

RESEARCH ARTICLE

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Aflatoxin Contamination, Mycoflora Diversity, and the Toxigenic Potential of *Aspergillus* spp. Associated with Maize Seeds and their Products

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Abstract

Aflatoxins are naturally occurring mycotoxins that are virtually impossible to eradicate from food and animal feed, and therefore contribute to the widespread contamination of maize products. These toxins are highly dangerous fungal substances that cause acute and chronic toxicity in humans and livestock. In the present study, 32 samples of different maize products were collected from different locations in Tamil Nadu, India, during 2021-2022 to assess aflatoxin contamination. Only two samples were free from aflatoxin contamination, whereas the other 30 maize samples were contaminated with different types of aflatoxins. In addition, maize seeds were collected from 15 locations covering 10 districts of Tamil Nadu, India, to study the mycoflora diversity. The results revealed the six main fungal genera, namely *Aspergillus*, *Fusarium*, *Penicillium*, *Rhizopus*, *Alternaria*, and *Macrophomina*, associated with maize seeds. Out of the 1152 mycoflora isolated from maize seeds, the genus *Aspergillus* contributed the most (82%), followed by *Fusarium* spp. (9%) and others (9%). Within the genus *Aspergillus*, *A. niger*, *A. flavus*, *A. fumigatus*, *A. tamarii*, and *A. oryzae* were recorded. Species composition studies revealed that *A. niger*, *A. flavus* and *A. tamarii* were present at all locations. Alpha diversity analysis of maize seed mycoflora and the genus *Aspergillus* using different indices revealed that abundance, dominance, and species richness were high at very few locations. Beta diversity analysis also revealed that there was no significant difference in the mycoflora between locations *i.e.* mycoflora distribution was even across all locations. Furthermore, the toxigenic potential of 24 *Aspergillus* isolates from different locations was assessed. The results revealed that the isolates Asp 16, Asp 17, Asp 20, and Asp 23 produced all four aflatoxins types, namely aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). Among the isolates, a maximum AFB1 of 10250.78 ppb/g of agar plug was recorded for the Asp 23 isolate, followed by Asp 20 (4246.584 ppb). Most isolates produced one or two aflatoxin types, and some non-toxigenic *Aspergillus* isolates were also recorded.

Keywords: Aflatoxin, Alpha and Beta Diversity, *Aspergillus*, Maize, Seeds

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INTRODUCTION

Maize (*Zea mays* L.) is an important cereal crop that is versatile in its adaptability to diverse agroclimatic conditions. Because it has the highest potential for genetic production among cereals, it is cultivated in approximately 160 different countries with a high variation in soil, temperature, biodiversity, and management practices. This versatile crop is in demand not only for its use in human and animal consumption, but also in food processing, poultry, dairy, and ethanol industries, thereby making it a rapidly expanding cash crop.

Maize productivity and quality are influenced by several biotic and abiotic variables. The most significant post-harvest issue is the contamination of maize kernels with mycotoxigenic fungi and their derivatives. According to the United Nations Food and Agriculture Organization (FAO), mycotoxins are found in 25% of harvested crops. Various molds generate harmful secondary metabolites, known as mycotoxins. The genera *Aspergillus*, *Fusarium*, and *Penicillium* are the primary producers of mycotoxins such as aflatoxin, ochratoxin, fumonisin, deoxynivalenol, T-2 toxin, and zearalenone.¹ Aflatoxins (AFs) derived from furanocoumarin are the most detrimental mycotoxins that contaminate maize-based feed and products and present substantial health hazards to both humans and animals because of their capacity to initiate extremely pathogenic cellular and physiological mechanisms.^{2,3} Several species of *Aspergillus* produce aflatoxins; however, *A. parasiticus*, *A. flavus*, *A. nomius*, and *A. pseudotamarii* are considered to be the major producers of aflatoxin.^{4,5} Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) constitute the four main aflatoxin variants. The predominant aflatoxin found in contaminated corn is AFB1.⁶

Food safety and security depend on three essential factors: food availability, safe food access, and the utilization of food for nutritional, cultural, and quality purposes. The method approved by the Association of Approved Analytical Chemists (AOAC) was used to identify aflatoxins in food and feed samples. Significant losses can be avoided with early detection.⁷ Alongside the ELISA (enzyme-linked immuno-sorbent assay),

chromatographic methods for example TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography), and LCMS (Liquid Chromatography Mass Spectrometry) are among the most commonly used procedures.⁸⁻¹⁰

Maize seeds harbor various ecto-endophytic bacteria, actinomycetes, and fungi. The isolated ectophytic mycoflora include *Diplodia*, *Aspergillus*, *Penicillium*, *Fusarium verticillioides*, *Gibberella zeae*, *F. proliferatum*, *F. glutinans*, *Botryosphaeria*, *Macrophomina phaseolina*, *Rhizoctonia*, *Nigrospora*, *Cladosporium*, *Trichoderma*, and *Rhizopus*.¹¹ Mycoflora infection in maize seeds results in decreased germination as well as rejection due to numerous abnormalities.¹² Further, poor storage structure can lead to infection of the entire seed stock by storage fungi. Sometimes, fungi growing on maize seeds can kill them, emit a bad odour/taste, and render the seeds inedible owing to the release of mycotoxins and changes in their chemical composition.¹³

The study of fungal diversity is crucial for evaluating ecosystems. Diversity metrics, such as species richness, evenness, population dynamics, and mycoflora dominance have been evaluated. Fungal species richness, indicative of niche diversity under limiting similarity that facilitates species cohabitation, is extensively used for habitat comparison.¹⁴ Fungal variety is affected by multiple factors, including temporal changes, climatic conditions, biological communities, topographical features, natural disturbances, anthropogenic disruptions, and pollution.¹⁵ Consequently, there is a growing need to predict features of fungal diversity and their spatiotemporal variations in both wild and controlled ecosystems. Alpha diversity refers to biodiversity within a specific community, whereas beta diversity assesses the variation in biodiversity between different locations. Examining species composition assists researchers in understanding species interactions and ecological functionality.¹⁶ Beta diversity reveals the distinctiveness of community composition across various landscapes.

Previous studies on maize have mainly focused on the rhizosphere microbiome, particularly the bacterial community. However, little attention has been paid to the structure and distribution of the fungal communities associated

with maize grains and the presence of mycotoxins. Understanding the composition and distribution of toxigenic fungi can provide basic information for the prevention of mycotoxin contamination, ultimately ensuring food safety in India and worldwide. Hence, the main aim of the present study was to evaluate aflatoxin concentrations in maize grains/products from various locations, the diversity of maize seed mycoflora, and the toxigenic potential of *Aspergillus* species from different locations in Tamil Nadu, India.

MATERIALS AND METHODS

Sample collection

Maize grains and maize-based products, such as grains, poultry feed, flour, corn flakes, and sweet and baby corn kernels, were collected from major districts of Tamil Nadu, India (Table 1). Samples were obtained from several stores and markets within a certain district, and composite samples were used to determine aflatoxin contamination. Maize grains, feed, and corn flakes were ground into a fine powder using a manual mill for analytical purposes. All obtained samples were preserved in sterile polythene bags, accurately labeled, and maintained at 4 °C until subsequent analyses. Similarly, seed samples were collected from the key maize-growing districts of Tamil Nadu, including Virudhunagar, Tuticorin, Madurai, Dharmapuri, Salem, Dindigul, Erode, Tirupur, Perambalur, and Coimbatore during 2021 to evaluate the mycoflora associated with the maize seeds. Fifteen seed samples were collected from the farmer's field for the evaluation of seed surface mycoflora. For one location, a 1 kg seed sample was collected from five different farmers' fields, and 500 g of the composite sample was then taken for further analysis. Seed samples were stored in sterile polythene bags and labeled appropriately.

Determination of aflatoxin contamination using RP-HPLC-FLD

Toxin extraction and column clean-up

The finely ground sample (25 g) and 2.5 g of sodium chloride (NaCl) were placed in a 100 ml conical flask, and 50 ml of 80% methanol was added. The material was then mixed using a high-speed blender or maintained on an orbital shaker for 30

min at 150 rpm. The material was subsequently filtered through Whatman No. 4 filter paper. A 2 ml aliquot was extracted from the filtered sample and diluted with 14 ml of phosphate-buffered saline (PBS). The filtrate, combined with PBS, was allowed to traverse an immuno-affinity column (AFLARHONE, R-Biopharm, USA) at a flow rate of 2 ml/min. Subsequently, the column was cleaned using 20 ml of PBS. Then, 1.5 ml of methanol and 1.5 ml of distilled water were passed through the column to elute the toxin, which was collected in an amber glass vial. The eluted toxin (50 µl) was subsequently subjected to HPLC. Aflatoxin detection was accomplished by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC-FLD) on an Agilent 1200 HPLC system (Agilent Technologies, USA) equipped with an autosampler and a fluorescence detector.

HPLC method validation

Linearity and sensitivity

A total aflatoxin standard was acquired at a concentration of 5 µg/ml (2 µg each for AFB1 and AFG1, 0.5 µg each for AFB2 and AFG2) and reconstituted in 10 ml of HPLC-grade acetonitrile. A 50% methanol solution was used to prepare working stocks at concentrations of 1, 5, 10, 15, 20, and 25 ng/ml. A multipoint calibration curve obtained by injecting concentrations of 1, 5, 10, 15, 20, and 25 ng/kg was used to assess the concentration-response linearity. Agilent Open Lab CDS (EZ Chrome Edition) software version A.04.08 (Agilent Technologies, USA) was used to acquire and process aflatoxin signals. For each toxin, LOD and LOQ were determined using signal-to-noise ratios of 3:1 and 10:1, respectively.

Recovery analysis of aflatoxin

Recovery analysis was performed for AFB1 and AFB2 to validate the procedure for extracting toxins from the sample. Briefly, 5 g of a healthy sample was spiked with different concentrations (2, 5, 10, and 20 ppb for AFB1 and 0.5, 1.25, 2.5, and 5 ppb for AFB2) of the total aflatoxin standard. Toxin extraction was performed using the immuno-affinity column as described above.¹⁷ Three replicates were maintained for each concentration, and the mean recovery percentage was calculated.

