.00. Data are the averages of three biological replications. The regulatory limits for fumonisin is 1 ppm (10 μg/kg).*

Table 1.
Levels of Total aflatoxins concentration in stored maize grains collected from the stores of traders of fifteen maize growing areas of Bangladesh.

determination, R^2 = 0.198 and coefficient of correlation, r = 0.44 which depicted that 1 % surges of toxigenic *A. flavus* in maize grains ultimately rised 50.137 μg/kg aflatoxin concentration. In terms of 5 % surges of toxigenic *A. flavus* in maize grains, the aflatoxin concentration was increased up to 2.75 μg/kg and when toxigenic *A. flavus* increased 20 % in maize grains, the aflatoxin concentration was escalated up to 11.0 μg/kg (**Figure 1**).

8.3 Identification of *A. flavus* from the stored maize grain samples collected from some selected growing areas of Bangladesh

Morphological identification of *A.flavus* was detected by using petridish and culture plate method as well as observing microscopic figures under compound and stereo microscope (**Figure 2A(a)–(d)**). Thirty five fungal isolates were identified using primers specific to ITS 1 and ITS 4 regions. PCR assays of *A. flavus* DNA with ITS 1 and ITS 4 primers amplified a single fragment of about 600 bp which revealed that all the isolates obtained were fungi. Sequence analysis of ITS region by BLAST program revealed that all the isolates obtained from maize were belong to *A. flavus* (**Figure 3A**).

8.4 PCR based identification and confirmation of aflatoxin producing *Aspergillus flavus* species obtained from maize grain samples

AF02_Ran, AF01_Lal, AF01_Bog, AF02_Bog, AF03_Jas, AF04_Jas, AF01_Chu, AF03_Kis, AF04_Kis, AF01_Man were identified by PCR amplification of ITS region using ITS1 and ITS4 primers and the results of PCR showed an amplification size 600 bp confirmed the fungal isolates (**Figure 3A**) and their several strains were found in Rangpur (*A. flavus* strain 64-A1), Lalmonirhat (*A. flavus* strain SGE22), Bogura (*A. flavus* strain SGE22 and *A. flavus* strain bpo4), Jashore (*A. flavus* and *A. flavus* isolate AA221), Chuadanga (*A. flavus* strain JN-YG-3-5), Kishoreganj (*A. flavus* strain 64-A1 and *A. flavus* strain ND26), Manikganj (*A. flavus* strain SU-16).

PCR products were then sequenced and analysis of sequence data of amplified ITS region using BLAST program revealed that fungal isolates AF01_Man, AF03_Jas, AF02_Ran obtained from maize grain samples collected from Manikganj, Jashore, Rangpur revealed the highest homology of 99.33 %, 99.17 %, 95.74 % with the *A. flavus strain* SU-16, *A. flavus, A. flavus strain* 64-A1. Other sevel isolates obtained from Lalmonirhat (AF01_Lal), Bogura (AF01_Bog), Bogura (AF02_Bog), Jashore (AF04_Jes), Chuadanga (AF01_Chu), Kishoreganj (AF03_Kis), Kishoreganj (AF04_Kis) showed significant homology with different strains of *A. flavus* (**Table 2**).

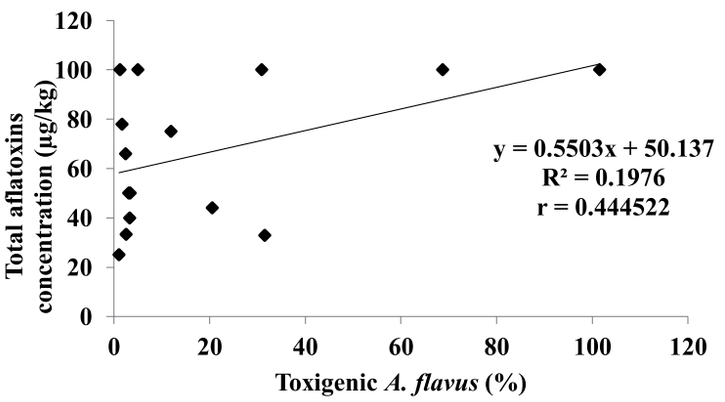

Figure 1.
Linear correlations between toxigenic A. flavus *infected maize grains and total aflatoxins concentration.*

