Science Discovery
2025, Vol. 13, No. 6, pp. 113–120
https://doi.org/10.11648/j.sd.20251306.12

Research Article

Prevalence of Aflatoxins in Wheat and Maize Grain Plant-Pest-Pathways at Three Zimbabwean Ports of Entries

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Abstract

The presence of aflatoxin producing fungi in food imports necessitates rigorous screening. A total of 42 maize and wheat pest pathways found contaminated with aflatoxins-causing- fungi; (*Aspergillus flavus and A. parasiticus)* during 2023-2024 phytosanitary inspections at three Zimbabwe ports of entries were subjected to aflatoxin screening at the Fertilizers, Farm Feeds and Remedies Institute (FFRI) using semi-quantitative Best Food Method (BFD) by means of Thin Layer Chromatograph (TLC) to detect aflatoxins contamination. Standard reference mixes containing aflatoxins concentration of 0.5 µg/kg each for B_1 and B_2, and 0.25 µg/kg each for G_1 and G_2 were checks. Controls with known concentration of aflatoxins B_1, B_2, G_1 and G_2 were visibly seen under Ultra Violet (UV) light read at 365 nm and differences of spots intensity for B_1 and B_2 aflatoxins concentration of 12 µg/kg, 24 µg/kg and 36 µg/kg and G_1 and G_2 aflatoxins concentration 6 µg/kg, 12 µg/kg and 18 µg/kg were noted. However, despite the presence of aflatoxin causing fungi in the 42 samples tested, aflatoxins were not detected by the TLC which has a detection limit of <1ppb. Spearman's rho correlation matrix showed perfectly ($\rho = 1.000$, $p < 0.01$) or near-perfectly correlation ($\rho \geq 0.999$, $p < 0.001$) on the four aflatoxin groups (B_1, B_2, G_1, G_2). Perfect correlations were revealed between aflatoxins B_1 and B_2 and between aflatoxins G_1 and G_2 whilst extremely strong correlations were revealed between aflatoxins B_1 and G_1; B_1 and G_2; B_2 and G_1 and B_2 and G_2. The test was highly significant ($p<0.001$) suggesting strong indication of no association between the four aflatoxin categories. Chi-Square goodness of fit test revealed statistically significant deviations from the expected frequencies (χ^2 (4) = 154.083, $p < 0.001$) suggesting that observed distributions of the aflatoxin were not caused by random variation. Due to the risks of mycotoxin co-occurrences in cereals, the research recommends expanding the testing beyond aflatoxins to include fumonisins caused by *Fusarium* species and other mycotoxins associated with *Penicillum* sp and *Macrophomina phaseolin* as these pests were also isolated from the cross border cereal grain-pest-pathways entering Zimbabwe. This research was limited by non-testing of the isolated fungi on their potential to cause aflatoxins contamination.

Keywords

Aflatoxins, Food Safety, Human Aided, Pest- Pathways, Phytosanitary Inspections

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Received: 12 September 2025; **Accepted:** 30 September 2025; **Published:** 8 December 2025

1. Introduction

The amalgamation of one health concepts provided a road map for strengthened sanitary and phytosanitary standards harmonisation and port of entry control systems. This research was an extension of a pest – pathways studies in cross border traffic into Zimbabwe. The research isolated number of pest threats that are associated with both plant and animal health safety apart from also being environmental biosecurity nuisance. The threat of transmitting plant pests across borders is not only a concern to global agriculture, and biodiversity, but it also affects food and feed safety and security across countries [1-7].

Different organisms associated with food and feed challenges are found in cross border pest-pathways across the globe. These hitch-hikers contaminating cross border phytosanitary traffic may include arthropods, weeds or other plants, nematodes, bacteria, fungi, and other eukaryotes and prokaryotes. Some of these organisms are associated with food safety threats. The systems of screening pest-pathways that are associated with cross border traffic provide opportunities for strengthened port of entry biosecurity controls by the country in the one health concept.

