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DETECTION OF AFLATOXIN B_1, B_2, G_1 AND G_2 IN RHIZOSPHERE AND RHIZOSPLANE OF *(ZEA MAYS)* MAIZE PLANTS USING LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

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ABSTRACT
An easy method for the determination of aflatoxins B_1, B_2, G_1, and G_2 in rhizosphere and rhizosplane of *zea mays* by LC coupled to mass spectrometry had been developed. Aflatoxins B_1, B_2, G_1 and G_2 in maize plants by LC coupled to mass spectrometry had been developed. Aflatoxins were extracted with a mixture of methanol and water and then it was purified by solid – phase clean – up using a polymeric sorbent. The eluted extracts were injected into the chromatographic system using a reverse – phase C18 short column with an isocratic mobile phase composed of methanol-water (30:70). A single quadruple mass spectrometry using an electro spray ionization source operating in the positive mode was used to detect aflatoxins. The method permits the detection of Aflatoxins with a detection limit of 0.01-14.90µg/g.

KEY WORDS: Aflatoxin, HPLC, LC-MS

INTRODUCTION

Agriculture is the major source of livelihood in the world. In India agriculture provides employment to 70% of population and contributes to about 25% of national economy. The exports from agricultural commodities contribute to about 12% of the total national exports (Directorate of economic statistics, 2004). In Tamil Nadu, maize is an important millet, generally cultivated under rain fed conditions and accounting for 71.2 percent (1, 60,159 ha) of the total area under other millets. It is mainly grown in Perambalur, Dindigul, Coimbatore, Salem, Erode, Virudhunagar, Villupuram, Theni, Tiruchirapalli and Thirunelveli districts. In addition to human use, this millet is also used as cattle and poultry feed. Fungi are major disease causing agents in maize and produces mycotoxins during the pre and post harvest period. Mycotoxins are fungal metabolites that can contaminate agricultural products and threaten food safety. Among the mycotoxins, aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* are the major concern for maize growing farmers. Aflatoxins are of prime importance owing to their ill effects on human, animal and poultry health. Biological effects of aflatoxin can be subdivided into its toxicity, carcinogenicity, mutagenicity, and teratogenicity. Outbreak of acute aflatoxicosis from highly contaminated food has been documented in India, Kenya and Thailand (Council for Agriculture Science and Technology, 2003). Aflatoxin is not rapidly degraded by the soil microflora. It is possible that substantial amounts of toxin may be absorbed by the root systems of crops and translocated to the foliage and developing fruits. Accumulation of aflatoxin by plants could not only represent a health hazard to the consumer, but also seriously affect the growth, development, and productivity of plants. (Mertz *et al., 1980*). Aflotaxin is reported to inhibit seed germination (sehoenetal *et al* 1965), growth (crisan: 1973, El-Khadem *et al* 1966, Reiss: 1969), and chlorophyll development and to induce chromosomal abbreations in plants. The potential hazard of aflatoxins to human health has led to worldwide monitoring programs for the toxin in various commodities as well as regulatory actions by nearly all countries. Levels varying from zero tolerance to 50 ppb have been set for total permissible aflatoxin content in foods and feed (Patterson, 1983). Hence, rapid detection of aflatoxin from contaminated samples is necessary. Detection of aflatoxin are usually done by chromatographic techniques, immunological techniques and molecular techniques. Among these, Chromatographic techniques such as, Thin layer chromatography(TLC), High Performance Liquid Chromatogrphy (HPLC), Liquid Chromatography and Mass Spectrometry (LCMS) are most widely used techniques. This investigation was undertaken to find out whether aflatoxin is absorbed by the root system of the plant transported to the stem, leaf and fruit bodies of corn plants *(*Mertz *et al* 1980*)*

Experimental

Sample collection

Rhizosphere soil together with maize plants were collected from corn fields in 10 different villages of Perambalur district in Tamil Nadu, India. including Kaikalathur, Sirunilla, Noothapur, Nerkkunam, Perunilla, Ayanarpalayam, Pathangi, Pillankulam, Pasumbalur and V. kalathur. Samples were taken in five different places of each field and pooled together to give the representative soil sample. Sampling of rhizoshere soil. The samples collected from the fields were dried in shade by spreading on a clean sheet of paper after breaking the large chumps with a wooden mallet. The dried soil samples were

subsequently sieved through a 2mm sieve. The material passing through the sieve was collected and stored in a clean container with proper labeling for laboratory analysis.

Determination of moisture content

A small amount of pre weighed soil sample transferred to the weighed Petri plate. This was kept in an oven at 105°C for 1 h. The sample containing Petri plate were removed from oven and weighed. The loss of weight was calculated using the following formula, and expressed as the moisture content in percentage.