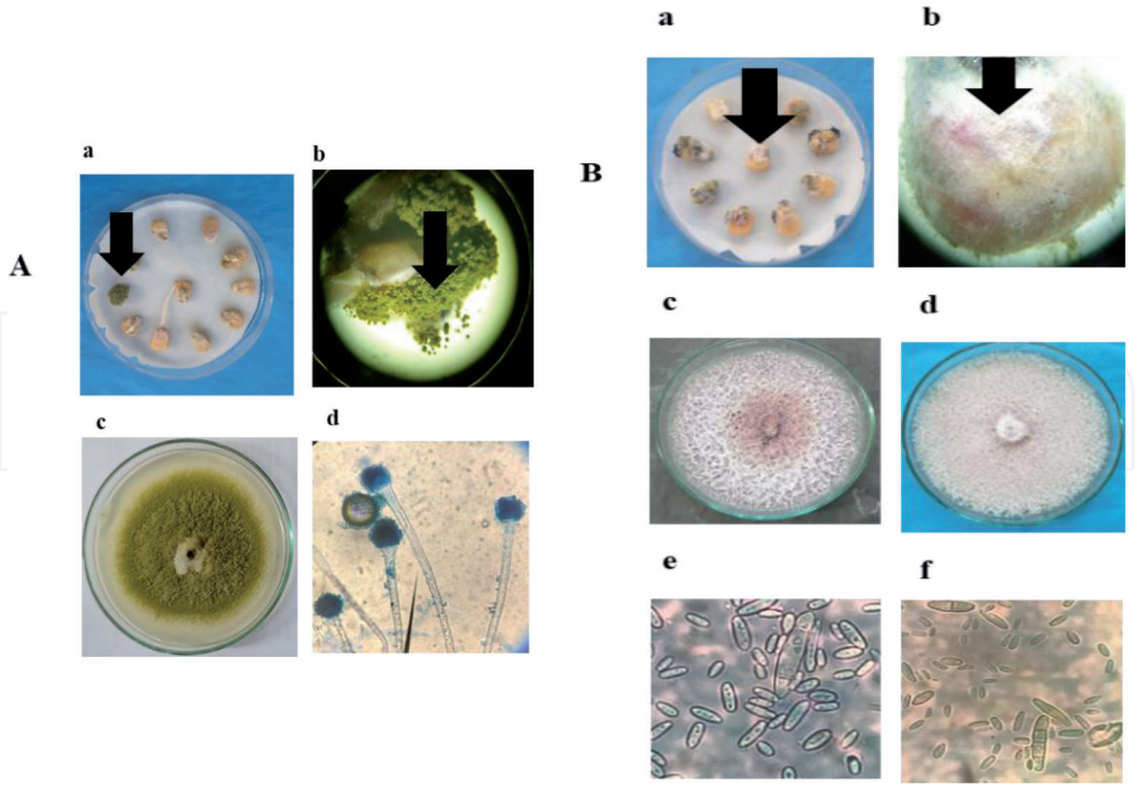

Figure 2.
(A) Composite photographs of Aspergillus *spp. in different sections. (a) Apparent growth of* Aspergillus *spp. on the maize grain surface, (b) enlarged view of individual maize grain showing the growth of* Aspergillus *spp., morphology of suspected* Aspergillus *spp. (c) Yellowish green colonies of* A. flavus *on PDA, (d) vesicle with less conidial ornamentation with conidiphores of* A. flavus. *(B) Composite photographs of* Fusarium *spp. in different sections. (a) Apparent growth of* Fusarium *spp. on the maize grain surface, (b) enlarged view of individual maize grain showing the growth of* Fusarium *spp., morphology of suspected* Fusarium *spp., (c) pinkish white growth of* F. proliferatum *on PDA, (d) microconidia of* F. proliferatum *without septum under microscope with 40× magnification, (e) whitish growth of* F. oxysporum *on PDA and (f) Micro and macro-conidia (with septum) of* F. oxyporum *without septum. Culture photographs were taken at 7 days after inoculation and microscopic photographs were taken with 40× magnification using compound light microscope equipped with a digital camera.*

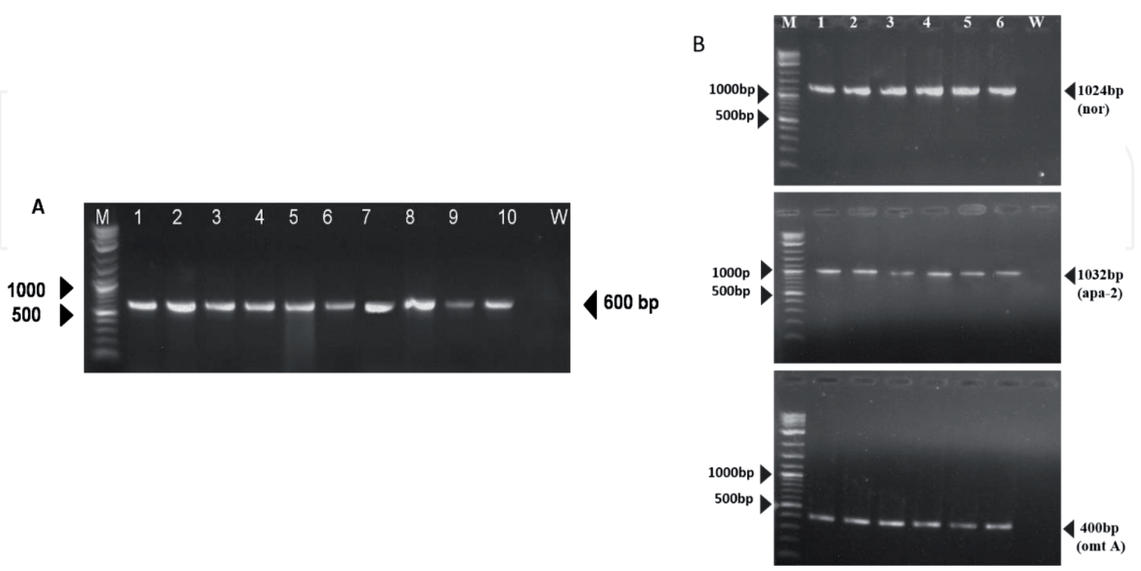

Figure 3.
(A) PCR amplification of ITS region from the genomic DNA of the fungal isolates using ITS-1 and ITS-4 primers and (B) PCR amplification of nor, omt, apa-2 gene from the genomic DNA of the fungal isolates obtained from obtained from fifteen maize growing areas of Bangladesh M: 1 kb plus DNA ladder, 1, AF02_Ran: Rangpur, 2, AF01_Lal: Lalmonirhat, 3, AF01_Bog: Bogura, 4, AF02_Bog: Bogura, 5, AF03_Jas: Jassore, 6, AF04_Jas: Jashore, 7, AF01_Chu: Chuadanga, 8, AF03_Kis: Kishoreganj, 9, AF04_Kis:Kishoreganj, 10, AF01_Man: Manikgan.

