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Article

Influence of climatic factors on spatio-temporal variabilities in the occurrence of aflatoxins in major food grains available at retail shops in Bangladesh

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Abstract: In Bangladesh, little is known about the role of climatic factors influencing spatio-temporal occurrence among different food grains of aflatoxins, which have been linked to liver cancer cases and other health hazards. In this study, 252 food grain samples of 10 types were collected from retail markets at 18 districts, pre-monsoon, monsoon and post-monsoon seasons during February-September, 2018. Occurrence and concentration of total aflatoxins in the samples were analysed by a direct competitive enzyme-linked immunosorbent assay, and compared with climatic data (temperature, humidity, rainfall, and sunshine hours). Contamination of aflatoxins was found in approx. 38% (n=96) of the total samples, with highest occurrence in groundnuts (82%), followed by corn grains (70%), chick pea (54.5%), wheat grain (50%) and green gram (42.9%). Aflatoxins occurrence was moderate in split chick pea (30.8%), yellow pea (28.2%), black gram (27.8%), and lentils (27.6%), while the lowest in grass pea (17.6%). Aflatoxins concentration was found to exceed the European Union permissible limit (≤5 µg kg^{-1}) in 33 (13.1%) samples. Overall, approximately 5.6%, 7.5%, and 25.0% samples were contaminated with high (10-19 ppb), medium (5-9 ppb) and low (1-4 ppb) concentrations of aflatoxins, respectively. High level concentration of aflatoxins was more frequent in groundnut (36%) and wheat grain (20%), in comparison to other types. Occurrence of aflatoxins during pre-monsoon was detected among 21% (19/91) samples, which increased to ca. 30% (36/119) during monsoon, and 98% (41/42) during post-monsoon. However, aflatoxins mean concentration during post-monsoon was comparatively low than the other seasons. Variations in aflatoxins monthly prevalence correlated significantly with relative humidity ($p \leq 0.01$) and rainfall ($p \leq 0.05$), when a 1-month time lag was considered. Therefore, predisposing climatic conditions, i.e., rainfall incidences and persistence of higher relative humidity in the previous month(s), have salient influence on aflatoxins occurrence, potentially impacting both pre-harvest and stored food grains. This study underscores the need of a more holistic monitoring of aflatoxins in agricultural products for a longer term, and adoption of proper intervention measures for food grains while being stored and before consumption.

Keywords: food grains; aflatoxins; seasonlity; climatic factors

1. Introduction

Aflatoxins are a type of small molecular weight mycotoxins mainly produced by *Aspergillus flavus* and *A. parasiticus*, the common filamentous fungi grown on a wide variety of plants, including crops, under conducive environmental conditions. The toxic by-products of aflatoxins are potentially carcinogenic and harmful to both human and animal health (Zain, 2011). Moreover, aflatoxins have immunosuppressive, hepatotoxic, teratogenic and mutagenic effects (Marchese *et al*., 2018; Bhatnagar-Mathur *et al*., 2015). Agricultural products including different kinds of grains and cereals are often found to be contaminated with aflatoxins. A number of environmental factors, including temperature, humidity, and water activity are considered to be influential for the growth of *Aspergillus* spp. in agricultural products, during both pre- and post-harvest (Johnsson *et al*., 2008; Lahouar *et al*., 2016). Production of aflatoxin in these fungal mold are naturally governed by weather conditions in the locality where foodgrains are produced and/or stored. Seasonal changes in atmospheric parameters and occurrence of rainfall and drought are considered as the key climatic drivers influencing the growth of *Aspergillus* mold and eventually aflatoxin production, as reported for different geographical regions (Cotty and Jaime-Garcia, 2007; Pratiwi *et al*., 2015; Lahouar *et al*., 2016; Bedaiko *et al*., 2019). In addition, crops damaged by insect infestation, interaction of field soil, drought or wet crops before harvest, and improper maintenance of the harvested products are also responsible for the occurrence and level of aflatoxin contamination in food products (Klich, 2007; Waliyar *et al*., 2015a; Bhatnagar-Mathur *et al*., 2015).

Health concern for aflatoxins initially gained prominence in the early 1960s after a mysterious "Turkey X disease", linked to peanut meal contaminated with *A. flavus*, killed approximately 100,000 turkey birds in England (Bennett *et al*., 2007). Among more than 20 kinds of alfatoxins distinguished so far four are considered as major types, namely B_1, B_2, G_1 and G_2, which produce potent toxic compounds in human and other animals (Kumar *et al*., 2017). After several evaluations of scientific evidences from human and experimental animals in different years, the International Agency for Research on Cancer (IARC) has classified all these four major aflatoxins as Group 1 carcinogens, i.e., they can cause cancers, particularly liver cancer, in human (Ostry *et al*., 2017). However, among these major types, aflatoxin B1 has the highest toxicity potential, and also found to be the most predominantly occurring in crops or food products (Kumar *et al*., 2017; Negash, 2018). Humans and other animals, including poultry and livestock, can easily become exposed to aflatoxins and other mycotoxins, through oral ingestion and adsorption (mucous/ cutaneous route) or by inhalation (respiratory route) of contaminated agriculture products, and eventually they may develop acute and chronic diseases, commonly termed as mycotoxicosis (Richard, 2007). Aflatoxins poisoning is also thought to be associated with kwashiorkor, a form of malnutrition, and growth retardation among children (Khlangwiset *et al*., 2011). In poultry animals, aflatoxin intoxication can cause a variety of impacts, e.g., growth retardation, hepatic necrosis, biliary hyperplasia, diarrhea, immunosuppression, and decreasing reproductive performance (Fouad *et al*., 2019). Aflatoxin and their metabolites are reported to be present in blood, tissues, eggs and different organelles in poultry and these kind of contaminated poultry products are a potential threat to the human consumer (Pandey and Chauhan, 2007; Fouad *et al*., 2019). In livestock animals, aflatoxin B1 and B2 is transformed into its hydroxylated forms, aflatoxin M1 and M2, which can be found in their secreted milk. These hydroxylated forms of aflatoxin are very stable toxins, i.e., remaining unaltered and potentially toxic even after pasteurization of milk and preparation of dairy products (Turna and Wu, 2021). Considering the toxic effects of aflatoxin on human and animal health, the permeable level of the content of total aflatoxin in food and feeds has been established by several countries. For example, U.S. FDA has set an action level for content of total aflatoxin of 20 and 100 μg/kg for human food and animal feed (https://www.regulations.gov/document/FDA-2020-D-1956-0001 (accessed on March 15, 2024; Bennett *et al.*, 2007). However, the European Union has a more stringent permissible limit with consideration of ≥5 μg kg^{-1} of total aflatoxins in food grains as unsafe for human consumption (EC, 2010).

Occurrence of acute and chronic diseases, including liver cancer, which could be related to the consumption of food contaminated with aflatoxins has been an alarming issue concerning public health in Bangladesh and neighbouring countries in South Asia (Koirala *et al*., 2005; Toteja *et al*., 2006; Mahfuz *et al*., 2019). An estimated of ca. 44% of total annual liver cancer cases in Bangladesh have been linked to the dietary aflatoxin exposure, together with synergistic impact of chronic hepatitis B viral infection (Turna and Wu, 2019). A study involving informal settlements in Dhaka city has observed majority (62%) of children under 5 years of age are susceptible to high aflatoxin exposure (Mahfuz *et al*., 2019). An earlier study detcted the presence of aflatoxin in 46% of urine samples of adult males and females in both rural and urban locations (Ali *et al*., 2016). Alarmingly, occurrence of aflatoxins and/or other mycotoxins have been reported in urine samples among ca. 96% of pregnant women in selected rural areas of Bangladesh (Kyei *et al*., 2022). Concerning public health view points and in order to ensure food safety and security, detail information on the geospatial and seasonal

variations in the occurrence and contamination level of aflatoxins in different kinds of food grains is integral. Aflatoxin-producing fungi are commonly encountered on food grains not only at the time of harvesting and crop maturation phase in the field but also during storage under improper conditions (Torres *et al*. 2014). Aflatoxins are highly persistent in the most conditions of storing, handling in market level and processing of food grains, while it is not easy to decrease them once the foodstuffs are contaminated (Ubwa *et al*., 2014).