Table 1. Different maize products collected from various districts of Tamil Nadu

No.	District	Place of collection	Sample type	Sample code
1	Coimbatore	Perur	Corn flour	MP 1
2		Thondamuthur	Poultry feed	MP 2
3		Kenathukadavu	Corn flour	MP 3
4		Kuniyamuthur	Corn flour	MP 4
5	Madurai	Vadipatti	Corn flour	MP 5
6		Thirupparankundram	Poultry feed	MP 6
7		Alanganallur	Corn flour	MP 7
8		Usilampatti	Corn flour	MP 8
9	Tiruppur	Thirumangalam	Corn flour	MP 9
10		Dharapuram	Corn flour	MP 10
11		Avinashi	Corn flour	MP 11
12	Chennai	T-Nagar	Corn flour	MP 12
13		Perambur	Corn flour	MP 13
14		Adyar	Corn flour	MP 14
15	Thirunelveli	Egmore	Corn flour	MP 15
16		Ambasamudram	Poultry feed	MP 16
17		Kadayanallur	Corn flour	MP 17
18	Krishnagiri	Sankaran kovil	Corn flour	MP 18
19		Hosur	Corn flour	MP 19
20		Kaveripattinam	Poultry feed	MP 20
21	Kancheepuram	Uttangarai	Corn flour	MP 21
22		Sriperumpudur	Corn flour	MP 22
23		Kancheepuram	Corn flour	MP 23
24	Trichy	Manapparai	Corn flour	MP 24
25		Thuraiyur	Corn flour	MP 25
26	Vellore	Thirupattur	Poultry feed	MP 26
27		Anaicut	Corn flour	MP 27
28	Virudhunagar	Rajapalayam	Corn flour	MP 28
29		Arupukottai	Corn flour	MP 29
30		Watrap	Poultry feed	MP 30
31		Srivilliputhur	Corn flour	MP 31
32		Sivakasi	Corn flour	MP 32

Assessment of mycoflora in maize seeds

The standard blotter technique was used to evaluate mycoflora on maize seed surfaces.¹⁸ Under a stereo zoom microscope, incubated seeds were visually evaluated to examine the growth pattern of mycoflora.¹⁹ One representative colony from each fungal genus in each seed sample was cultivated in potato dextrose agar (PDA) media and identified based on “habit characters”.

Computation for diversity indices

The method suggested by Tadych *et al.*²⁰ was used to calculate the relative densities (RD) of fungal species and genera. Alpha diversity can be determined using calculations of the Simpson diversity index (D),²¹ Shannon–Wiener

diversity index²² and species evenness index.²³

The values obtained for each index were ranked in the following steps. This study used Fernando’s biodiversity scale to score the indices (Table 2). Beta diversity refers to the fluctuation in species diversity between two regions. This was used for a comprehensive comparison of species diversity. β -diversity was calculated using the formula provided by Fontana *et al.*²⁴

Isolation of aflatoxigenic fungi and morphological characterization

To isolate aflatoxigenic fungi from maize seed samples collected from different locations, Pitt and Hocking’s²⁵ method was used. Maize seed samples were crushed into a fine powder using a

hand mill, and 1 g of the powdered sample was serially diluted in 10 ml of sterile distilled water until 10^{-4} and 10^{-5} dilutions were achieved. An aliquot (1 ml) was then plated in a sterile Petri plate containing Rose Bengal Chloramphenicol Agar medium.²⁶ The majority of *Aspergillus* sp. produces single spore colonies, which were then randomly selected and individually cultured on a PDA medium by single hypha transfer.²⁷ The plates were incubated at 30 °C for 7 days. After the incubation period, macroscopic characteristics such as colony morphology, colour, and sclerotia formation were observed. For microscopic observation, slides were prepared and the fungal structures were stained

using lactophenol cotton blue stain. Different *Aspergillus* species were identified based on the presence of conidiophores, vesicles, phialides, metulae, and conidia.

Molecular confirmation of *Aspergillus* isolates

Molecular confirmation of *Aspergillus* isolates was performed using internal transcribed spacer (ITS) 18S rRNA sequencing. DNA extraction was performed from the fungal mycelial mat using the CTAB method described by Doyle²⁸ and Allen *et al.*²⁹ The polymerase chain reaction (PCR) was conducted with 18S rDNA universal primers ITS 1 (TCCGTAGCTGAACCTGCCG) and ITS 4 (TCCTCCGCTTATTGATATGC) to verify the isolates.³⁰ The PCR procedure comprised 35 cycles, initiated with a denaturation phase at 94 °C for 5 min, followed by 60 s denaturation at 94 °C, annealing at 65 °C, 60 s extension at 72 °C, and a final extension of 5 min at 72 °C. Agarose gel electrophoresis (1.2%) was performed to observe the amplified products using a gel documentation unit (Alpha Imager 2000). Sanger dideoxy sequencing was used to sequence the amplified PCR products.

Table 2. Edwino Fernando's ranking of Biodiversity Indices

Relative values	Shannon (H') Index	Evenness (E)
Very high	3.5 and above	0.75-1.00
High	3.0-3.49	0.50-0.74
Moderate	2.5-2.99	0.25-0.49
Low	2.0-2.49	0.15-0.24
Very low	1.9 and below	0.05-0.14

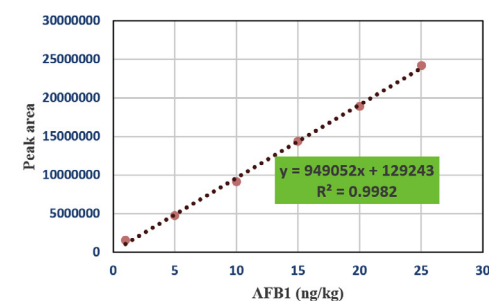


Fig. 1a

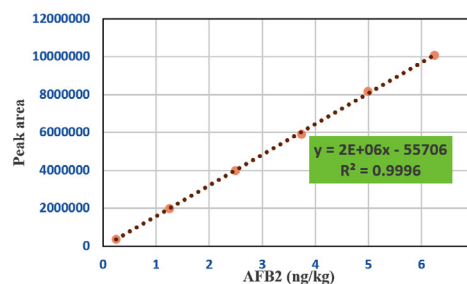


Fig. 1b

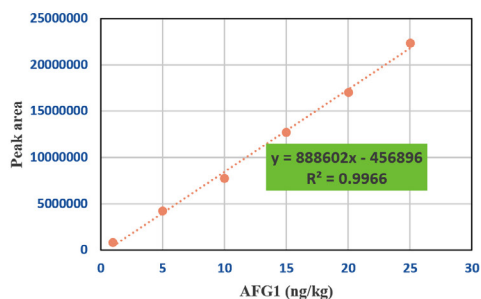


Fig. 1c

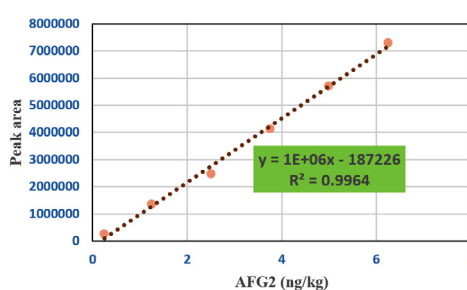


Fig. 1d

Figure 1. Multipoint calibration curve for AFB1 (1a), AFB2 (1b), AFG1 (1c) and AFG2 (1d)

Table 3. Sensitivity and linearity of aflatoxin B1, B2, G1 and G2 in HPLC analysis

Type of aflatoxin	Calibration curve	R ²	LOD (ppb)	LOQ (ppb)
B1	y = 949052x + 129243	0.9982	0.04	0.12
B2	y = 2E+06x - 55706	0.9996	0.029	0.086
G1	y = 888602x - 456896	0.9966	0.057	0.17
G2	y = 1E+06x - 187226	0.9964	0.030	0.091

LOD: Limit of Detection, LOQ: Limit of Quantification

Each concentration was injected thrice

Linearity was performed using linear regression analysis

Table 4. Recovery analysis of aflatoxin B1 and B2 in maize products by HPLC analysis

Particulars	Recovery (ng/kg)							
	AFB ₁				AFB ₂			
	Spiking amount (ng/kg or ppb)							
	2	5	10	20	0.5	1.25	2.5	5
Sample 1	1.05	4.46	8.2	17.96	0.21	0.91	1.92	4.18
Sample 2	1.06	4.69	9.0	19.1	0.21	0.96	2.11	4.44
Sample 3	1.1	4.5	9.26	19.67	0.22	0.92	2.17	4.57
Mean recovery	1.07	4.55	8.82	18.91	0.21	0.93	2.07	4.40
Std dev	0.02	0.10	0.45	0.71	0.00	0.02	0.11	0.16
Recovery %	53.50	91.00	88.20	94.55	42.67	74.40	82.67	87.93
Mean recovery	81.81%				71.92%			

Toxicogenic potential of *Aspergillus* isolated from maize kernels

The HPLC-FLD method was used to assess the aflatoxigenic capacity of the *Aspergillus* isolates. Five agar plugs were collected from a seven-day-old culture in a weighed microfuge tube. HPLC grade chloroform (1 ml) was added, vortexed, and centrifuged at 10,000 rpm for 15 min for extraction. The supernatants were transferred to three new microfuge tubes. The supernatant was dried in a vacuum concentrator. Then, 50% methanol (1 ml) was used to dissolve and store the air-dried residues in an amber glass vial at 4 °C until HPLC analysis.

Statistical analysis

All data were analyzed using descriptive statistics in the SPSS Statistics package (version 21.0; SPSS Inc. Chicago, IL, USA). Alpha diversity indices Shannon, Simpson, taxa, individual, Chao 1, beta diversity indices, and principal component analysis (PCA) were calculated using the R statistics

using package “vegan”, design of experiments by package “DOE”, heatmap by heatmap3.