Fungal pathological organism carried across borders in cereals-pest-pathways are noted as serious threats to food and feed safety due to the nature of poisons they secrete in food products. Fungi are associated with mycotoxins such as aflatoxins caused by some *Aspergillus* species and fumonisms caused by some Fusarium species. The Zimbabwe Food and Food Standards Act [Chapter 15: 04] [8] in harmony with the SADC and Codex Alimentarius guides the country's food safety standards [8, 9]. The maximum threshold limits for the total aflatoxins count for B_1, B_2, G_1 and G_2 of 10 parts per billion (ppb) or (10 microgram per kilogram) for SADC and 20 ppb for CODEX Alimentarius and a maximum of 5 ppb for total aflatoxins B_1 must be observed to guarantee public safety. Aflatoxins produced by the *Aspergillus* species are also highly, carcinogenic and causes sombre human and animal health catastrophes [10].

This research screened maize and wheat grains samples which were found to be contaminated with aflatoxins causing fungi, *Aspergillus flavus* and *A. parasiticus*, against aflatoxins B_1, B_2, G_1, B_5 and G_2. The samples were collected from Beitbridge, Forbes and Plumtree ports of entry by the plant health officials. The research also quantified the total aflatoxins that were found in maize and wheat grain cereals to ascertain the level of contamination and the associated threats to human and animal feed safety in accordance to the Zimbabwe Food and Food Standards Act [Chapter 15: 04] [8]. This study was also done to provide information that could lead to the improved harmonisation of port of entries biosecurity inspections in line with the one health concepts on sanitary and phytosanitary (SPS) systems on pest-pathways in cross border traffic.

2. Materials and Methods

2.1. Sources of the Biological Materials and Sample Collection

The maize and wheat samples used for the screening of aflatoxins B_1, B_2, G_1 and G_2 were obtained from maize and wheat pest-pathways that were found in association with aflatoxins causing fungi, *Aspergillus flavus* and *A. parasiticus*. These samples were randomly collected from human aided pest pathways and stratified by being cereals grain pathways. The pathways were collected from the Beitbridge, Plumtree and Forbes Border Ports during the period between 1st January 2023 and 31st December, 2024. The maize samples underwent pest screening at the Mazowe NPPO Laboratory. A stratified sampling technique was then used to select cereal pest pathways that were subjected to aflatoxins screening. The pathways (42 samples (27 = maize; 15 = wheat)) that were found contaminated with aflatoxin causing fungi (strata) were subjected to aflatoxin screening. The sample size submitted for aflatoxins screening was 200 grams each. Table 1 show the list and characteristics of the sampling sites used in this part of this research.

The samples used in these studies were handled aseptically to foil contamination, and laboratory practices were followed to maintain safety. The research complied with local and international plant biosecurity guidelines to ensure responsible management of potential harmful pathogens. [11] highlighted the worth of maintaining ethical standards and safety protocols during sample handling to prevent contamination and [12] advocated for obeying international guidelines in research practices.

Table 1. *List and characteristics of the sampling sites for the trapping of trans-boundary plant pests associated with cross border traffic into Zimbabwe for the period from 2023 to 2024.*

Name of entry point	Locations (GPS: Latitude, longitude)#	Category and Characteristic of entry port	Customs declarations characteristics	Operating times of the day	Bordering countries
Beitbridge Border Port	22 °13'05"S 29 °59'10"E	Land border	Commercial and non-commercial cargo	24 hours	South Africa

Name of entry point	Locations (GPS: Latitude, longitude)#	Category and Characteristic of entry port	Customs declarations characteristics	Operating times of the day	Bordering countries
Forbes Border	19 °00'18"S 32 °42'42"E	Land border	Commercial and non-commercial cargo	24 hours**	Mozambique**
Plumtree Border Port	20 °32'28"S 27 °44'15"E	Land border	Commercial and non-commercial cargo	0600 – 2200 hours	Botswana

**The entry point became a 24 hours border in 2023 from 0600 hours to 2200 hours border; [Source [13]]

Temporary storage of maize and wheat samples before screening.

Temporary storage of maize and wheat samples before screening was at the Mazowe Plant Quarantine Central Laboratory at Laboratory room conditions of 20 to 30 degrees Celsius and drier conditions until they were analysed. The storage time for the samples varied depending on when they were received. The least storage time was three weeks whilst the longest storage time was 17 months for both the wheat and the maize sampled found contaminated with aflatoxin. At this temperature condition and with humidity above 7 per cent, aflatoxins development occurs [14, 15].