In the predominantly hot and humid climatic conditions in Bangladesh, a variety of food grains serving as the major constitutent of the daily meals are potentially susceptible to contamination by aflatoxins (Bhuiyan *et al*., 2013). However, a few investigations have been conducted so far exploring the occurrence and contamination level of aflatoxins in only selected (one to four) kinds of food grains in particular regions (Roy *et al*., 2013; Bhuiyan *et al*., 2013; Khandaker *et al*., 2019; Rafik *et al*., 2020; Sultana *et al*., 2021; Jannat *et al*., 2022). Adequate knowledge on the comparative variations of aflatoxins contamination in a variety of agriculture products, including different types food grains, the key environmental drivers of aflatoxin occurrence at different geoclimatic settings, and associated health hazards has remained largely unexplored in this densely populated developing nation with agriculture-based economy. Hence, this study aimed to systematically assess the comparative occurrence, and spatiotemporal variations in contamination level of total aflatoxin content among majority of the common food grains, including cereal grains, pulses and nuts, commonly available at local markets in different geo-climatic regions of Bangladesh.

2. Materials and Methods

2.1. Ethical approval

No animal intervention was needed as only food grain samples were used in this study. A verbal consent was obtained from each of the owners of retail shops at local markets prior to the sample collection. The Ethical Committee of the Bangladesh Agricultural University approved the study under reference no. AWEEC/BAU/2020(27).

2.2. Study area and sample collection

Samples of common food grains were collected from retail markets at 18 districts in Bangladesh, representing her six geoclimatic zones, with variable range of annual rainfall (Figure 1). A total of 252 food grain samples stored at retail markets in different regions (North-East, Central, Central-East, South-Central, South-East and South-West) were collected at regular intervals between February, 2018 and September, 2018, capturing geoclimatic variabilities of all the major seasons, pre-monsoon, monsoon and post-monsoon (Table 1). The samples included 10 types of food grains: split chickpea (n=52), yellow pea (n=39), grass pea (n=34), lentil (n=29), ground nut (n=28), green gram (n=21), black gram (n=18), chickpea (n=11), corn (n=10), and wheat grain (10). During sample collection a variable number (n=7-33) and types (5-10) of samples were obtained which represented their temporal availability at a particular district. At least 300 g portion of each sample, representing a pooled amount by combining three randomly collected sub-samples (ca. 100 g each), was aseptically taken into a sterile polythene bag, which was sealed instantly. The collected samples were transported to a central laboratory in Dhaka` and analyzed within a week.

Table 1. Occurrence and concentration levels of aflatoxins in major food grain samples collected from retail markets at 18 selected districts during pre-monsoon, monsoon and post-monsoon seasons in Bangladesh.

Food grain type (n)	Negative samples [n (%)]	Positive samples with variable level of concentration				Total positive [n (%)]
		1-4 µg kg^{-1} [n (%)]	5-9 µg kg^{-1} [n (%)]	10-19 µg kg^{-1} [n (%)]	Mean (µg kg^{-1} ± SD)	
Ground nut (28)	5 (17.9)	10 (35.7)	3 (10.7)	10 (35.7)	7.95 ± 5.24	23 (82.1)
Corn (10)	3 (30.0)	4 (40.0)	3 (30.0)	0 (0.0)	4.16 ± 1.77	7 (70.0)
Chick pea (11)	5 (45.5)	4 (36.4)	2 (18.2)	0 (0.0)	4.18 ± 1.52	6 (54.5)
Wheat grain (10)	5 (50.0)	1 (10.0)	2 (20.0)	2 (20.0)	8.20 ± 5.67	5 (50.0)
Green gram (21)	12 (57.1)	7 (33.3)	1 (4.8)	1 (4.8)	4.60 ± 3.84	9 (42.9)
Split chick pea (52)	36 (69.2)	12 (23.1)	3 (5.8)	1 (1.9)	4.00 ± 2.66	16 (30.8)
Yellow pea (39)	28 (71.8)	9 (23.1)	2 (5.1)	0 (0.0)	2.69 ± 1.68	11 (28.2)
Black gram (18)	13 (72.2)	4 (22.2)	1 (5.6)	0 (0.0)	4.00 ± 2.24	5 (27.8)
Lentils (29)	21 (72.4)	7 (24.1)	1 (3.4)	0 (0.0)	3.13 ± 2.47	8 (27.6)
Grass pea (34)	28 (82.4)	5 (14.7)	1 (2.9)	0 (0.0)	2.83 ± 1.60	6 (17.6)
Overall (252)	156 (61.9)	63 (25.0)	19 (7.5)	14 (5.6)	4.95 ± 3.96	96 (38.1)