RESULTS

Sensitivity, linearity, and recovery analysis

HPLC analysis of aflatoxins was validated using a reference standard for mixed aflatoxins. In the present study, multipoint calibration curves for AFB1, AFB2, AFG1, and AFG2 were created by injecting six different concentrations of mixed standards. AFB1 and AFG1 multipoint calibration curves were linear from 1 ppb to 25 ppb, whereas AFB2 and AFG2 multipoint calibration curves had linearity ranging from 0.25 ppb to 6.25 ppb (Figure 1a, 1b, 1c and 1d). Linear regression analysis was used to evaluate the linearity between the instrument response and concentration (Table 3). The regression correlation coefficients above 0.99 for all four aflatoxin types (AFB1 = 0.998, AFB2 = 0.999, AFG1 = 0.996, and AFG2 = 0.996) showed good linearity. AFB1, AFG1, AFB2, and

Table 5. Determination of aflatoxin contamination in different maize products by HPLC analysis

No.	Sample code	Amount of aflatoxin (ppb)	Type of aflatoxin
1	MP 1	1.922	B1&B2
2	MP 2	8.896	B1&B2
3	MP 3	1.749	B1
4	MP 4	0.580	B1&B2
5	MP 5	4.590	B1&B2
6	MP 6	13.595	B1&B2
7	MP 7	4.151	B1&B2
8	MP 8	6.083	B1&B2
9	MP 9	4.999	B1&B2
10	MP 10	0.580	B1
11	MP 11	1.615	B1&B2
12	MP 12	0.849	B1&B2
13	MP 13	0.808	B1
14	MP 14	1.200	B1&B2
15	MP 15	0.868	B1
16	MP 16	6.451	B1&B2
17	MP 17	<LOD	-
18	MP 18	1.398	B1&B2
19	MP 19	1.252	B1
20	MP 20	9.932	B1&B2
21	MP 21	0.689	B1&B2
22	MP 22	1.492	B1&B2
23	MP 23	1.660	B1&B2
24	MP 24	1.173	B1&B2
25	MP 25	0.689	B1&B2
26	MP 26	9.191	B1&B2
27	MP 27	1.810	B1&B2
28	MP 28	5.731	B1&B2
29	MP 29	<LOD	-
30	MP 30	2.584	B1
31	MP 31	0.466	B1
32	MP 32	0.838	B1&B2

<LOD: less than the Limit of detection

AFG2 exhibited limits of detection (LOD) of 0.04, 0.029, 0.057, and 0.030 ppb and limits of quantification (LOQ) of 0.12, 0.086, 0.17, and 0.091 ppb, respectively.

The mean recoveries of AFB1 for spiking amounts of 2, 5, 10, and 20 ppb were 1.07, 4.55, 8.82 and 18.91 ng/kg with per cent recoveries of 53.50, 91.0, 88.2, and 94.55%, respectively, whereas the mean recoveries of AFB2 for spiking amounts of 0.5, 1.25, 2.5, and 5 ppb were 0.21, 0.93, 2.07 and 4.40 ng/kg and the per cent recovery were 42.7%, 74.4%, 82.67% and 87.93%, respectively (Table 4).

Table 6. Alpha-diversity analysis of maize seed mycoflora recorded in different locations

District Location	Virudhunagar		Tuticorin		Madurai		Dharmapuri		Salem		Dindigul		Erode		Tiruppur		Perambalur		Coimbatore	
	Watrap	Kunoor	Tuticorin	Tuticorin	Vadipatti	Vadipatti	Dharmapuri	Dharmapuri	Athur	Athur	Ottanchathiram	Ottanchathiram	Gobi	Bhavani	Udumalai	Veppanthattai	Perambalur	Perambalur	TNAU-SC-F	TNAU-NA-F
Taxa_S	6	6	6	6	7	6	6	6	5	6	9	9	10	8	6	7	3	5	5	6
Individuals	44	63	118	118	64	110	110	102	88	102	86	86	79	68	82	64	63	60	61	61
Dominance_D	0.187	0.265	0.247	0.247	0.254	0.342	0.342	0.285	0.300	0.285	0.152	0.152	0.148	0.186	0.447	0.241	0.646	0.207	0.200	0.200
Simpson_1-D	0.813	0.735	0.753	0.753	0.747	0.658	0.658	0.715	0.700	0.715	0.848	0.848	0.852	0.814	0.553	0.759	0.354	0.793	0.800	0.800
Shannon_H	1.587	1.409	1.539	1.539	1.521	1.236	1.236	1.434	1.314	1.434	1.927	1.927	1.950	1.718	1.039	1.511	0.636	1.517	1.568	1.568
Evenness_e^H/S	0.815	0.682	0.777	0.777	0.654	0.574	0.574	0.699	0.744	0.699	0.763	0.763	0.703	0.697	0.471	0.647	0.629	0.912	0.800	0.800
Equitability_J	0.886	0.787	0.859	0.859	0.782	0.690	0.690	0.801	0.816	0.801	0.877	0.877	0.847	0.826	0.580	0.777	0.579	0.943	0.875	0.875
Fisher_alpha	1.877	1.631	1.336	1.336	2.003	1.363	1.363	1.393	1.149	1.393	2.532	2.532	3.032	2.355	1.490	2.003	0.656	1.297	1.650	1.650
Berger-Parker	0.273	0.381	0.407	0.407	0.438	0.436	0.436	0.471	0.455	0.471	0.244	0.244	0.228	0.279	0.634	0.359	0.794	0.250	0.246	0.246
Chao-1	6	6	6	6	7	6	6	6	5	6	9	9	10	8	6.5	7	3	5	5	6

TNAU-SC-F: TNAU Seed Centre- Farm; TNAU-NA-F: TNAU-New Area- Farm; Taxa S: number of species recorded = species richness; Individuals: total number of mycoflora observed; Chao 1 is the expected species richness

Table 7. Beta-diversity analysis of maize seed mycoflora by Bray-Curtis method

District	Virudhunagar				Tuticorin		Madurai	Dharmapuri	Salem		Dindigul	Erode		Tiruppur	Perambalur		Coimbatore	
Locations	Watrap	Kunnor	Tuticorin	Vadipatti	Vadipatti	Dharmapuri	Salem	Athur	Ottanchathiram	Gobi	Bhavani	Udumalai	Veppanthattai	Perambalur	TNAU-SC-F	TNAU-NA-F		
Watrap	*	54.2056	53.0864	74.0741	42.8571	50	36.9863	60	63.4146	71.4286	42.8571	55.5556	31.7757	61.5385	60.9524			
Kunnor	*	*	50.8287	55.1181	45.0867	82.1192	56.9697	68.4564	64.7887	74.8092	45.5172	53.5433	52.381	53.6585	54.8387			
Tuticorin	*	*	*	69.2308	75.4386	54.3689	36.3636	53.9216	54.8223	61.2903	53	65.9341	43.0939	64.0449	63.6871			
Vadipatti	*	*	*	*	57.4713	59.2105	44.5783	68	71.3287	68.1818	41.0959	57.8125	37.7953	62.9032	62.4			
Dharmapuri	*	*	*	*	*	49.4949	30.1887	44.898	45.5026	55.0562	76.0417	59.7701	67.052	50.5882	50.2924			
Salem	*	*	*	*	*	*	74.7368	68.9655	65.8683	70.5128	44.7059	55.2632	43.7086	54.0541	53.6913			
Athur	*	*	*	*	*	*	*	69.1489	61.8785	49.4118	25	33.7349	24.2424	40.7407	41.7178			
Ottanchathiram	*	*	*	*	*	*	*	*	82.4242	70.1299	35.7143	50.6667	32.2148	60.274	61.2245			
Gobi	*	*	*	*	*	*	*	*	*	74.8299	40.9938	57.3427	35.2113	66.187	67.1429			
Bavani	*	*	*	*	*	*	*	*	*	*	57.3333	75.7576	44.2748	70.3125	69.7674			
Udumalai	*	*	*	*	*	*	*	*	*	*	*	71.2329	80	59.1549	60.1399			
Veppanthattai	*	*	*	*	*	*	*	*	*	*	*	*	51.9685	80.6452	80			
Perambalur	*	*	*	*	*	*	*	*	*	*	*	*	*	45.5285	45.1613			
TNAU-SC-F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	99.1736		
TNAU-NA-F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		

TNAU-SC-F: TNAU – Seed Centre - Farm, TNAU – NAF: TNAU-New Area-Farm

Table 8. Beta-diversity- similarity and distance matrices of mycoflora between locations

Step	Similarity %	Location Joined 1	Location Joined 2
1	99.1736	TNAU-SC-F	TNAU-NA-F
2	82.4242	Ottanchathiram	Gobi
3	82.1192	Kunnor	Salem
4	80.6452	Veppanthattai	TNAU-SC-F
5	80.0000	Udumalai	Perambalur
6	76.0417	Dharmapuri	Udumalai
7	75.7576	Bhavani	Veppanthattai
8	75.4386	Tuticorin	Dharmapuri
9	74.8299	Ottanchathiram	Bhavani
10	74.8092	Kunnor	Ottanchathiram
11	74.7368	Kunnor	Athur
12	74.0741	Watrap	Vadipatti
13	71.4286	Watrap	Kunnor
14	71.2329	Watrap	Tuticorin

Joined 1 is (Location 1) compared with Joined 2 (Location 2) and their similarity and distant indices.
This is the summary of the Table 7

Natural occurrence of aflatoxin contamination in maize products

In total, 32 maize-based product samples were collected from several major cities and towns to evaluate aflatoxin contamination using HPLC. Among the 32 samples tested, 30 (93.75%) were contaminated with aflatoxins, ranging from 0.296 to 13.595 ppb, and only two samples (6.25%) were free from aflatoxin contamination (Table 5). However, all samples were below the Indian regulatory limits (<15 ppb), whereas 10 samples (31.25%) were above the European regulatory levels (>4 ppb) (Figure 2). Furthermore, the majority of the samples (73.78%) contained both AFB1 and AFB2, and six samples (18.75%) of maize products contained only AFB1.

Mycoflora diversity on maize seeds

Mycoflora diversity on maize seeds collected from different regions of Tamil Nadu was assessed. The mycoflora populations of individual maize seeds were recorded. The microflora diversity in individual seeds varied from zero or no diversity to high diversity (Figure 3).

Proportion of fungal species in the mycoflora of maize seeds

A total of 1152 mycoflora isolates

encompassing 10 distinct species were identified, of which five belonged to the genus *Aspergillus*, accounting for 82% of the total mycoflora population (*A. niger* 29%, *A. flavus* 25%, *A. fumigatus* 17%, *A. tamarii* 10%, and *A. oryzae* 1%). The dominant mycoflora belonged to the genus *Aspergillus* followed by *Fusarium* spp. (9%). Other fungal genera included *Penicillium* spp. (5%), *Rhizopus* spp. (2%), *Alternaria* spp. (1%), and *Macrophomina* (1%) (Figure 4).

Fungal species composition at different locations

Two *Aspergillus* species, namely *A. niger* and *A. flavus*, were identified at all the localities, whereas *A. oryzae* was observed in only four regions. *Fusarium* spp. were detected in each region, excluding Virudhunagar. *Penicillium* spp. were detected in five regions, *Macrophomina* and *Rhizopus* spp. in three regions each, and *Alternaria* spp. in two regions. Tuticorin had the most species of mycoflora (118), followed by Dharmapuri (110), and other sites with 44 to 102 species (Figure 5).