Isolate ID	Location	Closest relatives	Accession numbers	Identity	Homology (%)	Aflatoxins biosysthesis genes			Comment
						nor	Omt A	apa	
AF01_Pan	Panchagarh	*A. flavus* isolate PA223	MN006634.1	422/428	98.6	—	+	+	Toxigenic
AF02_Pan	Panchagarh	*A. flavus* strain AF15	KX253943.1	194/204	95.1	+	—	—	Toxigenic
AF01_Tha	Thakurgoan	*A. flavus* strain SU-16	MT680400.1	95/99	95.96	+	—	—	Toxigenic
AF02_Tha	Thakurgoan	*A. flavus* isolate AA221	MN006401.1	171/178	96.07	+	—	—	Toxigenic
AF01_Din	Dinajpur	*A. flavus* isolate 2011F7	MT558941.1	595/598	99.5	+	+	—	Toxigenic
AF01_Nil	Nilphamari	*A. flavus* isolate Z15	MH237650.1	88/90	97.78	+	—	—	Toxigenic
AF02_Nil	Nilphamari	*A. flavus* strain SGE34	JQ776536.1	505/522	96.74	+	+	—	Toxigenic
AF01_Ran	Rangpur	*A. flavus* strain SU-16	MT680400.1	95/99	95.96	+	—	—	Toxigenic
AF02_Ran	Rangpur	*A. flavus* strain 64-A1	MT594359.1	90/94	95.74	+	+	+	Toxigenic
AF03_Ran	Rangpur	*A. flavus* strain SU-16	MT680400.1	416/427	97.42	—	+	—	Toxigenic
AF04_Ran	Rangpur	*A. flavus* strain 64-A1	MT594359.1	474/497	95.37	+	—	—	Toxigenic
AF01_lal	Lalmonirhat	*A. flavus* strain SGE22	JX232269.1	333/370	90	+	+	+	Toxigenic
AF01_Gai	Gaibandha	*A. flavus* isolate A3	MH237624.1	71/72	98.61	+	—	—	Toxigenic
AF01_Bog	Bogura	*A. flavus* strain SGE22	JX232269.1	403/446	90.36	+	+	+	Toxigenic
AF02_Bog	Bogura	*A. flavus* strain bpo4	MT492458.1	424/449	94.43	+	+	+	Toxigenic
AF03_Nat	Natore	*A. flavus* strain BLND1-1	MN396712.1	400/428	93.46	—	+	—	Toxigenic
AF01_Nat	Natore	*A. flavus* strain GFRS16	MT447484.1	591/608	97.2	+	—	—	Toxigenic
AF01_Kus	Kushtia	*A. flavus*solate V5F-13	HQ395774.1	310/321	96.57	+	—	—	Toxigenic
AF01_Jas	Jashore	*A. flavus* isolate BB-1	MT584825.1	577/600	96	+	—	—	Toxigenic
AF02_Jas	Jashore	*A. flavus* isolate AA221	MN006401.1	72/73	98.63	+	—	—	Toxigenic
AF03_Jas	Jashore	*A. flavus*	MN238861.1	599/604	99.17	+	+	+	Toxigenic

Isolate ID	Location	Closest relatives	Accession numbers	Identity	Homology (%)	Aflatoxins biosysthesis genes			Comment
						nor	Omt A	apa	
AF04_Jas	Jashore	*A. flavus* isolate AA221	MN006401.1	229/241	95	+	+	+	Toxigenic
AF05_Jas	Jashore	*A. flavus* strain BLND1-1	MN396712.1	157/164	95.73	+	—	—	Toxigenic
AF06_Jas	Jashore	*A. flavus* strain A1	CP051065.1	551/587	93.87	+	—	+	Toxigenic
AF07_Chu	Jashore	*A. flavus* strain FG38	EU030347.1	38/39	97.44	+	—	—	Toxigenic
AF01_Chu	Chuadanga	*A. flavus* strain JN-YG-3-5	MG554231.1	413/457	90.37	+	+	+	Toxigenic
AF01_Kis	Kishoreganj	*A. flavus* isolate AA221	MN006401.1	469/480	97.71	+	—	—	Toxigenic
AF01_Kis	Kishoreganj	*A. flavus* strain 64-A1	MT594359.1	144/150	96	+	—	+	Toxigenic
AF02_Kis	Kishoreganj	*A. flavus* strain JN-YG-3-5	MG554231.1	412/455	90.55	+	+	—	Toxigenic
AF03_Kis	Kishoreganj	*A. flavus* strain 64-A1	MT594359.1	146/154	94.81	+	+	+	Toxigenic
AF04_Kis	Kishoreganj	*A. flavus* strain ND26	MG659620.1	384/443	86.68	+	+	+	Toxigenic
AF01_Man	Manikganj	*A. flavus* strain SU-16	MT680400.1	591/595	99.33	+	+	+	Toxigenic
AF01_Cum	Cumilla	*A. flavus* isolate PA223	MN006634.1	304/317	95.9	+	—	—	Toxigenic
AF02_Cum	Cumilla	*A. flavus* strain train YLF-14	HQ400610.1	63/67	94.03	+	+	—	Toxigenic
AF03_Cum	Cumilla	*A. flavus* strain JN-YG-3-5	MG554231.1	304/358	85	—	+	—	Toxigenic