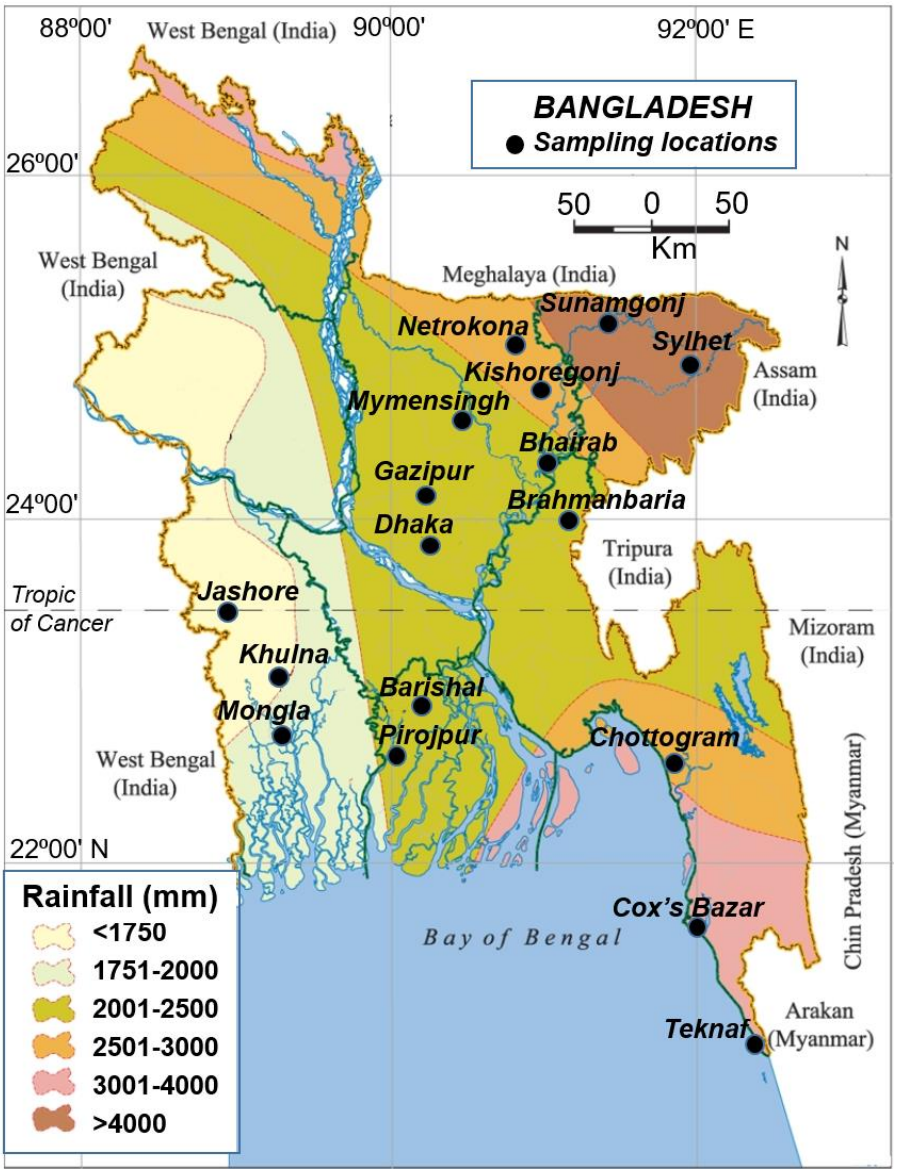

Figure 1. Map showing sampling locations at 18 districts in Bangladesh. Sampling locations represented her six geoclimatic regions based on variable ranges of annual rainfall. Various kinds of food grain samples, obtained from retail markets at the selected localities, were examined for the presence of aflatoxins. The map was adapted from Banglapedia (https://www.banglapedia.org).

2.3. Sample processing for extraction of aflatoxins

Preparation of samples and extraction were done according to the method described by Ubwa *et al*. (2014) with little modification. In brief, at least 50 g of each sample was ground properly using a Mixer Grinder machine so that >90% of ground sample would pass through a 20-mesh screen (aperture 0.9 mm). The grinder machine was properly cleaned after milling each sample before proceeding to the next sample. A 20 g portion of the ground sample together with 100 mL portion of methanol: water extraction solution (70:30, v/v) was taken in a clean conical flask (250 mL) and sealed properly. The sample and extraction solution, thus combined at a ratio of 1:5, respectively, were mixed well using a magnetic stirrer for 30 minutes at room temperature (ca. 25ºC), and afterwards the extracted sample was allowed to settle down. Finally, the supernatant was filtered using Whatman #1 filter, and the filtrate was collected as 1 mL fractions in sterile Eppendorf tubes (1.5 mL), which were kept in a refrigerator (4-5ºC) until further analysis for aflatoxin detection.

2.4. Detection and measuring the amount of total aflatoxins

Extracted solutions derived from different kinds of food grain samples were analyzed by a commercially available AgraQuant® Total Aflatoxin Assay (Romer Labs Singapore Pte Ltd.), a direct competitive enzyme-

linked immunosorbent assay (ELISA) measuring total aflatoxins (including B1, B2, G1 and G2). Among a number of established detection methods for aflatoxins, this ELISA-based assay was employed because this method was specifically intended for analyzing grains, cereals, nuts, and animal feeds, and reported to be highly sensitive, less complex to perform, and also comparatively less expensive than other methods, e.g., HPLC, TLC, and LC-MS/MS (Leszczyñska *et al*., 2001; Wacoo *et al*., 2014).

The ELISA kit included 6 standards, a microtiter plate containing 48 antibody-coated microwells and 48 colour coded dilution microwells, 8-channel pipette, substrate, conjugate (horse redoxy peroxide), and stop solution. The assay for total aflatoxins detection was performed according to Romer Labs Methods, as described in the manual of the ELISA kit, with little modifications. In brief, for each standard (0, 1.0, 2.0, 4.0, 10.0 and 20.0 ppb) or sample, an appropriate number of dilution microwells along with an equal number of antibody-coated microwells were used. Initially, a 200 µL of conjugate was dispensed into each dilution well and then 100 µL of each standard or sample was added. After mixing carefully by pipetting a 100 µL of the mixture contents from each dilution well were immediately transferred into a corresponding antibody-coated microwell and incubated at room temperature (ca. 25ºC) for 30 minutes. Afterwards, the contents of the antibody-coated microwell were emptied and washed gently with deionized water for four to five times. Residual water was then removed by tap drying the wells on absorbent paper towel. After addition of 100 µL portion of the substrate into each microwell, the microtiter plate was incubated for 15 minutes at room temperature in the dark. A 100 µL of stop solution was then added into each microwell to halt the reaction and associated changes in color intensity of this final solution. The resultant optical density (OD) (representing %absorbance) of the standards or test samples in the microtiter plate were read with a Stat fax ELISA reader using a 450 nm filter and a differential filter of 630 nm. Concentration (ppb or µg kg^{-1}) of total aflatoxins in the tested samples were estimated by comparing their OD values with those of the standards, applying regression analysis of calibration curve using the Romer® Log/Logit spreadsheet.

2.5. Climatic data collection and comparison

Among the climatic variables, daily data of temperature, rainfall, relative humidity (RH) and sunshine hours (SSH) of all the study regions (districts) for the tenure January-September, 2018, were obtained from the archives of AccuWeather (https://www.accuweather.com/). Temperature data comprised of daily minimum (Tmin), mean (Tmean), and maximum (Tmax) values. Climatic data were transformed from daily values into monthly mean values and compared with the monthly prevalence of aflatoxins in food grain samples. However, each of the climatic factors may influence the growth and aflatoxin production of *Aspergillus* spp. in agriculture products at various stages, including pre- and post-harvest period and during storage over time. Considering such uncertainity of time length for the climatic factors to regulate aflatoxin occurrence, a composite analysis was performed comparing the mean values at sliding bimonthly intervals, in addition to direct comparison of the mean values for the concurrent month. Comparison of this kind of overlapping bimonthly time frame is a robust method to capture intrinsic correlating pattern between two variables, if any, and particularly useful when considering diverse geo-climatic settings under composite influences of biological and environmental factors of different kinds and amplitude. Seasonal variabilities (pre-monsoon, monsoon and post-monsoon) in the overall mean values of each of the climatic factors and aflatoxins occurrence in food grain samples at different districts were also compared.

2.6. Data management and Statistical analyses

All data from the hard copies were imported to Microsoft Excel 2023 (Microsoft Corporation, Redmond, WA, USA) spreadsheet and cleaned, coded and checked for integrity of each data set. Firstly, descriptive statistical analysis, including estimation of mean and standard deviation, was done. The monthly variabilities in the occurrence of aflatoxin in food grain samples and individual climatic factor were correlated and linear regression analyses were performed using 'Xact' (ver. 7.21d, SciLab GmbH, St. Yrieix, France) to unveil any potential association. Differences in the seasonal patterns for the occurrence of aflatoxins and each of the climatic factors were determined by the Chi square test or Paired Samples t-test, as appropriate, using Statistica (ver. 10.0, StatSoft Inc., USA). A 'p' value of <0.05 was considered significant.

3. Results

Overall, contamination of aflatoxins was detected in approx. 38% (96 out of 252) of the total samples analyzed in this study (Table 1). Considering the diversity of food grains, all 10 types examined in this study were found contaminated with aflatoxins. The frequency of aflatoxins contamination was observed to be the highest (ca. 82%) in groundnut samples. Among the other types of food grains, aflatoxin contamination was detected at a

relatively higher frequency (ca. 70%) in corn grain while at a moderate frequency (43-55%) in green gram, wheat grain and chick pea samples. In the cases of split chick pea, lentils, black gram, and yellow pea, aflatoxins contamination was found in between 28 and 31% samples. The lowest rate (ca. 18%) of contamination was observed in grass pea samples.