Alpha diversity analysis of maize seed mycoflora

The Shannon–Weiner index (H') and Simpson's index are widely used to characterize alpha diversity. The Shannon–Weiner index (H') clarifies the impact of abundance. The Shannon index was <1.9 in every location, except for Gobi (1.95) and Ottanchathiram (1.927), based on Fernando's Ranking of Biodiversity Indices, indicating extremely low diversity of mycoflora (Table 6). Because the Shannon diversity index (H) is influenced by abundance, mycoflora abundance was absent in all locations. The Simpson diversity index was used to determine a measure of diversity that considers the number of taxa and their abundance, with more species observed and their abundances considered.

The highest Simpson index of 0.852 was recorded in Gobi, followed by Ottanchathiram (0.848), Bhavani (0.814), and Watrap (0.813%), representing the highest dominance of mycoflora, as the Simpson index is influenced by dominance.

Species richness, defined as the number of species or operational taxonomic units (OTUs) in a given area, is a fundamental metric of alpha diversity. Gobi exhibited the greatest species richness with ten species, followed by Ottanchathiram with nine species, and Bhavani

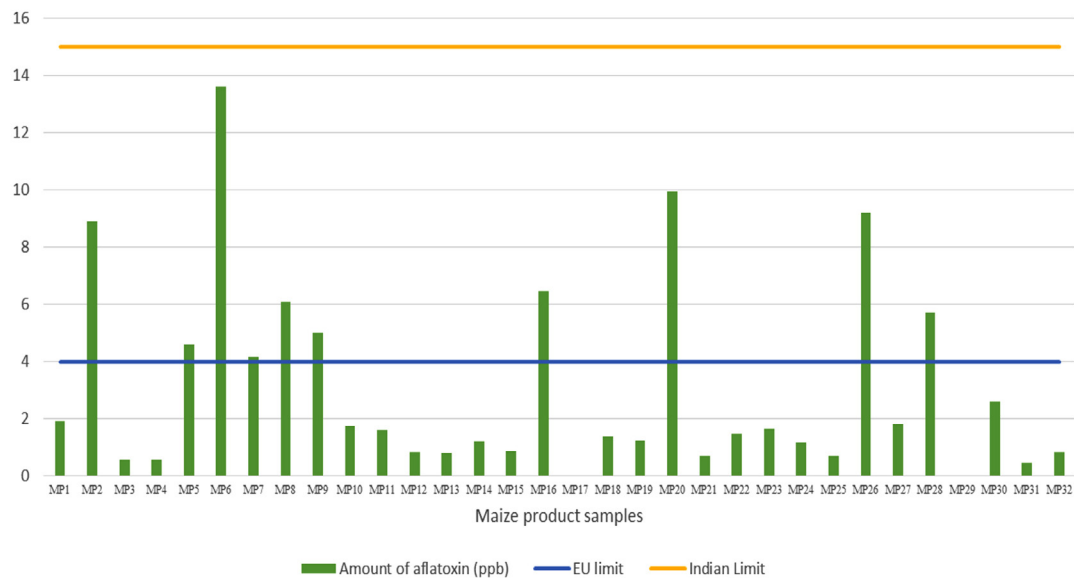


Figure 2. Natural occurrence of aflatoxin contamination in maize products

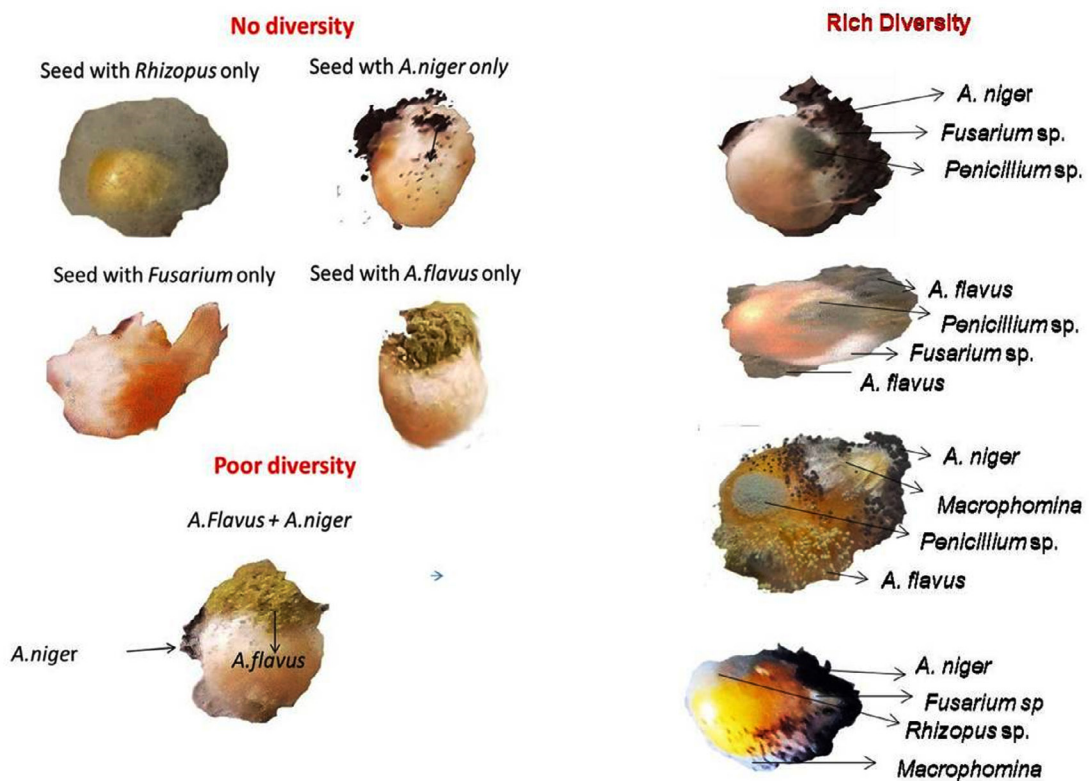


Figure 3. Mycoflora diversity on maize seeds collected from different locations

Table 9. Alpha diversity analysis of *Aspergillus* spp. at different locations

	Watrap	Kunnor	Tuticorin	Vadipatti	Dharmapuri	Salem	Athur	Ottanchathirram	Gobi	Bhavani	Udumalai	Veppanthattai	Perambalur	TNAU-SC-F	TNAU-NA-F
Taxa_S	4	4	4	5	4	4	3	4	5	5	4	4	2	3	3
Individuals	35	55	100	51	105	81	72	55	50	58	80	56	57	44	44
Dominance_D	0.2718	0.3468	0.3368	0.3833	0.3803	0.3556	0.5096	0.321	0.2824	0.261	0.4763	0.3233	0.7845	0.3337	0.3337
Simpson_1-D	0.7282	0.6532	0.6632	0.6167	0.6197	0.6444	0.4904	0.679	0.7176	0.739	0.5237	0.6767	0.2155	0.6663	0.6663
Shannon_H	1.34	1.169	1.213	1.164	1.093	1.15	0.8377	1.216	1.369	1.407	0.9618	1.195	0.3725	1.098	1.098
Evenness_e^H/S	0.955	0.8045	0.8411	0.6408	0.746	0.7896	0.7704	0.8434	0.7863	0.8169	0.6541	0.8261	0.7257	0.9995	0.9995
Equitability_J	0.9668	0.8431	0.8752	0.7234	0.7886	0.8296	0.7625	0.8772	0.8506	0.8743	0.6938	0.8622	0.5374	0.9995	0.9995
Fisher_alpha	1.164	0.9917	0.8342	1.373	0.8238	0.8831	0.6324	0.9917	1.383	1.312	0.8861	0.986	0.4034	0.7286	0.7286
Berger-Parker	0.3429	0.4364	0.48	0.549	0.4571	0.4938	0.6667	0.3818	0.36	0.3276	0.65	0.4107	0.8772	0.3409	0.3409
Chao-1	4	4	4	5	4	4	3	4	5	5	4	4	2	3	3

TNAU - SC-F: TNAU - Seed Centre - Farm, TNAU - NAF: TNAU-New Area- Farm; Taxa S - Number of species recorded = species richness; Individuals - Total number of mycoflora observed
Chao 1 is the expected species richness

Table 10. Beta-diversity analysis of genus *Aspergillus* spp. by Bray-Curtis method

Locations	Watrap	Kunnor	Tuticorin	Vadipatti	Dharmapuri	Salem	Athur	Ottanchathirram	Gobi	Bhavani	Udumalai	Veppanthattai	Perambalur	TNAU-SC-F	TNAU-NA-F
Watrap	*	64.4444	51.8519	74.4186	45.7143	56.8966	50.4673	73.3333	77.6471	75.2688	46.9565	59.3407	36.9565	68.3544	68.3544
Kunnor	*	*	50.3226	56.6038	45	80.8824	61.4173	78.1818	72.381	81.4159	45.9259	55.8559	48.2143	52.5253	52.5253
Tuticorin	*	*	*	66.2252	80.9756	54.1436	37.2093	54.1936	54.6667	62.0253	57.7778	69.2308	42.0382	61.1111	61.1111
Vadipatti	*	*	*	*	60.2564	60.6061	52.0325	77.3585	81.1881	67.8899	44.2748	57.9439	35.1852	61.0526	61.0526
Dharmapuri	*	*	*	*	49.4624	32.7684	83.6601	48.75	49.0323	56.4417	77.8378	60.8696	67.9012	53.6913	53.6913
Salem	*	*	*	*	*	*	*	77.9412	73.2824	74.8201	45.9627	56.9343	39.1304	52.8	52.8
Athur	*	*	*	*	*	*	*	70.8661	63.9344	56.9231	27.6316	35.9375	21.7054	37.931	37.931
Ottanchathirram	*	*	*	*	*	*	*	*	89.5238	77.8761	41.4815	54.0541	32.1429	58.5859	58.5859
Gobi	*	*	*	*	*	*	*	*	*	83.3333	47.6923	62.2642	35.514	63.8298	63.8298
Bhavani	*	*	*	*	*	*	*	*	*	*	60.8696	73.6842	45.2174	72.549	72.549
Udumalai	*	*	*	*	*	*	*	*	*	*	*	75	83.2117	66.129	66.129
Veppanthattai	*	*	*	*	*	*	*	*	*	*	*	*	53.0973	88	88
Perambalur	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TNAU-SC-F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	100
TNAU-NA-F	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Similarity indices in per cent; TNAU-SC-F: TNAU - Seed Centre - Farm, TNAU-NA-F: TNAU-New Area-Farm

with eight species. Salem and TNAU Seed Centre Farm had the lowest species richness, with five recorded number of species.