Determination of pH

Two grams of air dried soil was transferred to a clean 10 ml beaker and 2 ml distilled water was added. The content were stirred using a glass rod and was allowed to stand for half an hour. Soil suspension was again stirred just before taking the reading. The electrode was immersed in the beaker containing soil water suspension. The pH meter reading was recorded for all the soil samples.

Isolation of *aspergillus* species

Surface sterilization of the maize plants was done with 70% ethanol followed by several washes with sterile distilled water. They were homogenized by grinding with 2-3 ml sterile distilled water using mortar and pestle and one ml of plant extract was mixed with 100 ml of sterile saline (0.85%). Individual soil samples were similarly homogenized by 1g of sample in 100 ml of sterile saline (0.85%). Serial dilutions of homogenized plant and soil samples were done and appropriate dilutions i.e. 10^5, 10^6 and 10^7 in aliquots of 0.1 ml were plated in triplicate on pre poured *Aspergillus* differential media (Hi-media laboratories Ltd. Mumbai, India) plates. The plates were incubated at 28±2°C for 3-5 days.

Extraction of Aflatoxin from soil

Five grams of rhizosphere soil samples were taken in a stopper conical flask to which 50 ml of chloroform and few ml of distilled water were added. The samples were extracted for 1 h at room temperature on an orbital shaker set at 240 rpm. After extraction the samples were filtered with what man no 1 filter paper and filtrates transferred to a separating funnel. Water (one and half volume of chloroform) was added to the filtrate. Filtrate was shaken well and allowed to stand until two phases separated. The lower chloroform layer containing aflatoxin was separated. 10 g of anhydrous sodium sulphate was added to absorb excess water.

Extraction of aflatoxins from stem, leaf, cob and root

10g of each sample (stem, leaf, cob and root) was homogenized by grinding with 50 ml chloroform and few ml of distilled water using mortar and pestle. The homogenized samples were transferred to stopper conical flask and extracted for 1h at room temperature with vigorous shaking. The extracts were subsequently filtered with what man No.1 filter paper. To the filtered extract stem, leaf and cob 100 ml of 70% acetone and 7 ml of 20% lead acetate was added to remove chlorophylls, lipids and fatty acids. Filtrates were evaporated at reduced pressure using Buchi rotavapour. To the evaporated sample 10 ml of acetone, 20 ml of saturated ammonium sulphate (20%), and 10ml of hexane was added and transferred to a separating funnel. After removing lower layer, 2 volume of 25 ml chloroform was added and mixed well. Lower layer with chloroform and aflatoxin were collected. (Mertz *et al.,* 1980)

Column chromatography for aflatoxin

Final chloroform extract containing aflatoxin was further cleaned up by column chromatography using 10 g of pre activated silica gel (60-200 mesh). The chloroform extract (50 ml) was layered on a silica gel column (23 **X** 290 mm) and eluted first with 150 mL of hexane, followed by 150 ml of diethyl ether. Aflatoxin was finally recovered in 150 ml of chloroform/methanol (97:3).

Thin Layer Chromotography (TLC)

TLC plates were prepared with the help of a TLC spreader (CAMAG). The 20 X 20 cm glass plates were wiped with hexane to remove grease and fatty substances. 13 gm per plate of silica gel was taken and mixed with water at the ratio of 1:2. The mixture was vigorously shaken for 1-2 minutes and the prepared slurry was poured into the applicator and the TLC spreader was switched on for immediate coating of TLC plates with 300 mm thick layer of silica gel. The plates were allowed to dry for 1-2 hours at room temperature. Plates were activated for 1 hour at 100° C in a hot air oven and stored in a desiccating cabinet until use.Standard stock solutions of AFB1, AFB2, AFG1 and AFG2 were prepared by dissolving 1mg standard (Sigma, Sigma Chemical Co - St. Louis, MO) in 1mL benzene: acetonitrile (98:2, v/v) and made up to 100 ml in a volumetric flask using the solvent system, this was further diluted to 10 times to get a working solutions of concentration 1 µg/ml. The working solutions were stored under refrigerated conditions. All samples were spotted on TLC plates (5-10 µl) along with Aflatoxin standards B_1, B_2, G_1 and G_2. The developing solvent system (Acetone: chloroform (1:9)] was prepared fresh and TLC plates were developed in an equilibrated tank. The spots were visualized at 360 nm in UV chamber. (Camag U.V Betrachter, switerzaland)

Solid-phase clean up

Each sample was subjected to preparatory TLC. Sample spots were scrapped off from the TLC plates and used for clean up. 5 ml of chloroform was added to the powder samples and centrifuged for 5 minutes at 1000 rpm. Supernatant was taken and evaporated under nitrogen atmosphere. 50 µL of methanol was added to the residues. The samples were stored at 4°C until analysis by HPLC.