Table 2.
List of A. flavus *isolates identified by homology search of sequences of ITS region by BLAST program obtained from maize grain samples collected from fifteen growing areas of Bangladesh.*

When the isolates of *Aspergillus* Spp. were analyzed by PCR for aflatoxin producing ability using *nor*, *omtA*, *apa-2* genes based primers from fifteen maize growing areas. The result showed the amplified DNA fragment was 400 bp, 1024 bp, 1032 bp confirmed that the *A. flavus* isolates had the ability to produce aflatoxin that encode *nor*, *omtA*, *apa-2* genes (**Figure 3B**). Only six species showed a positive result with *nor*, *omtA*, *apa-2* genes set of primers. The result indicated *A. flavus* strains were aflatoxins producers as those were an evident from our investigation (**Figure 3B**).

PCR products were sequenced using ITS-1 primer and sequence data were analyzed by homology search using BLAST Nucleotide program. Isolates were identified as different *A. flavus* based on the homology percentage with their closest relatives available in the NCBI database.

9. Determination of total fumonisins contamination in stored maize grain samples collected from some selected growing areas of Bangladesh

The study was conducted at the Laboratory of Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh. Composite stored maize grains samples were collected from 15 maize growing areas of Bangladesh such as Panchagarh, Thakurgaon, Dinajpur, Nilphamari, Rangpur, Lalmonirhat, Gaibandha, Bogura, Natore, Kushtia, Jashore, Chuadanga, Kishoreganj, Manikganj, Cumilla.

Fumonisins were detected with the highest value recorded in Gaibandha (9.18 mg/kg) and the lowest in Cumilla (0.11 mg/kg) (**Table 3**). Panchagarh (1.47 mg/kg), Thakurgaon (1.27 mg/kg), Dinajpur (0.65 mg/kg), Nilphamari (1.28 mg/kg), Rangpur (1.65 mg/kg), Lalmonirhat (1.18 mg/kg), Bogura (1.29 mg/kg), Kushtia (1.44 mg/kg), Kishoreganj (1.54 mg/kg), and Manikganj (1.47 mg/kg) had moderately high fumonisin levels revealing statistically identical data. Other regions showed indentically dissimilar data except Natore (0.23 mg/kg) and Chuadanga (0.59 mg/kg) (**Table 3**).

Infection rate of *Fusarium* spp. had the highest value in Bogura (13.50 %) followed by Gaibandha (13.25 %), Nilphamari (12.50 %) depicted statistically similar data and the minimal was found in Chuadanga (0.50 %) and Kustia (0.56 %). Moderately higher levels of fumonisin detected in Panchagarh (2.63 %), Thakurgaon (6.06 %), Dinajpur (2.38 %), Rangpur (9.69 %), Jessore (2.25 %), Kishoreganj (17.88 %), Manikganj (6.94 %) and Cumilla (5.31 %) were in the group of ststistically identical data. Moderate but less high and statistically similar results showed in Thakurgaon (6.06 %) and Manikganj (6.94 %) (**Table 3**).

The outmost percent fumonisins concentration over standard limit was found in Rangpur (65 %) followed by Kishoreganj (53.5 %), Gaibandha (47.5 %), Manikganj (47 %), Kushtia (45 %), Panchagarh (46.5 %), Bogura (28.5 %), Thakurgaon (27 %), Nilphamari (27 %), Lalmonirhat (18 %) revealing that the aflatoxins contamination from those area were beyond the regulatory limit set by EU for fumonisins (1 ppm), conversely, fumonisins concentration from other five locations were below the regulatory limit of fumonisins (1 ppm) (**Table 3**).

9.1 Relationship between fumonisins producing *Fusarium* spp. and mean fumonisin concentrations

The regression analysis between *Fusarium* spp. infected maize grains and mean fumonisin concentrations which was positively correlated by observing regression equation where the slope was = 0.038 and y-intercept was = 0.882, coefficient of