Rare species are a group of mycoflora that are uncommon, scarce, and have small populations. According to the step graph, the rare species were *Alternaria*, *Macrophomina*, *Rhizopus*, and *A. oryzae* (Figure 6).

The evenness index lies between 0 and 1.0 and reflects the degree of equal abundance among different taxa within the community. In our study, all locations and species had even distributions, as indicated by the evenness values >0.5. The mycoflora showed equal distribution at all locations.

Beta diversity analysis of maize seed mycoflora between locations using the Bray-Curtis method

Beta diversity refers to the differences in species composition between samples. It measures the variation in the overall taxonomic composition between two samples. Bray-Curtis similarity measures the degree of species composition similarity between two sites, ranging from 0 (no species shared) to 1 (identical composition). TNAU New Area Farm had a similarity index of 99% with TNAU Seed Centre Farm; they had a

Bray-Curtis similarity of 1 (100%), that is, they had similar mycoflora composition with the same abundance (Table 7 and 8). Gobi had a similarity index of 82.42% with Ottanchathiram, and Salem with Dindigul had a similarity index of 82.12%; these locations share species, which means that they have similar mycoflora with exactly the same abundance. A very low similarity index of 24.24% was observed between Perambalur and Athur, followed by 25% between Udumalai and Athur, because they do not share mycoflora. Pairwise comparisons of the similarities between other locations ranged from 25% to 88%.

Diversity of the genus *Aspergillus* spp.

Genus *Aspergillus* composition in different locations

Five *Aspergillus* spp. were recorded: *A. niger*, *A. fumigatus*, *A. tamarii*, *A. flavus*, and *A. oryzae*. Among these, *A. niger* contributed the most (36%), followed by *A. flavus* (31%), *A. fumigatus* (20%), and *A. tamarii* (12%), whereas *A. oryzae* contributed the least (1%) (Figure 7). All five *Aspergillus* species were recorded in Gobi, Bhavani, and Vadipatti. Only two species, namely *A. flavus* and *A. niger*, were recorded in Perambalur. The highest number of *Aspergillus* isolates of 105

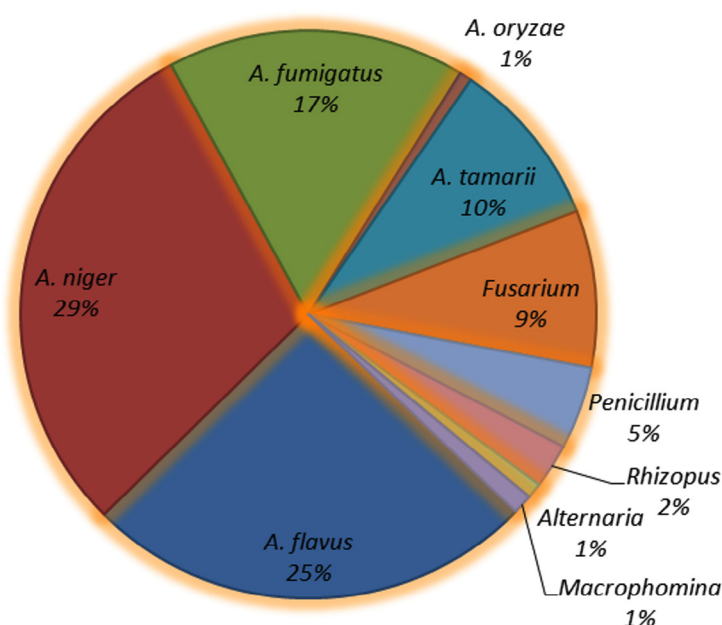


Figure 4. Percent mycoflora population on maize seeds

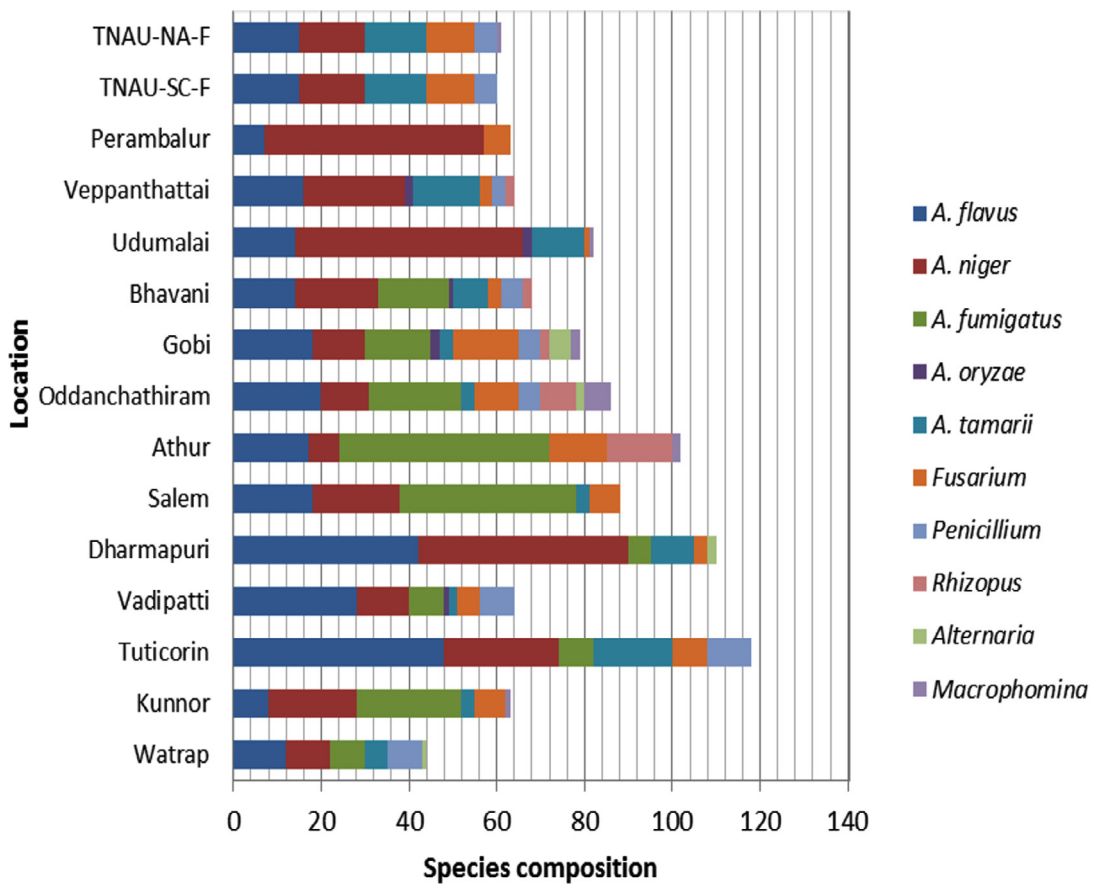


Figure 5. Fungal species composition of mycoflora in maize seeds at different locations

Table 11. Beta-diversity-similarity and distance matrices of *Aspergillus* spp. between locations

Step	Clusters	Similarity%	Joined 1	Joined 2
1	14	100	TNAU-SC-F	TNAU-NA-F
2	13	89.5238	Ottanchatram	Gobi
3	12	88.0000	Veppanthattai	TNAU-SC-F
4	11	83.6601	Salem	Athur
5	10	83.3333	Ottanchathiram	Bhavani
6	9	83.2117	Udumalai	Perambalur
7	8	81.4159	Kunnor	Ottanchathiram
8	7	81.1881	Kunnor	Vadipatti
9	6	80.9756	Tuticorin	Dharmapuri
10	5	80.8824	Kunnor	Salem
11	4	77.8378	Tuticorin	Udumalai
12	3	77.6471	Watrap	Kunnor
13	2	75.0000	Tuticorin	Veppanthattai
14	1	73.6842	Watrap	Tuticorin

Joined 1 is (Location 1) compared with Joined 2 (Location 2) and their similarity and distant indices.
This is the summary of the Table 10