2.2. Screening of Maize and Wheat Samples for Possible Aflatoxins Contaminations

The semi-quantitative Best Food Method (BFD) that uses the Thin Layer Chromatograph (TLC) analytical procedure was used to analyse the imported grain samples to aflatoxins B1, B2, G1 and G2. The method used was similar to the method used by [16-21]. Standard samples containing known concentration of aflatoxins of 0.5 µg/kg for B_1 and B_2, and 0.25 µg/kg for G_1 and G_2 were used as benchmarks. Comparison was also done using Certified Reference Material (CRM) Aflatoxins Proficiency Testing for Eastern and Central Africa testing programs obtained from the University of Texas of the United States of America. The method used was similar to the ones mention by [22-26].

2.3. Quantification of Aflatoxins in Test Samples and Controls

The quantification of aflatoxins contamination in samples followed the TLC which is similar to densitometry analysis as described by [27] with minor adjustment made. These methods state *Desaga CD6 TLC Densitometer (Bucharest, Romania) which is equipped with data acquisition and processing software is used for quantitative evaluation of the plates.* With this estimation methods for aflatoxins, the separated mycotoxins on the TLC plate are detected and quantified in fluorescence mode ($\lambda_{excitation}$ = 365 nm, slit of 0.06 x 4 mm for aflatoxins), with a scanning resolution of 0.025 mm and 16 readouts/point [27]. The control standards of aflatoxins (B_1, B_2, G_1, and G_2) applied to the TLC plate was 4, 8 and 12 uL. Aflatoxins B_1, B_2, G_1, and G_2 quantification followed formulae as described by the FFRI Annual, (2023). This method is similar to the densitometry analysis described by [27]. The formula used is given by the equations box 1 below:

Box 1. Formulae to quantify aflatoxins concentration in standard spotted on TLC as checks.

$Y = C_S \times (VS_s/Vs_s) \times (D_F/EW_t) \times 1000$ parts per billion (ppb)

Where:

Y = concentration of aflatoxins B1, B2, G1 or G2 in the sample screening by TLC

C_S = Standard concentration of aflatoxins (B1, B2, G1 and G2)

VS_s = Volume of the aflatoxins standard spotted on the TLC plate

Vs_s = Volume of sample spotted on the TLC plate

D_F = Dilution factor of sample

EW_t = Effective weight of the sample used.

Whereas the quantification of the aflatoxins concentration in the sample followed the densitometry analysis (box 2) similar to the formulae described by [27].

Box 2. Formulae to quantify aflatoxins in test samples as described by FRRI (2023).

$C_p = C_s (V_f/V_s)(1/m_s)$

Where:

C_p = Concentration of mycotoxin (ug/kg) in the cereal sample

Cs = Quantity determined by densitometry (ng mycotoxin/spot)

Vf = Final volume of the extract after concentration (1000ul/L) or (ppb)

Vs = Volume of extract spotted on TLC plate (10uL)

Ms = Sample weight (8.33g)

Finally, standard linear curves representing the peak areas in the function of the applied quantities of separated mycotoxins standards on the TLC were drawn as part of the results.

2.4. Data Analysis

The variables collected were the contamination levels of the aflatoxins screened used the TLC. The picture of the TLC plate read under UV light (365 nm wavelength) were obtained in order to visualise the aflatoxin contamination levels. Microsoft Excel was used to draw statistical inference

regarding the contamination levels by the aflatoxins. A Chi-square test of association computed to check the relationship between the aflatoxin groups. Spearman's rho correlation matrix was computed to check the correlation of all the four aflatoxin groups (B_1, B_2, G_1, G_2).