Independently of its variable occurrence in different food grain samples, the concentration level of aflatoxin in the positive samples showed variations, i.e., low (1-4 ppb), medium (5-9 ppb) and high (10-19 ppb). Among an estimated 25.0% of the total samples the level of aflatoxin concentration was low, while approximately 7.5% and 5.6% of the samples were contaminated with medium and high levels of concentration, respectively (Table 1). A significantly higher frequency, i.e., ca. 36% and 20%, respectively, of groundnut and wheat grain samples were found to be contaminated with high level (10-19 ppb) of toxin concentration in comparison to other types of food grains. Likewise, among the positive samples of groundnut and wheat grain the mean concentration of aflatoxin was also higher (ca. 8 ppb) when compared to the overall mean of total positive samples (ca. 5 ppb). A small fractions of green gram and split chick pea samples, comprising ca. 5% and 2%, respectively, were also found contaminated with high level of aflatoxin concentration. The highest concentration of aflatoxin detected in ground nut and wheat grain samples was ca. 18 and 16 ppb, respectively.

Occurrence in food grain samples of aflatoxin showed a remarkable spatial variations, when comparing the detection rates of different localities/districts and regional dimensions, particularly during pre-monsoon and monsoon seasons (Table 2). For example, during pre-monsoon, aflatoxin occurrence was detected in higher frequency at Mymensingh (33.3%) in the Central region, followed by Mongla (28.6%) in the South-West, in comparison to other districts in the South-Central, South-West and North-East regions where its occurrence was detected between 14.3 and 21.4% of the samples. During monsoon a distinctively higher occurrence of aflatoxin was detected in samples from the North-East districts, Sylhet (54.5%) and Sunamgonj (42.9%) when compared to samples from the Central and South-East districts (11.1-24.0%). In contrast, during post monsoon, a ubiquitous occurrence of this toxin was noted in food grain samples, irrespective of their types and locations of their collection in different regions. A 100% positive rate of afltaoxin contamination was noted for all kinds of food grain samples in three districts at North-East, Central and Central-East regions during this season. In comparison, in Bhairab district at the Central-East region, the presence of this toxin during post-monsoon was detected in a slightly lower frequency, comprising 91.7% of the tested food grain samples.

Table 2. Spatiotemporal variations of aflatoxin-positive samples in different food grains.

Season / Samples (n): [positive/ total (%)]	Location (District/ Region)	Tested samples: total (n); *food grain type (^{n})**	Positive samples: total (n); *food grain type (^{n})**	Percentage of positive
Pre-monsoon	Barishal (S-C)	**7**; *GN^1, GP^1, LN^1 YP^2, SC^2,*	**1**; *GN^1*	14.3
19 / 91 (20.88%)	Jashore (S-W)	**21**; *GN^1, CR^1, CP^1, BG^1, LN^2, YP^2, GG^2, SC^3, WG^4, GP^4*	**3**; *GN^1, CP^1, WG^1*	14.3
	Khulna (S-W)	**14**; , *CP^1, GP^1, BG^1, LN^1, GN^2, CR^2,YP^2, SC^4*	**3**; *GN^1, CR^2*	21.4
	Mongla (S-W)	**7**; *GN^1, GP^1, LN^1, GG^1, SC^1, YP^2*	**2**; *GN^1, GG^1*	28.6
	Pirojpur (S-C)	**7**; *GN^1, CR^1, GP^1, LN^1, WG^1, SC^2*	**1**; *GN^1*	14.3
	Mymensingh (C)	**18**; *CR^1, GG^1, GN^1, WG^1, SC^2, CP^2, GP^2, YP^2, BG^3, LN^3*	**6**; *CR^1, GG^1, GN^1, WG^1, CP^2*	33.3
	Netrokona (N-E)	**17**; *YP^1, GG^1, WG^1, CR^1, GN^2, SC^2, GP^2, BG^2, LN^2, CP^3*	**3**; *CR^1, YP^1, GN^1*	17.6
Monsoon	Chittagong (S-E)	**22**; *WG^1, BG^1,GN^2, GP^3, LN^3, GG^3, YP^4, SC^5*	**3**; *WG^1, GN^2*	13.6
36 / 119 (30.25%)	Cox's Bazar (S-E)	**25**; *CR^1, CP^1, GG^2, GN^3, LN^3, GP^5, YP^5, SC^5*	**6**; *GN^3, YP^2, SC^1*	24.0
	Teknaf (S-E)	**16**; *CR^1, GG^1, GN^2, LN^2, YP^2, GP^4, SC^4*	**2**; *CR^1, GN^1*	12.5
	Dhaka (C)	**9**; *GN^1, GP^1, LN^1, BG^1, GG^1, YP^2, SC^2*	**1**; *GN^1*	11.1

Table 2. Contd.

Season / Samples (n): [positive/ total (%)]	Location (District/ Region)	Tested samples: total (n); *food grain type (^{n})**	Positive samples: total (n); *food grain type (^{n})**	Percentage of positive
	Sunamgonj (N-E)	**14**; *GP[1], CP[1], BG[1], GN[2], LN[2], YP[2], GG[2], SC[3]*	**6**; *GP[1], CP[1], GN[1], LN[1]*	42.9
	Sylhet (N-E)	**33**; *GP[3], BG[3], GN[4], LN[4], YP[5], GG[5], SC[9]*	**18**; *GN[2], LN[3], YP[1], GG[5], SC[7]*	54.5
Post-monsoon	Brahmanbaria (C-E)	**9**; *GN[1], CR[1], GP[1], LN[1], BG[1], SC[1], WG[1], YP[2]*	**9**; *GN[1], GP[1], LN[1], BG[1], SC[1], WG[1], YP[2],*	100
41 / 42 (97.62%)	Bhairab (C-E)	**12**; *GN[1], GP[2], LN[1], BG[2], SC[2], YP[4]*	**11**; *GN[1], GP[2], LN[1], BG[2], SC[2], YP[3]*	91.7
	Gazipur (C)	**10**; *LN[1], BG[1], GG[1], CP[1], GN[2], GP[2], SC[2]*	**10**; *LN[1], BG[1],CP[1], GG[1], GN[2],GP[2], SC[2]*	100
	Kishoregonj (N-E)	**11**; *GN[1], CR[1], BG[1], GG[1], CP[1], WG[1], YP[2], SC[3]*	**11**; *GN[1], CR[1], BG[1], GG[1], CP[1], WG[1], YP[2], SC[3]*	100

*GN, Ground nut; CR, Corn; YP, Yellow pea; WG, Wheat grain; CP, Chick pea; GP, Grass pea; LN, Lentils; GG, Green gum; SC, Split chick pea; BG, Black gram

A seasonal influence of aflatoxins occurrence in food grain samples was demarcated. When comparing the types of food grains, the presence of aflatoxin was observed only during post-monsoon for the black gram samples (Figure 2). In the cases of grass pea, lentils and split chick pea, this toxin could be detected during both monsoon and post-monsoon. In the other kinds of food grain species, i.e., ground nut, corn, chick pea, yellow pea, green pea, and wheat grain, aflatoxin was detected in all the three seasons but at variable rates (Figure 2). However, the prevalence of aflatoxin was found to be widespread and exceptionally higher for the case of ground nut, since contamination was detected in most of its samples irrespective of spatiotemporal variations.