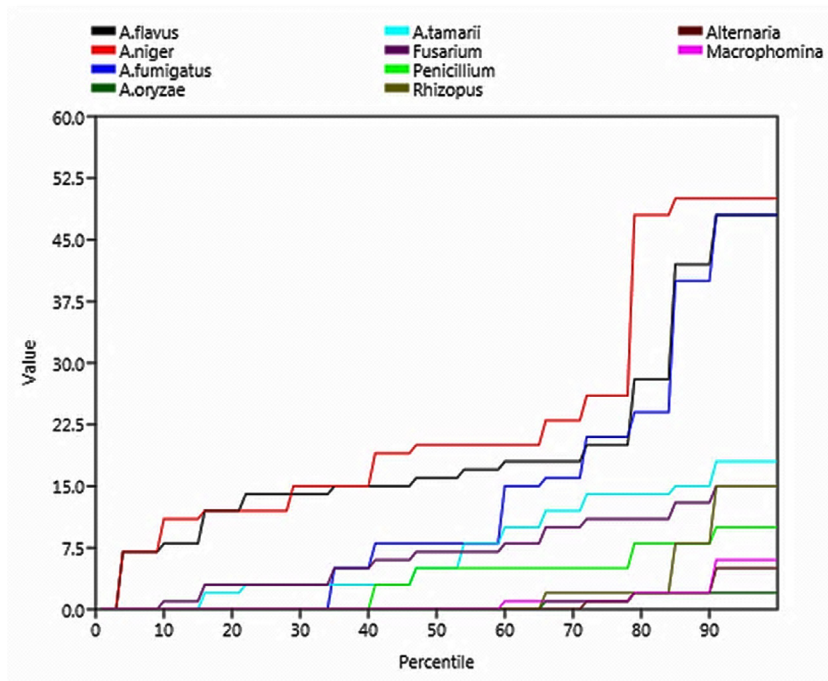


Figure 6. Step graph showing species richness and rareness of mycoflora

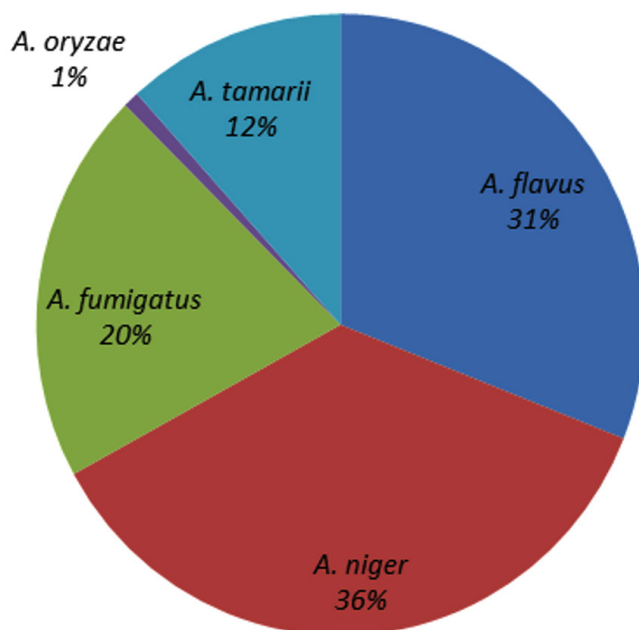


Figure 7. Species composition of genus *Aspergillus* in maize seeds

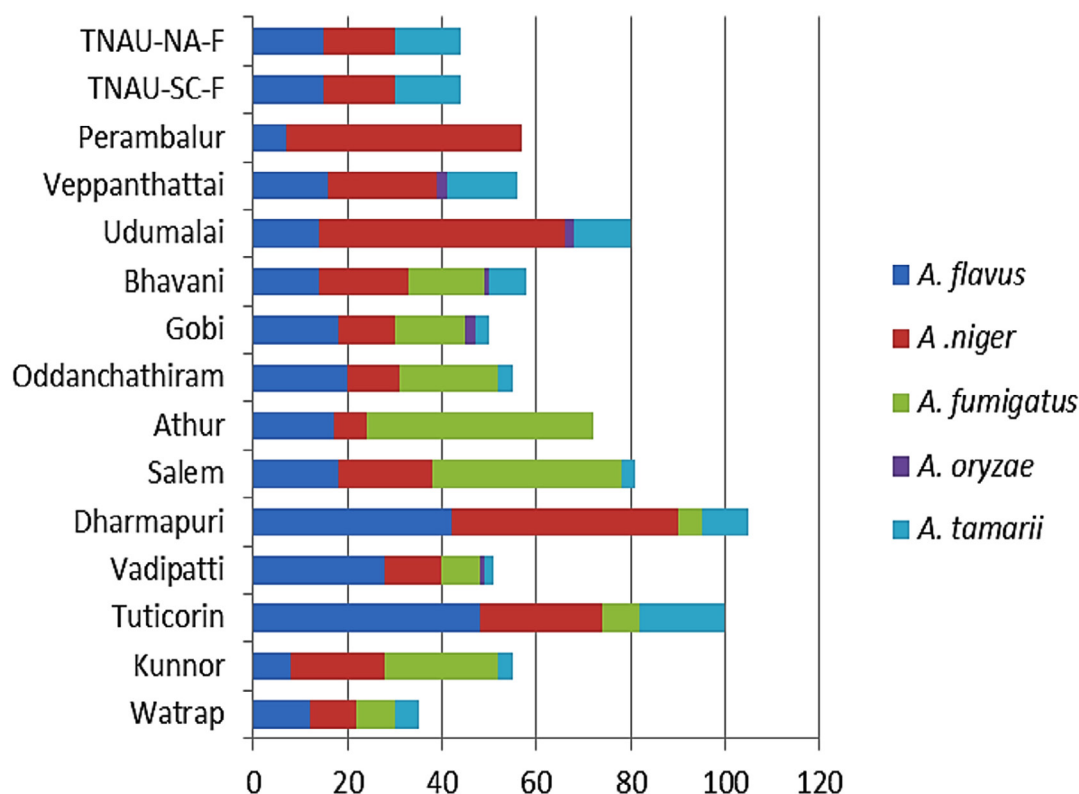


Figure 8. Species composition of genus *Aspergillus* at different locations

was recorded in Dharmapuri, followed by Tuticorin (100), and the lowest number of 35 isolates was recorded in Virudhunagar. *Aspergillus oryzae* was observed only at four locations, namely Vadipatti, Gobi, Bhavani, and Udumalai (Figure 8).

Alpha diversity indices of *Aspergillus* spp.

Based on Fernando's ranking, all sites had a Shannon index <1.9, indicating no significant presence of *Aspergillus*, owing to the influence of this index on abundance.

The Simpson index 1/D ranged from 0.2155 to 0.739. A maximum of 0.739 was recorded in Bhavani, followed by Watrap (0.728), representing a high dominance of *Aspergillus* spp. Perambalur had a minimum Simpson index 1/D of 0.2155, meaning no dominance of mycoflora. Chao 1 is an estimate that quantifies abundance and denotes species richness. The highest abundance

of five species was recorded in Gobi, Bhavani, and Vadipatti (Table 9).

The highest evenness of *Aspergillus* (0.995) was recorded at the TNAU Seed Centre Farm, TNAU New Area Farm, and Watrap. At other locations, it ranged from 0.6408 to 0.8434, meaning that *Aspergillus* spp. were evenly distributed in all tested locations. *Aspergillus oryzae* is a rare species, recorded in only 5 out of the 15 locations tested (Vadipatti, Gobi, Bhavani, Udumalai, and Veppanthattai).

Beta diversity analysis of genus *Aspergillus* between locations using the Bray–Curtis method

A complete similarity of *Aspergillus* species (100%) was recorded between the TNAU Seed Centre Farm and TNAU New Area Farm, followed by Ottanchathiram and Gobi (89.52%). The similarity of *Aspergillus* spp.

Table 12. The toxigenic potential of different *Aspergillus* (Asp 23) isolated from maize seeds

No.	Location	Isolate	Aflatoxin (ppb/g of agar plug)			
			B1	B2	G1	G2
1	Vadipatti	Asp 1	1497.190	50.789	0	0
2	Vadipatti	Asp 2	4.097	0.688	0	0
3	Kunnor	Asp 3	13.563	0	0	0
4	Kunnor	Asp 4	2059.392	135.858	0	0
5	Tuticorin	Asp 5	1.636	0.307	0	0
6	Tuticorin	Asp 6	3.432	0	0	0
7	Dharmapuri	Asp 7	0.094	0	0	0
8	Dharmapuri	Asp 8	6.189	0	0	0
9	Bhavani	Asp 9	0.119	0	0	0
10	Bhavani	Asp 10	0.217	0	0	0
11	Veppanthattai	Asp 11	0.300	0	0	0
12	Gobi	Asp 12	14.365	0	0	0
13	Gobi	Asp 13	0.221	0	0	0
14	Ottanchathiram	Asp 14	2288.611	84.088	0	0
15	Watrap	Asp 15	102.041	5.824	0	0
16	Watrap	Asp 16	1887.62	30.37	13.52	39.49
17	Udumalai	Asp 17	861.35	483.11	68.14	109.8
18	Udumalai	Asp 18	0	0	0	0
19	Coimbatore	Asp 19	2065.08	617.94	0	0
20	Coimbatore	Asp 20	4246.584	33.203	6.768	35.207
21	Athur	Asp 21	66.157	4.056	0	0
22	Salem	Asp 22	0.882	0	0	0
23	Salem	Asp 23	10250.78	88.750	22.462	91.867
24	Perambalur	Asp 24	515.404	2.661	0	0

Values are mean of three replications

between Veppanthattai and TNAU Farm was 88%, whereas 83% similarity was observed between Ottanchathiram and Bhavani, and Udumalai and Perambalur. A minimum similarity of 21.7% was observed between the Perambalur and Athur (Tables 10 and 11).

Isolation, morphological, and molecular characterization of *Aspergillus* spp.

Twenty-four different *Aspergillus* spp. were isolated from maize seed samples collected from various regions of Tamil Nadu. The fungal isolates were identified based on their cultural and morphological features. Additionally, 18S rDNA gene sequence analysis was conducted using ITS 1 and ITS 4 primers to identify *Aspergillus* isolates, and a homology search was performed using the NCBI nucleotide database via BLAST. All fungal isolate DNA was amplified with an amplicon range

of 550-600 bp (Figure 9). Among them, 17 isolates were identified as *A. flavus*, four as *A. parasiticus*, and three as *A. oryzae*.

Toxigenic potential of *Aspergillus* isolates

The toxigenic capacity of *Aspergillus* isolates was evaluated using HPLC. Among the 24 *Aspergillus* isolates, Asp 16, Asp 17, Asp 20, and Asp 23 produced all four forms of AFG1, AFB1, AFB2, and AFG2 (Table 12; Figure 10 and 11). Isolates Asp 1, Asp 2, Asp 4, Asp 5, Asp 14, Asp 15, Asp 19, Asp 21, and Asp 24 produced only AFB1 and AFB2. None of the aflatoxin types were detected in isolate Asp 18. The highest amount of AFB1 (10250.78 ppb/g of agar plug) was recorded in isolate Asp 23 followed by Asp 20 (4246.584 ppb/g of agar plug). The AFB1 toxin levels ranged from 0.0 to 10,250.78 ppb, whereas the AFB2 levels ranged from 0.0 to 617.94 ppb. Asp 2, Asp 3,

Asp 5, Asp 6, Asp 7, Asp 9, Asp 10, Asp 11, Asp 13, Asp 18, and Asp 22 were identified as nontoxigenic *Aspergillus* isolates.

Four clusters were identified based on the heatmap. Asp 23 and Asp 20 formed a separate cluster that produced the highest quantity of AFB1. The third cluster comprised low-toxigenic or non-toxigenic isolates such as Asp 24, Asp 3, Asp 12, Asp 11, Asp 10, Asp 13, Asp 18, Asp 7, Asp 9, Asp 6, Asp 22, Asp 8, Asp 2, Asp 6, Asp 16, and Asp 21. The fourth cluster comprises Asp 19, Asp 14, Asp 4, Asp 16, Asp 1, and Asp 17 which produced all four types of aflatoxins or high levels of AFB1 and AFB2 (Figure 12).