Location	Total fumonisins (mg/kg)	Percent maize grains infected with *Fusarium* species	Percent total Fumonisins concentration over standard limit
Panchagarh	1.47 ± 0.14^{b}	2.63 ± 1.20^{e}	46.5
Thakurgoan	1.27 ± 0.13^{b}	6.06 ± 2.07^{cd}	27
Dinajpur	0.65 ± 0.01^{d}	2.38 ± 0.54^{ef}	—
Nilphamari	1.28 ± 0.11^{b}	12.50 ± 0.89^{a}	27
Rangpur	1.65 ± 0.27^{b}	9.69 ± 2.33^{b}	65
Lalmonirhat	1.18 ± 0.17^{bc}	0.00 ± 0.00^{h}	18
Gaibandha	9.18 ± 1.02^{a}	13.25 ± 1.39^{a}	47.5
Bogura	1.28 ± 0.33^{b}	13.50 ± 1.5^{a}	28.5
Natore	0.23 ± 0.06^{de}	0.00 ± 0.00^{h}	—
Kushtia	1.44 ± 0.1^{b}	0.56 ± 0.41^{fgh}	45
Jashore	0.75 ± 0.10^{cd}	2.25 ± 0.43^{efg}	—
Chuadanga	0.59 ± 0.07^{de}	0.50 ± 0.50^{gh}	—
Kishoreganj	1.54 ± 0.20 ^{b}	7.88 ± 0.82^{bc}	53.5
Manikganj	1.47 ± 0.22^{b}	6.94 ± 0.91^{cd}	47
Cumilla	0.11 ± 0.01^{e}	5.31 ± 0.35^{d}	—
Level of significance	**	**	
LSD	0.52	1.86	
CV (%)	12.99	15.97	

**Significant at 5% level of significance. Least significant difference (LSD) at P = 0.05 was used for comparing means and the P values were 0.00.*
***Significant at 1% level of significance. Least significant difference (LSD) at P = 0.05 was used for comparing means and the P values were 0.00. Data are the averages of three biological replications. The regulatory limits for fumonisin is 1 ppm (1 mg/kg).*

Table 3.
Levels of total fumonisins concentration in stored maize grains collected from the stores of traders of fifteen maize growing areas of Bangladesh.

determination, R^2 = 0.198 and coefficient of correlation, r = 0.45 which depicted that 1 percent surges of *Fusarium* in maize grains ultimately rised 0.038 mg/kg fumonisins concentration. In terms of 5 % surges of *Fusarium* in maize grains, the fumonisins concentration was increased up to 0.19 mg/kg and when *Fusarium* increased 20 % in maize grains, the fumonisins concentration was escalated up to 0.76 mg/kg (**Figure 4**).

9.2 Identification of *Fusarium* species from the stored maize grain samples collected from some selected growing areas of Bangladesh

Morphological identification of *F. oxysporum* and *F. proliferatum* were detected by using petridish and culture plate method as well as observing microscopic figures under compound and stereo microscope (**Figure 2B(a)-(f)**). Fifteen fungal isolates were identified using primers specific to ITS 1 and ITS 4 region. PCR assays of *F. oxysporum* DNA with ITS 1 and ITS 4 primers amplified a single fragment of about 600 bp which revealed that all the isolates obtained were fungi (**Figure 5A**). Sequence analysis of ITS region by BLAST program revealed that all the isolates obtained from maize were belong to *F. oxysporum* and *F. proliferatum*.

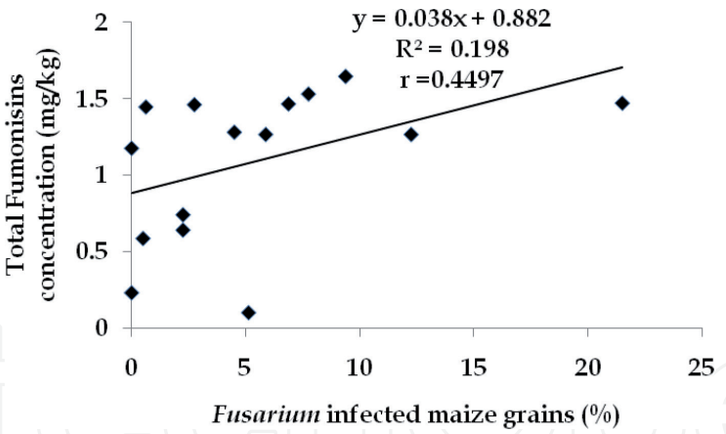

Figure 4.
Linear correlations between Fusarium *infected maize grains and total fumonisin concentration.*

10. PCR based identification and confirmation of fumonisins producing *Fusarium* species obtained from maize grain samples

F01_Pan, F02_Tha, F03_Din, F04_Nil, F05_Ran, F06_Lal, F07_Gai, F08_Bog, F09_Nat, F010_Kus, F011_Jes, F012_Chu, F013_Kis, F014_Man and F015_Cum were identified by PCR amplification of ITS region using ITS1 and ITS4 primers and the results of PCR showed an amplification size 600 bp confirmed the *Fusarium*. PCR products were then sequenced. (**Figure 2A**). Out of fifteen maize growing areas, *F. oxysporum* was found in Panchagarh (*F. oxysporum* strain EP19), Thakurgaon (*F. oxysporum* strain En3), Dinajpur (*F. oxysporum* strain EP19), Nilphamari (*F. oxysporum* strain EP19), Rangpur (*F. oxysporum* strain En3), Natore