During pre-monsoon only ca. 21% (i.e., 19 out of 91) samples were detected positive for this toxin (Figure 3A). The frequency of aflatoxin-positive samples increased to ca. 30% (36 out of 119) during monsoon while ca. 98% (41 out of 42) of the samples were found contaminated with aflatoxin during post-monsoon. In comparison to pre-monsoon, the increased detection rate in food grain samples of aflatoxin contamination during monsoon was not found to be statistically significant. However, the frequency of aflatoxin occurrence during post-monsoon was significantly higher ($p<0.0001$, chi-square test) in comparison to both pre-monsoon and monsoon. Interestingly, in contrast to the observed trend of seasonal impacts on the occurrence or detection rate (% of positivity) of aflatoxin in the tested food grains, its concentration level in the positive samples showed an opposite trend (Figure 3B). For example, during pre-monsoon the mean concentration of aflatoxin in positive samples was ca. 7.3 ppb, which decreased to ca. 5.7 ppb during monsoon. In comparison, the mean concentration of aflatoxin in the positive samples during post-monsoon (ca. 3.2 ppb) was significantly lower than that of pre-monsoon ($p<0.01$, t-test) and monsoon ($p<0.05$, t-test).

Seasonal variations in the climatic factors were computed from their monthly mean values corresponding to each season using t-test (2-tail, two sample unequal variance). Basically, the monthly mean values (with ± standard deviations) in the climatic factors represented the overall regional variations, considering the sampling locations in different districts, and have been depicted in Figure 4 (A). Monthly mean values of Tmax, Tavg and Tmin showed an increasing trend during the pre-monsoon but afterwards they did not fluctuate significantly. In the case of Tmax, the monthly mean values during pre-monsoon was significantly lower ($p<0.05$) than that of monsoon and post-monsoon seasons (Figure 4B). However, in both the cases of Tavg and Tmin, the difference in monthly mean values was highly significant ($p<0.01$) when comparing pre-monsoon (19-29ºC and 14-25ºC, respectively) with those of monsoon and post-monsoon seasons (29-30ºC and 26-27ºC, respectively). A similar trend in the monthly means of relative humidity with comparatively lower values ($p<0.01$) during pre-monsoon (52-66%) than those of the monsoon and post-monsoon seasons (73-81%) was noted (Figure 4B). During pre- and post-monsoon seasons, the monthly precipitation ranged from 0.7 to 63 mm and 87 to 110 mm, respectively (Figure 4A). A conspicuous increase in rainfall was evident for the monsoon season when the monthly precipitation ranged between 115 and 240 mm. Consequently, in comparison to pre-monsoon, the mean monthly precipitation was significantly higher ($p<0.01$) during both monsoon and post-monsoon seasons (Figure 4C). On the other hand, the mean monthly precipitation was significantly lower ($p<0.05$) during post-monsoon than that of the monsoon season. During the study period, the monthly sunshine hours ranged between 230 h during Jan-

Feb and 340 h in May (Figure 4A), however, no significant difference in mean sunshine hours was noted when compared between any two of the three seasons (Figure 4 C).

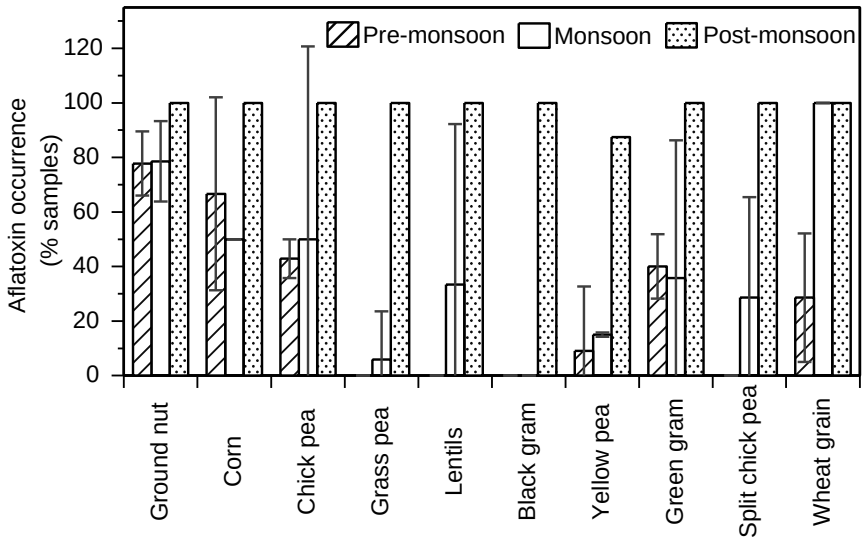

Figure 2. Aflatoxins occurrence in different kinds of food grain samples during pre-monsoon, monsoon and post-monsoon seasons. A variable number of samples for the selected kinds of food grains depending on their seasonal availability at a particular district were obtained (Table 1). Aflatoxins occurrence in individual food grains represented the mean percentage of samples detected positive for these toxins in different districts during a particular season and their standard deviations are shown as vertical bars.

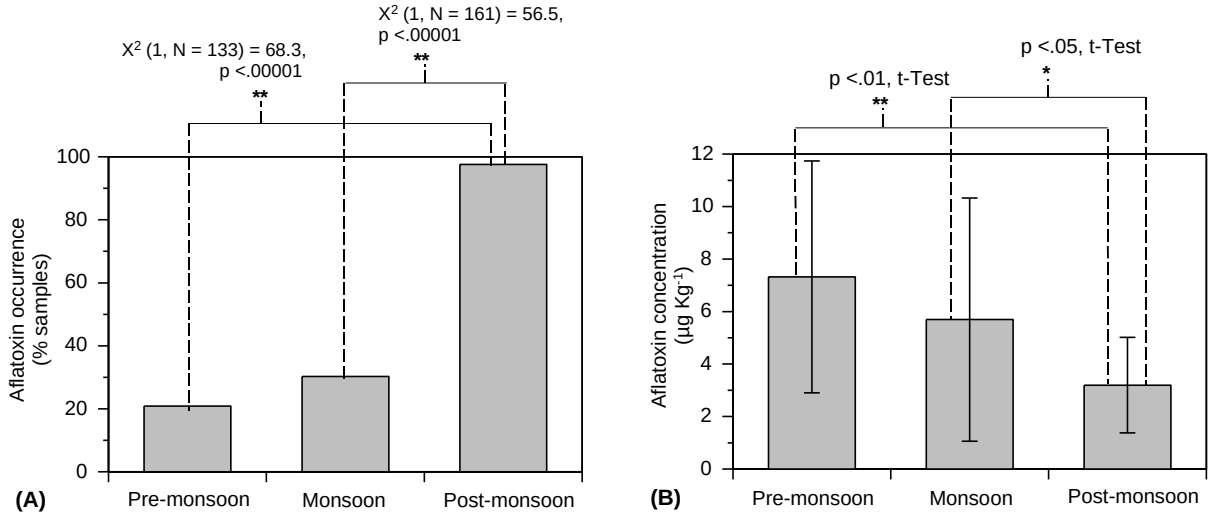

Figure 3. Seasonal variabilities in the overall occurrence and concentration level of aflatoxins in different food grain samples. (A) Variations in aflatoxins seasonal occurrences are shown in horizontal bars, representing the overall percentage of positive samples in different kinds of food grains examined during each of the pre-monsoon, monsoon and post-monsoon seasons. During these seasons a total of 91, 119 and 42 samples, respectively, were screened for the presence of aflatoxins. Chi-square test was performed to determine the significant difference in aflatoxins occurrence between two seasons. (B) Variations of aflatoxins concentrations in the positive samples (n=19, 31 and 41 during pre-monsoon, monsoon and post-monsoon seasons) are shown by their mean values in horizontal bars and their (±) standard deviations are shown as vertical error bars. Paired samples t-test (2-tailed, unequal variance) was performed to estimate any significant difference in the concentration level of aflatoxins between two seasons. Statistical results comparing the variablilities in aflatoxins occurrence and concentration levels are shown (on top) for the comparable seasons. The level of significance as p-values <0.05, and <0.01 were considered as significant and highly significant, respectivey, for each of the comparison.