DISCUSSION

Various biotic and abiotic factors affect maize productivity. Both internally and externally, they support various microbiomes. Mycotoxins, particularly aflatoxins released by *Aspergillus* spp., pose a serious threat to maize and its

products. Aflatoxin in food matrices has been detected and quantified using an array of analytical techniques, including TLC, HPLC with a fluorescent detector, and LCMS.³¹⁻³³ HPLC technique validation parameters such as sensitivity, linearity, and recovery rate should be within established limits to guarantee accurate measurement of aflatoxin contamination. Based on our experimental results, AFB1 and AFG1 multipoint calibration curves were linear from 1 ppb to 25 ppb, whereas AFB2 and AFG2 multipoint calibration curves had linearity ranging from 0.25 ppb to 6.25 ppb. This confirms that the chromatographic conditions used in this study were appropriate for the quantification of total aflatoxins. The linearity assessment of AFB1 in HPLC revealed a regression value of 0.9987, similarly to Kollia *et al.*³⁴ The LOD and LOQ under the chromatographic conditions in this experiment were identical to those reported by Lee *et al.*³⁵: LOD of 0.03 ppb and LOQ of 0.09, 0.04, 0.20 and 0.09 ppb for AFG1, AFB1, AFB2, and AFG2, respectively. According to Khan *et al.*,³⁶ the LOQ

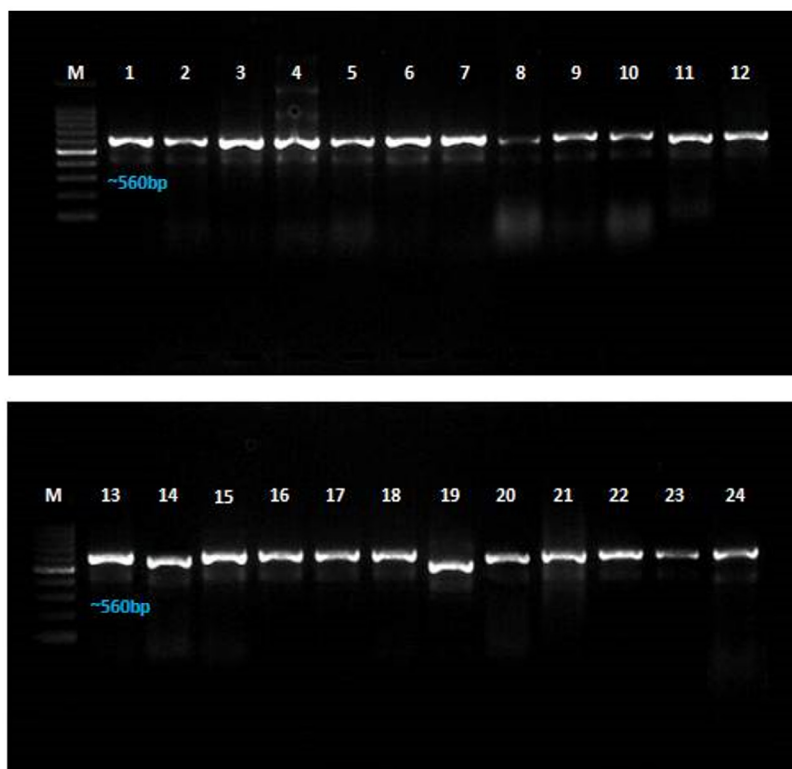


Figure 9. Molecular characterization of *Aspergillus* spp. isolated from maize

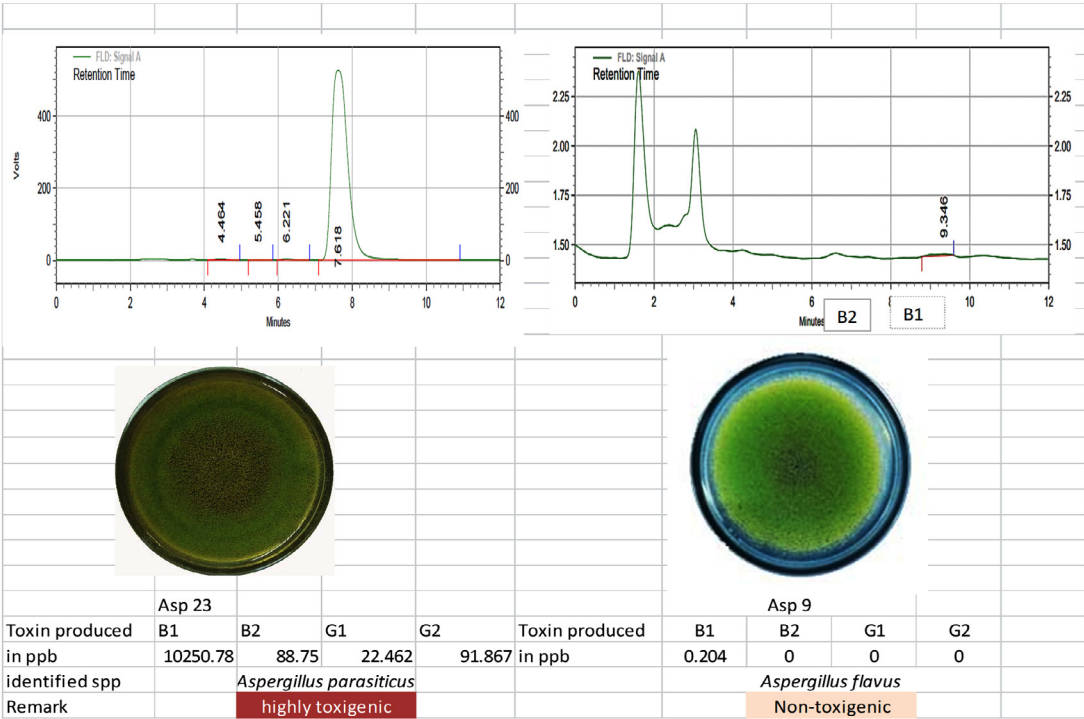


Figure 10. Toxicogenic *Aspergillus* (Asp 23) and non-toxicogenic *Aspergillus* (Asp9) Culture and its HPLC chromatogram

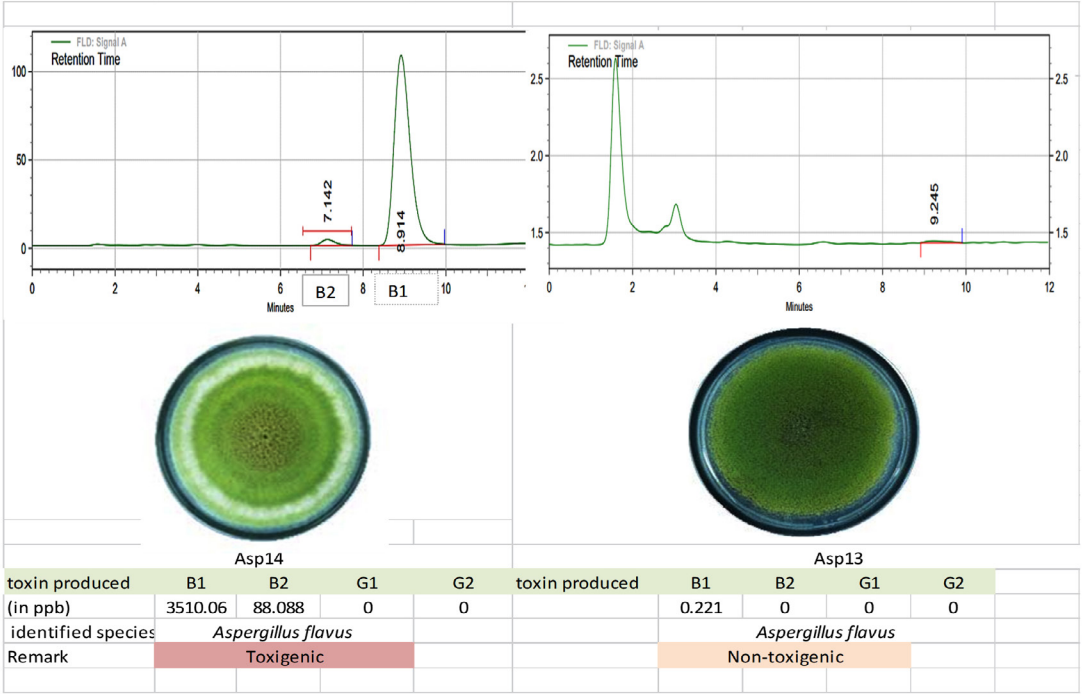


Figure 11. Toxicogenic *Aspergillus* (Asp 14) and non-toxicogenic *Aspergillus* (Asp 13) culture and its HPLC chromatogram

for AFB1 and AFB2 are 0.220 and 0.189 ppb, respectively, whereas the limits of detection were 0.072 and 0.062 ppb, respectively.

By manually spiking different known concentrations of AFB1 and AFB2, the aflatoxin extraction and column clean-up processes used in this study produced a mean recovery rate of 81.81% for AFB1 and 71.92% for AFB2. The AOAC (2002) recommended recovery limits of 70%-125% and 70%-110% for spiking levels of 10 ppb and 10-100 ppb, respectively, and these results fall within those ranges. Using immuno-affinity column clean-up techniques, Ozkan *et al.*³⁷ was able to achieve 89.3% and 85.95% recovery rates for AFB1 and AFB2, respectively, in non-contaminated red chilli pepper samples. Similarly, Choochuay *et al.*³⁸ used immuno-affinity column cleaning for the sample and obtained a recovery in the range of 82.50%-109.85% feed using HPLC with pre-column derivatization and fluorescence detection.