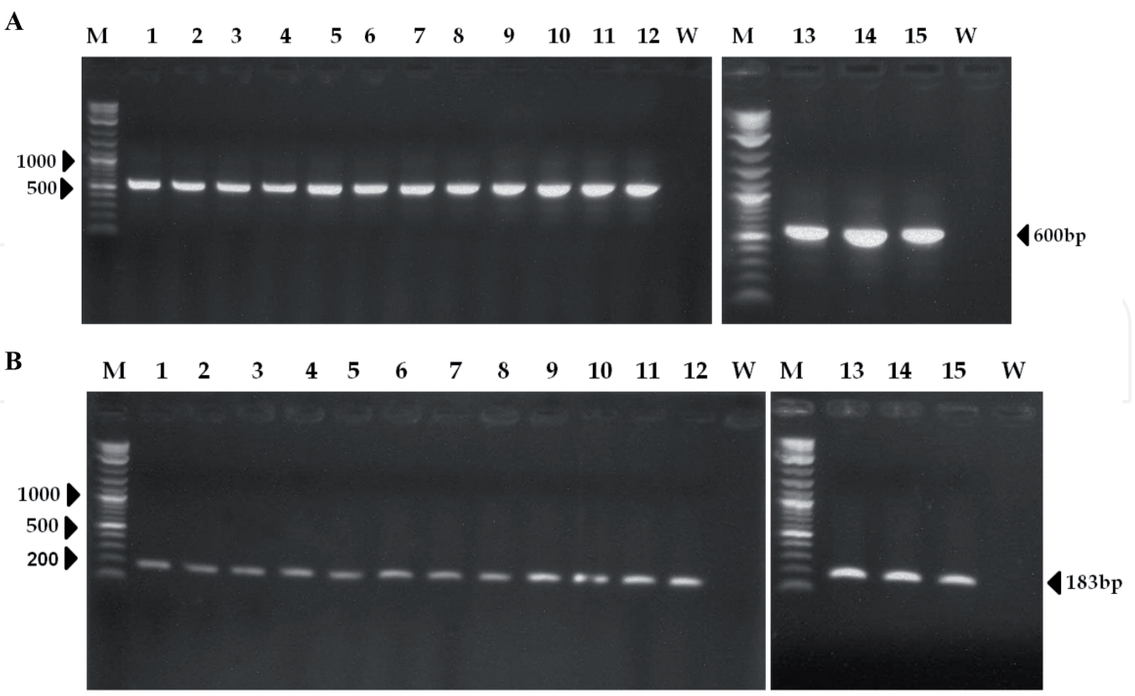

Figure 5.
A. PCR amplification of ITS region from the genomic DNA of the fungal isolates using ITS-1 and ITS-4 primers and B. PCR amplification of FUM1 gene from the genomic DNA of the fungal isolates obtained from obtained from fifteen maize growing areas of Bangladesh M: 1 kb plus DNA ladder, 1, F01_Pan: Panchagarh, 2, F02_Tha: Thakurgoan, 3, F03_Din: Dinajpur, 4, F04_Nil: Nilphamari, 5, F05_Ran: Rangpur, 6, F06_Lal: Lalmonirhat, 7, F07_Gai: Gaibandha, 8, F08_Bog: Bogura, 9, F09_Nat: Natore, 10, F010_Kus: Kushtia, 11, F011_Jes: Jashore, 12, F012_Chu: Chuadanga, 13, F013_Kis: Kishoreganj, 14, F014_Man: Manikganj and 15, F015_Cum:Cumilla.

(*F. oxysporum* isolate FH10 18S), Kushtia (*F. oxysporum* strain EP19), Jashore (*F. oxysporum* strain En3), Chuadanga (*F. oxysporum* isolate H200714-017) Manikganj (*F. oxysporum* strain EP19), Cumilla (*F. oxysporum* strain En3) and *F. proliferatum* was found in Lalmonirhat (*F. proliferatum* strain TH11-3), Gaibandha (*F. proliferatum* strain TH11-3), Bogura (*F. proliferatum* strain TH11-3) and Kishoreganj (*F. proliferatum* strain TH11-3).

Fungal isolates F06_Lal, F07_Gai, F08_Bog and F013_Kis obtained from maize grain samples were collected from Lalmonirhat, Gaibandha, Bogura and Kishoreganj showed the highest homology with *F. proliferatum* strain TH11-3 (**Table 4**). The fungal isolates obtained from maize grain samples collected from

Isolate ID	Location	Closest relatives	Accession number	Identity	Homology (%)
F01_Pan	Panchagarh	*F. oxysporum* strain EP19	MN704852.1	486/534	91.01
F02_Tha	Thakurgoan	*F. oxysporum* strain En3	MN726603.1	491/537	91.43
F03_Din	Dinajpur	*F. oxysporum* strain EP19	MN704852.1	445/530	83.96
F04_Nil	Nilphamari	*F. oxysporum* strain EP19	MN704852.1	486/534	91.01
F05_Ran	Rangpur	*F. oxysporum* strain En3	MN726603.1	477/539	88
F06_Lal	Lalmonirhat	*Fusarium proliferatum* strain TH11-3	MT563411.1	472/508	92.91
F07_Gai	Gaibandha	*Fusarium proliferatum* strain TH11-3	MT563411.1	472/508	92.91
F08_Bog	Bogura	*Fusarium proliferatum* strain TH11-3	MT563411.1	491/544	90
F09_Nat	Natore	*F. oxysporum* isolate FH10 18S	KU361495.1	257/305	84.26
F010_Kus	Kushtia	*F. oxysporum* strain EP19	MN704852.1	486/534	91.01
F011_Jes	Jashore	*F. oxysporum* strain En3	MN726603.1	477/539	88
F012_Chu	Chuadanga	*F. oxysporum* isolate H200714-017	MT974426.1	477/541	88.17
F013_Kis	Kishoreganj	*F. oxysporum* strain TH11-3	MT563411.1	472/508	92.91
F014_Man	Manikganj	*F. oxysporum* strain EP19	MN704852.1	486/534	91.01
F015_Cum	Cumilla	*F. oxysporum* strain En3	MN726603.1	477/539	88