3. Results

As shown in Figure 1, the fluorescence of aflatoxins screened using the TLC as read under 365 nm Ultra Violet light. Controls with known concentration of aflatoxins B_1, B_2, G_1 and G_2 were visibly seen under the UV light. The differences of spots intensity for B_1 and B_2 aflatoxins concentration of 12 µg/kg, 24 µg/kg and 36 µg/kg and G_1 and G_2 aflatoxins concentration 6 µg/kg, 12 µg/kg and 18 µg/kg were also visible. All the 42 test samples of maize (27) and wheat grain (15) pest-pathway from ports of entry tested negative to aflatoxins as shown on the TLC plate read under 365 nm UV light (Figure 1) and as shown as well on Table 2 and 3. Despite absence of detectable aflatoxin concentration, further analysis of the results with Spearman's rho correlation matrix confirmed that all four aflatoxin groups (B_1, B_2, G_1, G_2) were nearly perfectly correlated ($\rho \geq 0.999$, $p < 0.001$), (Table 4). Perfect correlations were revealed between aflatoxins B_1 and B_2 ($\rho = 1.000$, $p < 0.01$) and between aflatoxins G_1 and G_2 ($\rho = 1.000$, $p < 0.01$). Extremely strong correlations were revealed between aflatoxins B_1 and G_1 ($\rho = 0.999$, $p < 0.01$); B_1 and G_2 ($\rho = 0.999$, $p < 0.01$); B_2 and G_1 ($\rho = 0.999$, $p < 0.01$) and B_2 and G_2 ($\rho = 0.999$, $p < 0.01$).

A Chi-square test of association performed on the four aflatoxins (B_1, B_2, G_1, G_2) produced identical Chi-Square values for all the four aflatoxins groups; (X= 154.083), (df = 4), and p-values = 0.000 (Asymptomatic Significance)). This further reinforced Spearman's correlation findings that the aflatoxins were perfectly or near-perfectly related. The test was highly significant ($p<0.001$) suggesting strong indication that there were no association between the four aflatoxin categories. The Chi-Square goodness of test revealed statistically significant deviations from the expected frequencies ($\chi^2(4) = 154.083$, $p < .001$) for aflatoxins B_1, B_2, G_1, and G_2 buttressed by Monte Carlo simulation with 99% confidence intervals ($p = 0.000$) for each variable. This result suggested that the observed distributions of aflatoxin B1, B_2, G_1, and G_2 may not have been caused by random variation. Notably, all the expected cell frequencies exceeded the minimum threshold (no cells were less than 5 and the minimum expected frequency was 9.6), hence satisfying the assumptions of the Chi-Square test.

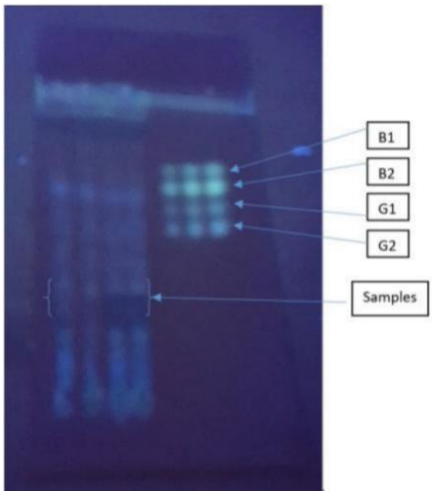

Figure 1. *The image of TLC plate read under the 365 nm UV light showing aflatoxins bands of check versus the test samples.*

Table 2. *Quantitative results of the aflatoxins contamination in cross border maize and wheat cereals grain pest pathways screened by Thin Layer Chromatograh (TLC) at the Fertilisers, Farm Feeds and Remedies Institute Food Testing Laboratory during the period 2023 to 2024 in Zimbabwe.*

Entry	Aflatoxin_B_1 (Ppb)	Aflatoxin_B_2 (Ppb)	Aflatoxin_G_1 (Ppb)	Aflatoxins G_2 (Ppb)
27 x Maize (10 uL)	<1	<1	<1	<1
15 x Wheat (10 uL)	<1	<1	<1	<1
Control @ 4 uL	5.66	5.66	2.34	2.34
Control 2 @ 8 uL	3.46	3.46	2.45	2.45
Control 3 @ 12 uL	4.90	4.90	3.46	3.46
Control 4 Certified Reference Material (CRM) @ 10 uL	6.00	6.00	6.00	6.00
Mean	3.34	3.34	2.36	2.36
Standard deviation	2.52	2.52	2.12	2.12
Standard error of mean	0.389	0.389	0.327	0.327