Aflatoxin contamination occurs in diverse array of regional crops and food products. Aflatoxins primarily contaminate food and feed such as corn, rice, spices, dried fruits, nuts, and figs.³⁹ The four major aflatoxins, AFB1, AFB2, AFG1, and AFG2, are commonly found in a wide range of food commodities and are synthesized by *Aspergillus* spp. Among various products, maize has the highest concentration of carcinogens that affect human health. Due to the global prevalence of maize products in traditional diets across the world, a large proportion of the population is highly exposed to aflatoxins.⁴⁰

The legal restrictions for aflatoxin may vary by nation and intended application, with thresholds as low as 4 parts per billion (ppb), 10 ppb in Tanzania, and 20 ppb in India (USFDA, 2000; European Commission, 2010). Two (6.25%) of the 32 samples of maize products tested for

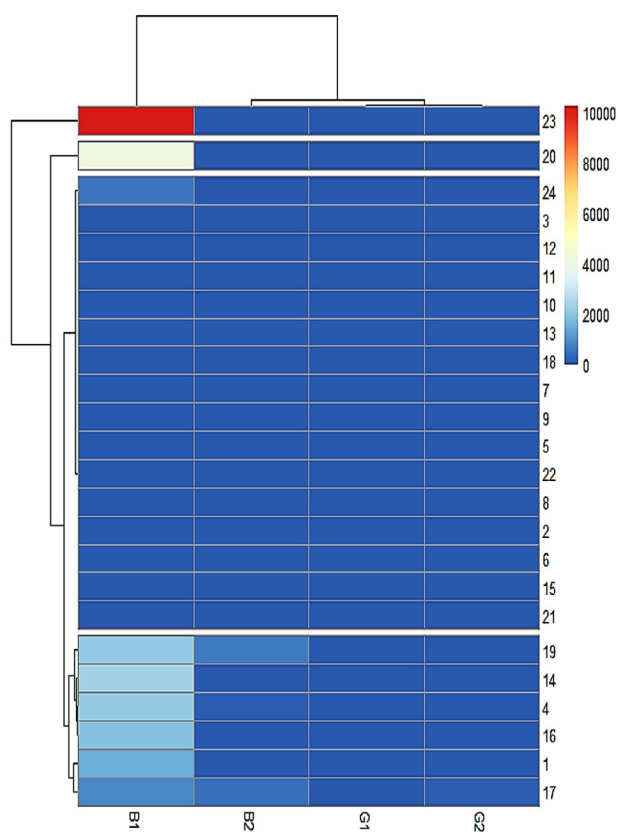


Figure 12. Heat map of aflatoxins (AFB1, AFB2, AFG1, AFG2) produced by *Aspergillus* isolates and dendrogram (white bar represent division of clusters)

aflatoxin contamination were free of the toxin, whereas the remaining samples had levels below the regulatory thresholds established by India. Ten samples (31.25%) exceeded the regulatory thresholds in Europe (>4 ppb). Additionally, a contamination range of 0.296-13.595 ppb was observed in 93.75% of samples. Our findings are in line with those of Krishnan *et al.*,⁴¹ who reported that out of 40 corn flour samples collected from Chennai, Tamil Nadu, 23 samples (57.5%) were positive for aflatoxin (50.0% were positive for AFB1, 32.5% for AFB2, 35.5% for AFG1, and 5% for AFG2). Overall, the test findings showed that corn flour had a very high level of AFB1 contamination. Comparable research was conducted in Rwanda by Matsiko *et al.*,⁴² and in rural Kenya by Nabwire *et al.*⁴³ on maize kernels and freshly milled maize flour, respectively. Indian exporters were probably furious after Canadian officials found aflatoxin in corn from India. This information is also likely to cause many countries that purchase grain that is "Grown in India" to become concerned.

Seed-borne fungi are primarily responsible for the declining quality and short shelf life of seeds and grains. This results in a variety of anomalies in the seeds, such as being discolored, harmed, shrunken, undersized, and rotting, which reduces germination. The worst illnesses are those that affect seeds because they damage the initial development of the seed and the plant as a whole. Similar to other crops, maize is plagued by several insect illnesses. It is recognized that the majority of illnesses are seed-borne in nature. The goal of this study was to critically estimate the quality of maize seeds used by Tamil Nadu farmers to determine their health. The mycoflora populations of individual seeds were recorded. Mycoflora diversity in individual seeds varied from 0 (no diversity) to high.

A total of 1152 mycoflora isolates from 10 distinct species were detected in our study, of which five were from the *Aspergillus* genus and accounted for 82% of the total mycoflora population. *Aspergillus* spp. were the dominant mycoflora, followed by *Fusarium* spp. (9%). *Penicillium* spp. (5%), *Rhizopus* spp. (2%), *Alternaria* spp. (1%), and *Macrophomina* (1%) were among the other identified mycoflora genera. This finding is comparable to that of El-Shanshoury *et al.*,⁴⁴ who isolated and identified

eight fungal taxa belonging to the *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Mucor*, *Rhizopus*, *Trichoderma*, and *Cladosporium* species. According to Ghiasian *et al.*,⁴⁵ *Fusarium* species exceed other fungal genera, with a dominance rate of 38.5%, followed by *Aspergillus* (8.7%), *Rhizopus* (4.8%), *Penicillium* (4.5%), and *Mucor* species (1.1%), and four other fungal genera. Tsedaley and Adugna⁴⁶ collected 110 fungi from three different types of maize, with *Aspergillus*, *Fusarium*, and *Penicillium* being the most prevalent. Kumar *et al.*¹⁹ reported fungal species belonging to the genera *Aspergillus*, *Alternaria*, *Bipolaris maydis*, *Botryosphaeria*, *Cephalosporium*, *Cladosporium*, *Diplodia*, *Fusarium*, *Nigrospora*, *Macrophomina*, *Mucor*, *Penicillium*, *Rhizoctonia*, and *Trichoderma* in maize seeds. Li and Liu⁴⁷ reported that the ten most prevalent fungal genera in the strawberry rhizosphere were *Aspergillus* (6.98%), *Acremonium* (4.10%), *Fusarium* (4.27%), *Chaetomium* (3.07%), and *Gibberella* (2.39%). We found that 82% of the mycoflora was composed of five distinct species of *Aspergillus*. The most prevalent *Aspergillus* species were *A. flavus* and *A. niger*. According to Gulbis *et al.*,⁴⁸ the most prevalent genera in Latvian maize grains are *Alternaria*, *Fusarium*, and *Penicillium* spp.

Alpha diversity assessment was used to evaluate community species diversity according to its richness and/or evenness. The Shannon index considers both the species richness and abundance. In our study, the Shannon index was <1.9 in all but two of the 15 locations, indicating that there was no mycoflora abundance. Our findings are comparable to those reported by Kumar *et al.*,¹⁹ in which *A. niger*, *A. flavus*, and *A. tamarii* were the most prevalent mycoflora in maize seeds, based on the Shannon-Weiner index of diversity (H), Simpson index of dominance (D), and evenness (E). Gu *et al.*⁴⁹ used the alpha diversity indices Chao 1 and Shannon to examine fungal populations in Chinese chives. Based on the findings, Ascomycota, Basidiomycota, and Mortierellomycota accounted for 59.34%-66.78%, 15.1%-29.32%, and 6.79%-11.57% of the total, respectively.

Beta diversity is another term used for sample dissimilarity. This quantifies the differences in the overall species composition between the two locations. In our study, a similarity in species

composition was observed between the locations. However, Wang *et al.*⁵⁰ studied bacterial and fungal species diversity in drylands, where beta diversity revealed species differences between all locations. The dominance of mycoflora species varies by location and is affected by several environmental factors including species variety, farming methods, and soil conditions. Liu *et al.*⁵¹ estimated diversity and revealed that fungal communities differed across sampling sites. Moreover, there was no variation in the heterogeneity of communities among the study sites. At the phylum level, the conditions were sufficiently comparable such that there was minimal variation in Ascomycota, Basidiomycota, and Mucoromycota dispersal across the sites.

A total of 24 *Aspergillus* species were isolated based on conidial coloration and purified on PDA medium. Houshyar-Fard *et al.*⁵² identified 226 *Aspergillus* section Flavi isolates in maize seeds. Molecular identification approaches based on internal transcribed regions are useful for identifying the species of fungal isolates. In this study, we used 18S rDNA sequence analysis to identify fungal isolates from maize seeds belonging to *Aspergillus* section Flavi. Previous research revealed that DNA sequence studies of ribosomal DNA ITS regions are crucial for identifying fungal contamination linked with food commodities.⁵³ Similarly, Zulkifli and Rasit⁵⁴ used ITS sequence analysis to identify *Aspergillus* isolated from maize grains. Our findings revealed that the majority of isolates from the *Aspergillus* section Flavi shared a significant level of sequence similarity with *A. flavus* and *A. parasiticus*. These outcomes are in agreement with earlier studies by Adjovi *et al.*,⁵⁵ who discovered that 16 of 20 isolates from cassava samples belonged to *A. flavus*.

We examined the aflatoxigenic capacity of randomly chosen isolates of *Aspergillus* section Flavi isolated from maize seeds using the agar plug technique and HPLC analysis to assess the probability of the isolates producing aflatoxins. The capacity of the tested strains to produce aflatoxins varied, with concentrations ranging from 0 to 10250.78 ppb/g. *Aspergillus* isolate Asp 23 was the most toxigenic, producing all four aflatoxins with a maximum production capacity of 10250.78 ppb/g of AFB1. Of the isolates examined, 95.8%

generated AFB1, 54.2% produced both AFB1 and AFB2, 16.7% produced all four aflatoxin types (AFB1, AFB2, AFG1, and AFG2), and 42% were nontoxigenic. Similarly, Rizzo *et al.*⁵⁶ found that 50% of the studied isolates from dried medicinal and aromatic herbs were toxigenic and generated aflatoxins at concentrations ranging from 10 to 2000 ng/g. Ritter *et al.*⁵⁷ isolated 30 *Aspergillus* isolates, 12 of which tested positive for aflatoxin production. Del Palacio *et al.*⁵⁸ used HPLC to identify aflatoxigenic isolates of *Aspergillus* section Flavi isolated from sorghum silage and discovered that 30% of the tested isolates produced aflatoxins.

Our results align with those of Yan *et al.*,⁵⁹ who evaluated the aflatoxigenic potential of randomly selected *A. flavus* and *A. parasiticus* strains isolated from herbal tea samples, revealing that *A. flavus* sourced from ginseng generated the highest aflatoxin levels (498 µg/kg). Similarly, our results are similar to those of Nikolic *et al.*,⁶⁰ where 50% of the tested isolates either failed to generate AFB2, AFG2, AFB1, and AFG1, or did so at very low concentrations.

CONCLUSION

The current study clearly demonstrated that most maize samples obtained from various places in Tamil Nadu were contaminated with different types of aflatoxins; nonetheless, the toxin levels remained below the Indian regulatory limits. In addition, the information gathered from this study is crucial for seed-certifying organizations to avoid losses after harvest. Because certain fungal genera, such as *Aspergillus*, *Fusarium*, *Penicillium*, produce mycotoxins, colonization by these genera makes them inappropriate for human consumption. This affects the health and economy of the population. The study revealed that the most common mycoflora found in the samples were *Fusarium*, *Aspergillus*, and *Penicillium*, all of which are known to generate mycotoxins. In addition, the toxigenic potential of *Aspergillus* isolates varied from one location or product to another, which may be due to improper handling and storage conditions. To reduce contamination, similar studies should be performed across diverse storage structures because the majority of contamination arises from inadequate storage and handling practices.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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