PCR products were sequenced using ITS-1 primer and sequence data were analyzed by homology search using BLAST Nucleotide program. Isolates were identified as different Fusarium species based on the homology percentage with their closest relatives available in the NCBI database. F01_Pan: Panchagarh, F02_Tha: Thakurgoan, F03_Din: Dinajpur, 4, F04_Nil: Nilphamari, F05_Ran: Rangpur, F06_Lal: Lalmonirhat, F07_Gai: Gaibandha, F08_Bog: Bogura, F09_Nat: Natore, F010_Kus: Kushtia, F011_Jes: Jashore, F012_Chu: Chuadanga, F013_Kis: Kishoreganj, F014_Man: Manikganj and F015_Cum: Cumilla.

Table 4.
List of Fusarium isolates identified by homology search of sequences of ITS region by BLAST program obtained from maize grain samples collected from fifteen growing areas of Bangladesh.

Panchagarh (F01_Pan), Thakurgaon (F02_Tha), Dinajpur (F03_Din), Nilphamari (F04_Nil), Rangpur (F05_Ran), Lalmonirhat (F06_Lal), Gaibandha (F07_Gai), Bogura (F08_Bog), Natore (F09_Nat), Kustia (F010_Kus), Jessore (F011_Jes), Chuadanga (F012_Chu), Kishoreganj (F013_Kis), Manikganj (F014_Man) and Cumilla (F015_Cum) showed significant homology with different strains of *F. oxysporum* (**Table 4**).

When the isolates of *Fusarium* species were analyzed by PCR for fumonisins producing ability using *FUM1* gene based primers from fifteen maize growing areas. The result showed the amplified DNA fragment was 183 bp confirmed that the *Fusarium* had the ability to produce fumonisin that encode *FUM1* gene (**Figure 5B**). Only two *Fusarium* species showed a positive result with *FUM1* gene set of primers. The result was contrary as *F. proliferatum* and *F. oxysporum* (**Table 4**) were fumonisin-producers as it was evident from our investigation.

11. Discussion

The experiment was conducted at Plant Bacteriology and Biotechnology Laboratory of Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh during the period of 2019–2020. The purpose of the experiment were to detect the levels of fumonisins and aflatoxins and to identify the aflatoxin and fumonisins producing *Aspergillus* and *Fusarium* in maize associated with maize by PCR using nor, omtA, apa-2 and *FUM1*. Genes involving *afl R*, *ver-1*, *omt-1* and *apa-2* associated with biosynthetic pathway regarding aflatoxins production [73–76]. *Apa-1*, *Nor-1*, *Omt-1* and *Ver-1* gens belong to four primers were applied to detect aflatoxins contamination [77, 78]. *A. flavus* was quantified by *nor-1* gene in several contaminated food samples and cereals using PCR assay [77]. Besides, [56] mentioned that *FUM1* gene with an expected amplicon size of 183 bp can easily detect the fumonisin and non-fumonisin producing *Fusarium,* moreover other researchers also identified the fumonisin by using *FUM1* gene which is in accordance with our study [79–81]. We gathered samples from 15 maize growing areas to measure the aflatoxins and fumonisins level but not all the *Aspergillus* strains are capable of engendering mycotoxins, thus screening is crucial and we detected by Agra Quant Total Aflatoxin and Fumonisin Test Kit following ELISA approach for detection and this method also used by [82–87] for detecting aflatoxins and fumonisin. In our experiment, we detected the aflatoxins contamination Agra Quant Total Aflatoxins 96 well microtiter plate ELISA test kit produced in Romer Labs, Packers and Stockyards Administration (GIPSA) in US Department of Agriculture (USDA) which ability to detect individual aflatoxins very precisely and accurately with a range of 0–320 ppb in accrodance with an experiment conducted by [82]. A number of approaches have been widely used to detect mycotxin naming high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and thin layer chromatography (TLC) [83, 84] and served as a reliable method for detecting aflatoxins and fumonisins [85, 88, 89]. In Gaibandha and Cumilla region fumonisin contamination were highest and lowest compared to other areas revealing moderate amount of fumonisins. In this study, all of the 15 samples were found positive with fumonisins producing *Fusarium* and aflatoxin producing fungi *Aspergillus* which in accordance with the findings of [90, 91]. We found positive correlation for both aflatoxins and fumonisins contamination between their toxin percentages which were matched with the findings of [92] who found apositive correlation has been identified between the proportion of *FUM1* transcripts and the proportion of fumonisins biosynthesized by the *F. verticillioides* and *F. proliferatum* species.