Table 3. *Qualitative results of the aflatoxins contamination in cross border maize and wheat cereals grain pest pathways screened by Thin Layer Chromatograph at the Fertilisers, Farm Feeds and Remedies Institute Food Testing Laboratory during the period 2023 to 2024 in Zimbabwe.*

Sample	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
27 x Maize (10 µL)	Not Detected	Not Detected	Not Detected	Not Detected
15 x Wheat (10 µL)	Not Detected	Not Detected	Not Detected	Not Detected
Control 1 (4 µL)	Detected	Detected	Detected	Detected
Control 2 (8 µL)	Detected	Detected	Detected	Detected
Control 3 (12 µL)	Detected	Detected	Detected	Detected
Control 4 (CRM 10 µL)	Detected	Detected	Detected	Detected

Table 4. *Spearman's rho correlation matrix of aflatoxin groups (B_1, B_2, G_1, G_2) of aflatoxins contamination in cross border maize and wheat cereals grain pest pathways screened by Thin Layer Chromatograph at the Fertilisers, Farm Feeds and Remedies Institute Food Testing Laboratory during the period 2023 to 2024 in Zimbabwe based on estimates.*

Aflatoxin group / test			Aflatoxin_B_1 (Ppb)	Aflatoxin_B_2 (Ppb)	Aflatoxin_G_1 (Ppb)	Aflatoxins G_2 (Ppb)
Spearman's rho	Aflatoxin_B_1 (Ppb)	Correlation Coefficient	1.000	1.000**	0.999**	0.999**
		Sig. (2-tailed)	0.0	0.0	0.000	0.000
		N	48	48	48	48
	Aflatoxin_B_2 (Ppb)	Correlation Coefficient	1.000**	1.000	0.999**	0.999**
		Sig. (2-tailed)	0.0	0.0	0.000	0.000
		N	48	48	48	48
	Aflatoxin_G_1 (Ppb)	Correlation Coefficient	.999**	.999**	1.000	1.000**
		Sig. (2-tailed)	.000	.000	.	.
		N	48	48	48	48
	Aflatoxin G_2 (Ppb)	Correlation Coefficient	.999**	.999**	1.000**	1.000
		Sig. (2-tailed)	.000	.000	.	.
		N	48	48	48	48

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

4. Discussion

The non-detection of aflatoxins in the samples of cereal pest pathways is good indicator that the cereal grain pest-pathways were free from aflatoxins. According to [10], aflatoxins levels that are deemed safe range from 4 to 30 µg/kg for human consumption with the European Union (EU) using standard much lower than the Codex Alimentarius guidelines [24]. The EU gazetted 2 µg/kg aflatoxins concentration as the maximum limit for aflatoxin B_1 and 4µg/kg as the total aflatoxins limit. Zimbabwe in harmony with COMESA accepts maximum aflatoxins limit 10µg/kg. Some countries mostly use the 20 µg/kg of total aflatoxins as the standard set by CODEX Alimentarius [10]. The contamination levels of aflatoxins found in the wheat and maize grain pathways during this study that were found to be associated with the aflatoxins causing fungi, *Aspergillus flavus and A. parasiticus*, were below the poison threshold limit and are within the set limits for all the known maximum aflatoxins contaminations across the globe [28]. In 2023, [29] recorded 18% incidence of aflatoxins in wheat and 71% in the maize samples corn samples grown in Albania which is contrary to the findings of this research. Topi et al, (2023) posted that Aflatoxins B_1 concentration ranged from 0.2 to 0.4 µg/kg

whilst contaminated maize samples reached up to 3550 µg/kg.

Aflatoxins are most prevalent in storage conditions that are not supported by good agricultural, good manufacturing or good storage practices [10]. The result of this work are similar to the results which were obtained by Mupunga (2013) in his Master of Science Thesis in Life sciences where aflatoxins were not detected in samples imported from Botswana. However, the current study were found opposite to those obtained by [10, 30] where Zimbabwean maize and wheat products as well as those similar products found in Nigeria; had high aflatoxins contamination levels above the 20 µg/kg as per CODEX standard. Contrary, livestock feed in Bulawayo had detected total aflatoxins contamination levels reaching 250.9 µg/kg for B_1, B_2, G_1 and G_2 [31].