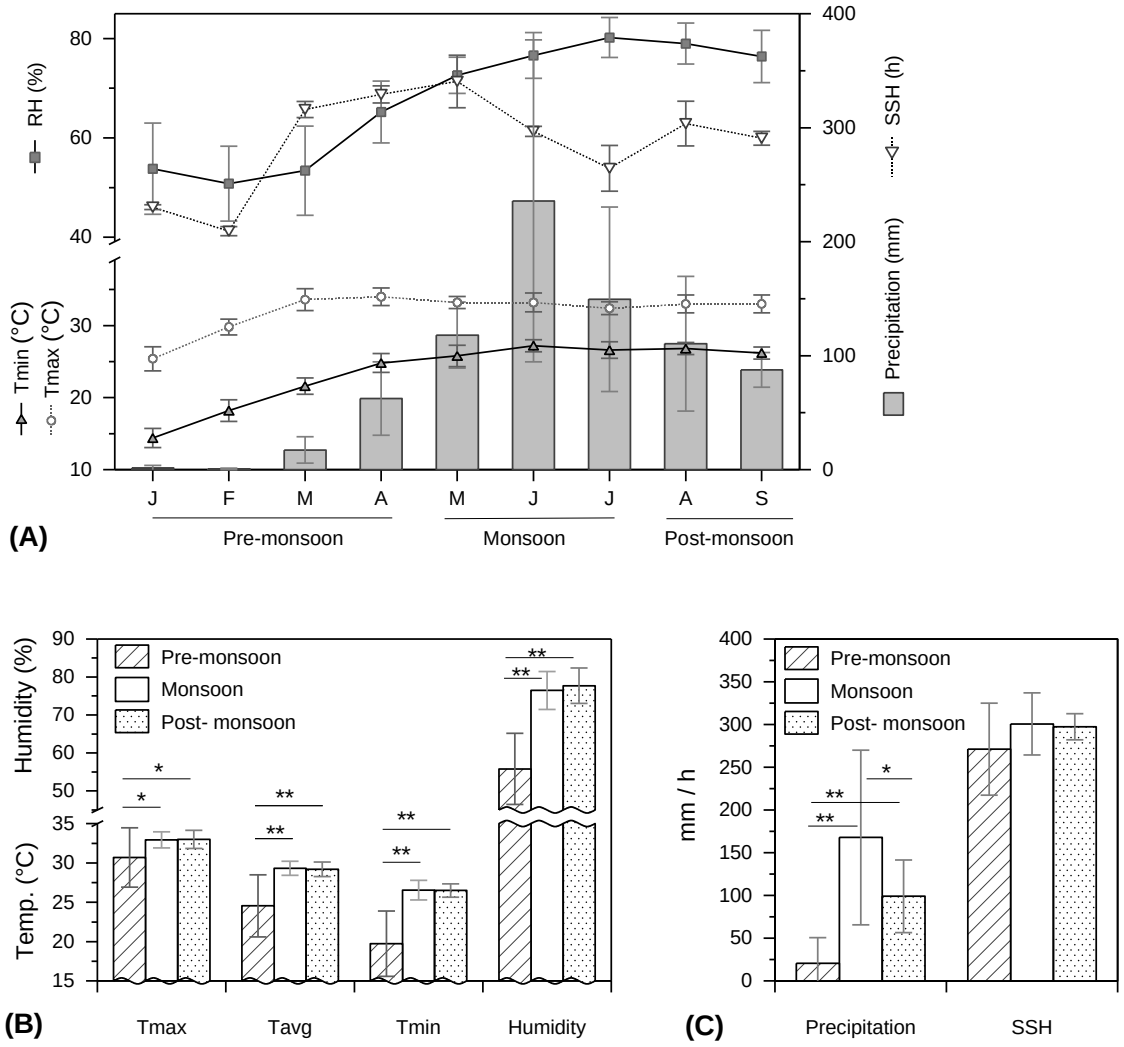

Figure 4. Monthly dynamics and seasonal variations of major climatic factors in the sampling regions during the study period. (A) Variations in the monthly means of atmospheric temperature minimum (Tmin), temperature maximum (Tmax), and relative humidity (RH) are shown as horizontal lines in the left Y-axis while those of precipitation and sun shine hours (SSH) are shown as horizontal column and line, respectively, in the right Y-axis. The daily means of these climatic factors in different study regions were transformed into overall monthly means and their (±) standard deviations are shown as vertical error bars. (B and C) Seasonal variations in mean values of atmospheric temperature indicators (including average temperature, Tavg), humidity, precipitation and SSH are shown as horizontal columns, with (±) standard deviations as vertical error bars. Paired samples t-test (2-tailed, unequal variance) was performed to estimate any significant difference between two seasons in the mean values of each climatic factor. The level of significance as p-values <0.05, and <0.01 were considered as significant (*) and highly significant (**), respectively, which are shown (on top) for individual comparison between two seasons.

A direct correlation between the monthly mean prevalence of aflatoxin in the tested food grains and any of the climatic factors was not statistically evident. However, time-series analysis comparing the mean values at sliding bimonthly intervals of aflatoxin occurrence in food grains and individual climatic factors revealed a significant positive correlation between aflatoxin contamination rate and two of the climatic factors, i.e., relative humidity and rainfall, when a 1-month time lag was considered. More explicitly, mean prevalence of aflatoxins in food grain samples during the sliding bimonthly time series of February-March, March-April, and so on, showed a significant correlation with mean values of relative humidity ($r = 0.92$, $p = 0.01$) and also with rainfall ($r= 0.85$, $p= 0.029$) when compared for a corresponding 1-month lead sliding bimonthly time series, i.e., January-February, February-March, and so on (Figure 5). In a similar time-series analysis, an increasing trend in the mean bi-monthly prevalence of aflatoxin corresponded to higher bimonthly mean values of Tmin, but

their comparative variation did not yield a highly significant statistical correlation (r = 0.779, p = 0.067). On the other hand, when comparing the mean bimonthly preavelence of aflatoxin in food grain samples with corresponding 1-month lead bimonthly mean values of Tmax, Tavg, and SSH, none showed any significant statistical relation.

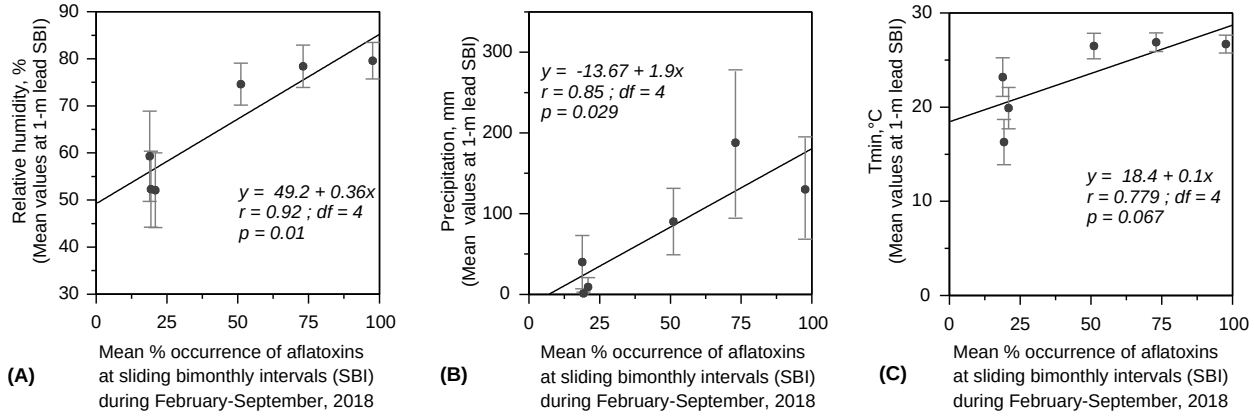

Figure 5. Correlation of aflatoxins occurrence in stored food grain samples with relative humidity (A), precipitation (B), and temperature minimum, Tmin (C). The mean values of aflatoxins occurrence (% samples) at sliding bimonthly intervals (SBI) during February-September, 2018 were correlated with the mean values of each climatic factor at SBI of a one-month lead time series during January-August, 2018. Standard deviations (±) of each climatic factor are shown as vertical error bars. Lines represent linear regressions; relevant information including regression equation, 'r' and 'p' values are shown inside each box.