In case of Percent total Fumonisins concentration over standard limit, five regions were under the regulatory limit and other ten regions were exposed higher limit than the regulatory limit exhibiting 65 % followed by 53.5 %, 47.5 %, 47 %, 46.5 %, 45 %, 28.5 %, 27 %, 27 %, 18 % over the standard limit (1 ppm) in the area of Rangpur, Kishoreganj, Gaibandha, Manikganj, Panchagarh, Kustia, Bogura, Nilphamari, Thakurgaon, Lalmonirhat respectively. On the other hand, highest and lowest aflatoxin concentration was recorded in Chuadanga and Dinajpur regions and in terms of percent aflatoxin concentration over standard limit, eight regions were below the permissible limit of aflatoxins, conversely, five regions exposing 915.7 % followed by 587.3 %, 214.8 %, 208.85 %, 19.5 % aflatoxin concentration beyond permissible limit of 10 μg in the region of Chuadanga, Gaibandha, Kustia, Kishoreganj and Cumilla respectively. Refs. [15, 93] recorded that surges of aflatoxin contamination levels beyond regulatory limit due to increased droughts, pest damages, temperatures, host susceptibility.

As we observed that both aflatoxin and fumonisin concentration were fluctuate one region to another region which have been also monitored that due to association of several significant factors like temperature, water activity, storage conditions, drought, humidity, insect damage, flowering stage, plant characteristics [94–98]. Ref. [48] revealed that aflatoxin production comprised of several factor including existence of certain genes and in intact that means deletions or insertions within the gene regions, crop stress [99] and in fumonisins two factors temperatures and water potential are fundamental to produce fumonisins [99] along with rainfall patterns, longer durations of drought which has been prominent in Mediterranean regions [100–103]. These all conditions significantly impact on the variation of the population of mycotoxin producing fungi both *Fusarium* and *Aspergillus* [103]. In our experiment, we recorded over all three regions (Chuadanga, Kishoreganj, Gaibandha) were engendering higher amount of aflatoxins and fumonisins production respectively, thus we speculated in Chuadanga, temperature fluctuation influences the mycotoxin production, in Kishoreganj which exposed with flood and severe water stress and the region Gaibandha with drought problems, these might have the feasible factor for *Aspergillus* and *Fumonisins* to produce gigantic amount of mycotoxins compared to other areas. Aflatoxin levels rise as a result of drought, insect damage, and heat during fungal growth [25]. Marasas [104] found that, the presence of fumonisins is linked to weather conditions, with larger instances occurring during hot and dry conditions. Abbas et al. [105] revealed that *A. flavus* grows supreme around 28–37° C with a humidity level of at least 80 %.

Post-harvest factors are also exacerbate mycotoxin production and generate a favorable condition for fungus related to their growth and mycotoxin production and those include storage fungus, insect infestation, contaminant mold respiration, insects and mites, water availability and temperature ultimately deteriorate grain quality [106–108]. As [109] also observed that interaction between these factors triggered the mycotoxigenic species growth, mycotoxin production, niche occupation and competitiveness, [110] also revealed the moisture and surrounding air conditions also influenced mycotoxin production by initiating biological and biochemical activity. Maize is a hygroscopic crop which easily absorbs or release moisture and humidity in the surrounding ambience until getting the adjustment with equilibrium conditions which led to swift degradation in storage. *Fusarium* species can damage stored grain by causing seedling illnesses, root rots, stalk rots, and ear rots in maize which ultimately hazardous to plants and animal [111–116]. Due to all correlating factors with aflatoxin production, high amount of aflatoxins were found in Bangladeshi markets [23] and 82 % contamination in South Asia [49]. Decomposing potentiality of AFs are very slow several approaches including

physical, chemical have been investigated [19] and monitored changing in sensory property and nutrient diminishment which led to mount food safety problems ultimately. A number of microorganisms have been identified fruitfully working as a biocontrol agents to control mycotoxins such as *Bacillus subtilis*, *Pseudomonas*, *Trichoderma*, atoxigenic strains of *A. flavus* and *A. parasiticus* [117–119]. Thus, suppressing mycotoxins by biocontrol agent would be a fruitful approach though several experiments need to be conducted precisely in future.

12. Conclusion

Aflatoxins and fumonisins are the major source of disease outbreaks due to a lack of knowledge and consumption of contaminated food and feed in Bangladesh. Excessive levels of aflatoxins and fumonisins in food in Bangladesh is a major concern because still majority of the people have not any idea that they are consuming food and feed which crossed the permissible limit set by EU. Another significant factor is no sign of regulating any acceptable limit for this country and that's why people are easily contaminated with several mycotoxins without properly knowing any acceptable limit as well as industries are also not ensuring any precise step to diminish mycotoxins concentration in terms of engendering several products. As our study clearly conceded that most of the regions (Rangpur, Gaibandha, Kushtia, Chuadanga, Kishoreganj, Manikganj, Cumilla) were at higher risk for aflatoxin as well as the regions (Panchagarh, Thakurgoan, Nilphamari, Rangpur, Lalmonirhat, Gaibandha, Bogura, Kushtia, Kishoreganj, Manikganj) were exposed with fumonisins contamination more than that of acceptable limit of fumonisins which ultimately effects animal and mankind by entering our food chain. Thus, several effective approaches (physical, chemical, biological, and genetic engineering techniques) need to be employed as early as possible to suppress the ruinous consequences of mycotoxin contamination of Bangladesh.

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Conflict of interest disclosure

Authors do not have any conflict of interests to declare.

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