The proliferation of aflatoxins is exacerbated by tillage systems, storage conditions of the products and other factors as described by [10] where he mentioned practices as the major factors in managing development of aflatoxins in food and feeds [32]. In this particular study, the wheat and grain samples were stored at laboratory room conditions with drier conditions and temperature ranges between 20 to 30 degrees Celsius in Mazowe in Zimbabwe. The storage temperature together with humid conditions above 7 per cent was enough to stimulate production of the toxins during temporary storage at the Mazowe Plant Quarantine Central Laboratory [32, 14, 15]. However, no toxins were detected despite the presence of the aflatoxin causing fungi that were isolated from the specified maize and wheat grain pathways.

Detection of aflatoxins is not dependent with the testing Analytical Method employed, High-Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), LC-MS/MS, Enzyme-Linked Immunosorbent Assay (ELISA) and LC-QTOF-MS all detected aflatoxins levels with similar accuracy [33]. Though, Miklós et al., (2020) [34], cited the use of HPLC as analytical methods amongst the most sensitive for aflatoxins detection, [35] equated several thin layer chromatographic (TLC) and high performance liquid chromatographic (HPLC) methods as similar and more suitable for quantification of aflatoxins. [36] re-iterated that sample preparation techniques are the most important factors in aflatoxins analysis as the test relies more on liquid–liquid extraction or solid-phase extraction [36]. Thus, the little differences regarding the aflatoxins detection methods suggest the important results found regarding absence of detectable levels of aflatoxins contaminations in the maize and wheat pathways associated with human aided cross border traffic into Zimbabwe.

The management of biosecurity pathways across ports of entry thus requires cooperation between port entry staff for plant health, animal health and food safety. In countries like South Korea and United States of America, biosecurity and port security takes care of animal, plant, health and food safety inspections with actions resembling one health initiative [37]. The results of this suggest the use of one health initiative and observation of one set of biosecurity at port of entry as properly defined system for plant, animal and environmental biosecurity protection.

5. Conclusion

Despite the presents of aflatoxins causing fungi, *Aspergillus flavus and A. parasiticus*, in the wheat and maize grain pest pathways entering Zimbabwe, there was no aflatoxins detected suggesting that there was no significant risk of contamination of food and feed by transboundary plant pests associated with pest-pathways to Zimbabwe. There is however, a risk of core-occurrence of different mycotoxins associated with cereal products and hence there is need to expand the scope of mycotoxins screening to include fumonisms caused by *Fusarium* species and other toxins associated with *Penicillum* and *Macrophomina phaseolina* which were also isolated from the cross-border cereals pest-pathways entering Zimbabwe.

6. Recommendations

The isolation of aflatoxin producing fungi indicated a potential for food and feed poisoning accidents in cross-border maize and wheat cereal pathways. However, the current research did not test the isolated fungi for their aflatoxin-producing potential by inoculating them on other grains and testing for aflatoxins contamination, hence, such analysis is required for future research.

Abbreviations

BFD	Best Food Method
COMESA	Common Market for East and Southern Africa
CRM	Certified Reference Material
ELISA	Enzyme-Linked Immunosorbent Assay
FFRI	Fertilisers, Farm Feeds and Remedies Institute
HPLC	High-Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LC-QTOF-MS	Liquid Chromatography-Quadrupole Time-of-Flight Tandem Mass Spectrometry
MUAST	Marondera University of Agricultural Sciences and Technology
N	Populations Size
NAPPO	North American Plant Protection Organization
ppb	Parts per Billion
SADC	Southern African Development Community
TLC	Thin Layer Chromatograph/ Thin Layer Chromatography

UV	Ultra Violet
µg/kg	Microgram per Kilogram

Acknowledgments

The Department of Research and Specialist Services in the Ministry of Lands, Agriculture, Fisheries, Water and Rural Development for the kind support in aflatoxins analysis, pathways sampling and storage. MUAST is acknowledging for technical guidance and project supervision.

Conflicts of Interest

The authors declare no conflicts of interest.

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