4. Discussion

Risk of health hazards due to consumption of foods contaminated with aflatoxins has been reported from many parts of the world. In Bangladesh, dietary exposure of aflatoxins through contaminated agriculture products have been linked to a significant portion of total annual liver cancer cases (Turna and Wu, 2019). Metabolic derivatives of aflatoxins have been detected in majority or a significant percentage of the urine samples of children, pregnant women and adult populations in both rural and urban localities in this country (Ali *et al*., 2016; Mahfuz *et al*., 2019; Kyei *et al*., 2022). Despite being an enormous concern to public health, little efforts have been made exploring spatio-temporal variations and the role of climatic factors in the occurrence and concentration level of aflatoxins in a variety of common food grains available at local markets. Systematic investigations made in this study, comparing the variabilities in geoclimatic situations and aflatoxins contamination level in food grains at retail shops, clearly demonstrates that people all over in Bangladesh are highly vulnerable, particularly during post-monsoon season, to aflatoxins exposure and associated chronic health hazards.

The estimated overall contamination rate of total aflatoxin (38.1%) in different food grains in this study is in line with results reported previously by several other studies in the region (Koirala *et al*., 2005; Bhuiyan *et al.*, 2013). Observed variations in the contamination rate in different types of food grains is notable. Individual food grain wise comparison in this study has observed the highest occurrence of aflatoxin in groundnut (82.1%), which is in congruence to reported large-scale contamination of this kind of nuts in different parts of the world (Monyo *et al*., 2012; Iqbal *et al*., 2013; Ding *et al*., 2015). Ocassionally, a relatively less frequent occurrence of aflatoxins in raw and roasted groundnuts or peanuts, whereas an alarmingly higher occurrence of these mycotoxins in processed peanut butter, likely due to insufficient protection measures during industrial processing, handling, and storage, have been also reported (Ok *et al*., 2007; Chen *et al*., 2013; Norlia *et al*., 2019). Between the two kinds of cereal grain samples analyzed in this study, the observed higher frequency in aflatoxins contamination for corn samples (70.0%) in comparison to wheat grains (50.0%) is similar to previous findings in the Indian subcontinent (Koirala *et al*., 2005; Bhuiyan *et al*., 2013). Among the different kinds of common pulse grains, explorations made in this study has demonstrated the occurence of aflatoxins in a considerable portion of chick peas (55%) and green grams (40%), while observing its relatively less frequent contamination rate (between 18 and 31 %) in other kinds, e.g., split chick peas, yellow peas, black grams, lentils and grass peas. Systematic comparison on the variable occurrence and distribution of aflatoxins in diverse pulse

grains widely used as staple food in many countries including Bangladesh have largely remained unexplored, except for a few earlier studies on selected other type(s) of pulse grains in different regional settings (Matumba *et al*., 2017; Nazir *et al*., 2019; Acuña-Gutiérrez *et al*., 2022).

When comparing the amounts of total aflatoxins in the contaminated food grains of different types, a higher mean concentration for the positive samples of groundnut and wheat grain (7.95 and 8.2 µg kg^{-1}, respectively) in comparison to other food grains (between 2.7 and 4.6 µg kg^{-1}) is a distinct observation of this study. A mean concentration of ca. 9.5 ppb aflatoxin has been reported for layer feed grains in poultry farms in Bangladesh (Lubna *et al*., 2019). However, a number of previous studies in the Indian subcontinent observed a higher level of aflatoxin contamination (>30 µg kg^{-1}) in a portion of groundnuts, corns, and lentils (Dawlatana *et al*., 2002; Koirala *et al.*, 2005; Bhuiyan *et al*., 2013; Roy *et al*., 2013; Firdous *et al*., 2014). According to the food safety regulation of United States, all samples analysed in this study were within the permissible limit, i.e., 20 µg kg^{-1} food products, for human consumption (Wu *et al*., 2014). However, considering the EU regulation of a permissible concentration ≤5 µg kg^{-1} for total aflatoxin, 33 of 252, i.e., 13.1% of the food grain samples exceeded the limit. On the other hand, if considering the Indian food safety regulation, prescribing a maximum limit of 10 ppb aflatoxin for ready-to-eat nuts, a substantial fraction (35.7%) of ground nut, whereas a considerable fraction (20%) of wheat grain, and a negligible fraction of green gram (4.8%) and split chick pea (1.9%) samples of this study could pose health hazards.

A conspicuous seasonal variations in the occurrence and concentration level of aflatoxins observed for the studied samples point out a salient role of the climatic factors. It is widely-acknowledged that aflatoxin contamination in agricultural produce is primarily influenced by a number of environmental drivers, including the interacting climatic factors, crop type, soil condition, the timing and method of harvesting, insect infestation and storage conditions (Cotty and Jaime-Garcia, 2007; Bediako *et al*., 2019). In the monsoon influenced tropical climate of Bangladesh, most of the pulses and wheat grains are cultivated during the dry period (November-May) before and after rainy monsoon season, whereas corn and ground nuts are grown in all three seasons (pre-monsoon, monsoon and post-monsoon). These variabilities in cropping, harvesting, and storage times of different food grains may partially explain the observed finding of no direct correlation between the monthly dynamics of any of the climatic factors and the concurrent aflatoxin occurrence in stored food grains. Nonetheless, considering a one-month time lag while comparing the sliding bimonthly mean values of the variables, a significant correlation of both relative humidity and precipitation with the observed incidences of aflatoxin contamination in food grain samples was evident. This strongly indicates that aflatoxin occurrence in stored food grains is saliently influenced by pre-existing environmental conditions, particularly, rainfall incidences in the previous month(s) and persisitence of higher relative humidity over a long time-period. Infestation of *Aspergillus* spp. and other fungi in stored food grains is known to be primarily stimulated by high levels of humidity (>70%), temperature (>25ºC), aeration, and longer storage time (Waliyar *et al*., 2015a). Together with higher relative humidity or water activity, a stimulating influence of increased temperature on *Aspergillus* populations, eventually enhancing aflatoxin occurrence, during crop harvesting and/or storage over seasons has been observed in some countries (Pratiwi *et al*., 2015; Lahouar *et al*., 2016). However, among the temperature indicators (Tmax, Tmean, Tmin), Tmin showed the strongest positive correlation (r=0.779), but statistically less significant (p=0.067), with aflatoxin occurrence in stored food grain samples analyzed in this study. Controlled field experiments in the sub-tropical regions of the USA also revealed that among the potential climatic drivers, Tmin had the strongest influence on aflatoxin occurrence in corn grains before harvest (Damianidis *et al*., 2018). A correlation between sunshine duration and air temperature has been observed in many parts of the sub-tropical and temperate regions, e.g., in European countries (Van den Besselaar *et al*., 2015). However, in the tropical climatic conditions of Bangladesh, no discernible significant correlation between SSH and aflatoxin occurrence for the observed food grain samples could be largely attributable to the masking influence of the wet monsoon season over temperature or SSH.

In combination with the seasonal dynamics in climatic factors, the spatial variations in aflatoxins occurrence noted in this study further illustrate an influential role of regional geo-climatic settings. The monsoon wind, which bring enormous amount of moisture from the Bay of Bengal, usually crosses over Bangladesh from the South-West coasts towards the North-East region on the way to the Himalaya. Eventually, in comparison to other regions, localities in the North-East being closer to the Himalayan mountains experience distinctively higher precipitation events and higher relative humidity, which may be related to the observed higher occurrence of aflatoxins in stored food grains in this region. In comparison, a number studies in other regional settings have reported a contradictory situations, i.e., low rainfall or drought conditions but increased tempertature as the major contributors for aflatoxin occurrence, which could be partially attributable to the diverisity in geoclimatic settings, spatiotemporal variabilities in cropping patterns, and timing of rainfall with

respect to differential maturation phases of the growing crop (Cotty and Jaime-Garcia, 2007; Giorni *et al*., 2016; Smith *et al*., 2016; Temba *et al*., 2021). In this study, however, the observed ubiquitous occurrence of aflatoxin contamination in all kinds of tested food grains during post-monsoon (August), irrespective of spatial geoclimaitc variabilities, clearly reflects a salient influence of higher (>75%) relative humidity after heavy monsoon rainfall along with prevailing high temperature (Tmin >25ºC). This inference is also in congruence to previous studies observing similar climatic conditions as the key stimulating factors for infesting fungi in stored ground nuts (Waliyar *et al*., 2015b). Unfortunately, at most of the retail markets in Bangladesh, including those selected in this study, food grains are usually stored without maintaining proper conditions to withstand atmospheric alterations, e.g., keeping at low temperature (<10ºC, Galván *et al*., 2021) and in air-protected condition to resist the influence of atmospheric moisture or humidity (Bauchet *et al*., 2021).

An interesting observation of this study is the significantly lower mean value of aflatoxin concentration in the contaminated samples during post-monsoon (ca. 3.2 ppb) than that of pre-monsoon (ca. 7.3 ppb) and monsoon (ca. 5.7 ppb). This is inconsistent to the observed seasonality in aflatoxin occurrence, i.e., significantly higher incidence rate during post-monsoon in food grain samples screened in this study. In certain kinds of food grain(s), the mean concentration level of aflatoxin may fluctuatate following a trend simiar to its incidence rate in different seasons at particular geoclimatic settings; however, large variations in mean aflatoxin concentration, independently of its temporal occurrence rate in stored food grains and poultry feed have been also reported (Khan *et al*., 2005; Taheri *et al*., 2012; Bhuiyan *et al*., 2013). A study in Kenya investigating corn samples stored ca. 2-months after harvest has observed that pre-occurrence of the long rain season (like the monsoon) significantly coincide with lower aflatoxin levels than those from the short rain season (Obonyo and Salano, 2018). At storage conditions of food grains, the production rate of aflatoxins by *Aspergillus* spp. are thought to be influenced by a number of intrinsic and extrinsic factors, including nutritional factors in available substrate, temperature (25ºC-35ºC as optimum), relative humidity or water activity, aeration and duration of storage time (Schmidt-Heydt *et al*., 2010; Mannaa and Kim, 2017; Mahato *et al*., 2019). Moreover, variabilities in aflatoxin production in different strains, intra- and inter-species competitive interaction of *Aspergillus* spp., composition of coexisting fungal communities, and occurrence of antagonistic microbes producing volatile organic compounds may retard or cause differential aflatoxin concentration in contaminated food grains (Huang *et al*. 2011; Okoth *et al*. 2018; Jaibangyang *et al*., 2020). Results obtained in this study and others underscores the necessity of a more careful evaluation of the wide-scale seasonal variabilities in environmental factors, considering diversity in cropping patterns, harvesting time, storage duration and conditions, and concomitant microbial flora when comparing aflatoxin occurrence in stored agricultural products at different geo-climatic settings.

The widespread occurrence of afltaoxins in stored food grains, as evident from this study and others, strongly point out an imperative need of appropriate interventions at different levels of food value chain. Adopting good agriculture practice including planting and harvesting crops in a timely manner, using the pest-resistant varieties, attempting to lower mold growth using biocontrol agents in field soils or harvested crops, disposing of visibly moldy or damaged kernels and improved sun drying before storage are among the potential intervention strategies for the producers or farmers (Torres *et al*., 2014; Bediako *et al*., 2019). Controlling moisture (≤8% moisture level), temperature, infestation of insects rodents and fungus, and avoiding soil contact or cross-contamination during storage can prevent aflatoxin development (Waliyar *et al*., 2015a; Mannaa and Kim, 2017). More importantly, intervention measures to minimize aflatoxin contamination before consumption of the food grains at household-level on a regular basis are highly recommended. Simple methods such as sorting, washing, crushing, and grain dehulling, may reduce aflatoxin levels to some extent (Stasiewicz *et al*., 2017). In case of unshelled peanuts, traditional boiling with salt (5% NaCl) or cooking in the presence of calcium chloride or citric acid may significantly reduce aflatoxin contamination (Yazdanpanah *et al*., 2005; Rastegar *et al*., 2017). A combination of methods proved to be more fruitful, e.g., reduction of aflatoxins in corn grits by frying after boiling was more effective than boiling alone (Milani and Maleki, 2014). However, food producers and consumers in general are totally unaware of aflatoxin mediated health hazards and potential intervention strategies to reduce its contamination level in food products. Prioritization of aflatoxins as an important threat to public health and the associated risk issues, including food-mediated and environmental, are yet to be properly communicated to government authorities and other important stakeholders in Bangladesh.

5. Conclusions

The present study demonstrates that a variety of food grains sold at retail markets constitute a significant source of aflatoxin exposure and related health hazards in Bangladesh. It is evident that in the predominantly hot and humid climatic conditions in Bangladesh, contamination of aflatoxin in food grains not only occurs sporadically

at the agricultural field during or before harvest but may significantly increase while being stored in conventional conditions at retail markets, particularly during post-monsoon. Proper treatment measures, including control of humidity while storage and adequate processing before consumption of food grains are strongly recommended. Notably, special attentions should be devoted while dealing with high risk food grains like ground nuts, corn and wheat grains, particularly at situations with persistent humid climate. The differential risks of aflatoxin contamination of food grains observed in this study would aid in developing multi-level interventions and awareness building programs, integrating producers, marketers and consumers, to tackle the associated health hazards at diverse geoclimatic settings. A more holistic monitoring the spatio-temporal variations of aflatoxins in agricultural harvests and stored food products for a longer term, together with clinical surveillance programs involving the vulnerable community members, would provide further insights to our understanding of the multifaceted threats and health hazards associated with these silent killing agents.

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Data availability
Detail information on the datasets and materials used in this study are available from the corresponding author on reasonable request. Confidentiality of data is maintained anonymously.

Conflict of interest
None to declare.

Authors' contribution
S. M. Lutful Kabir, Sucharit Basu Neogi and Ashis Ratan Sen designed the study. Md. Nasir Uddin, Mohammad Arif, Priyanka Rani Paul, Ashis Ratan Sen, Md. Jahidul Islam Saddam and A.K.M. Ziaul Haque collected the samples from the field and performed laboratory analysis. Sucharit Basu Neogi, Seksun Samosornsuk and Worada Samosornsuk analyzed the data and prepared the draft manuscript. S. M. Lutful Kabir and Sucharit Basu Neogi supervised the study programs, provided technical support, and finalized the manuscript. All authors have read and approved the final manuscript.

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