High Performance Liquid Chromotography (HPLC)

HPLC characterizations of samples were done; using Shimadzu Liquid Chromatography LC-10A (shimadzu, Japan) fitted with supelco C_{18}, 5 µm, and 25cm X 4.6 mm column. The mobile phase was water- acetonitrile-methanol (60:30:10). The mobile phase was delivered at the flow rate of 1 ml/min with an isocratic mode. 20 µL of samples were injected and the elution profile was detected by a UV detector at 365 nm.

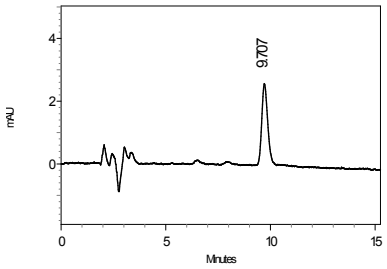

Fig: 1 Standard Aflatoxin B_1

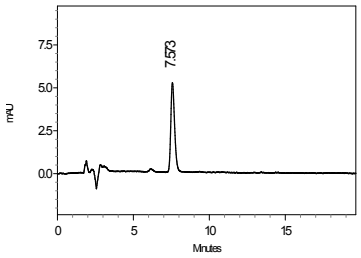

Fig: 2 Standard Aflatoxin B_2

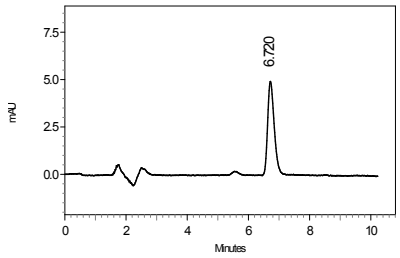

Fig:3 Standard Aflatoxin G_1

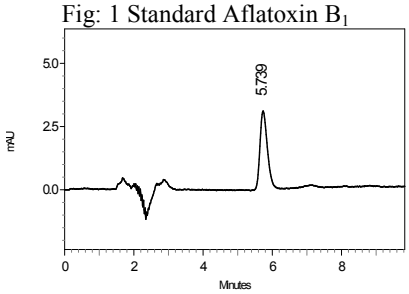

Fig: 4 Standard Aflatoxin G_2

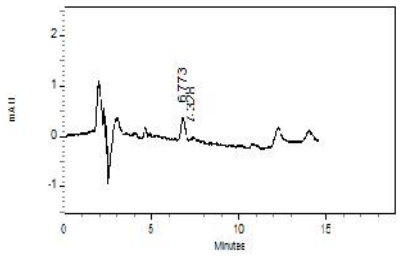

Fig: 5 HPLC profile of Aflatoxin in soil

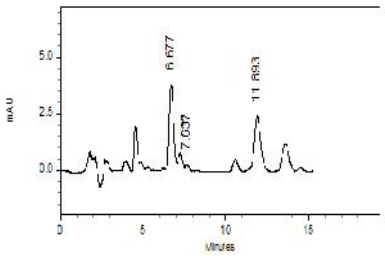

Fig: 6 HPLC profile of Aflatoxin in root

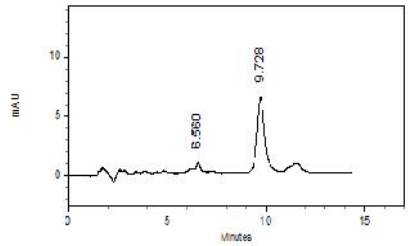

Fig: 7 HPLC profile of Aflatoxin in stem

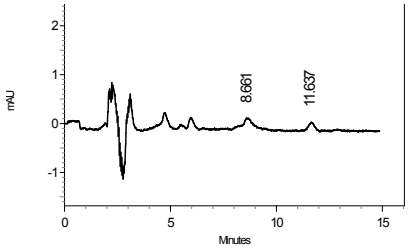

Fig: 8 HPLC profile of Aflatoxin in leaf

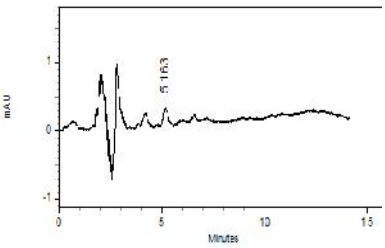

Fig: 9 HPLC profile of Aflatoxin in cob

HPLC graphs showing aflatoxin level in soil, root, stem, leaf and cob

Liquid Chromatography Mass Spectrophtometry (LC - MS) Determination

Liquid chromatography

The determination and confirmation of aflatoxin were determined in Liquid Chromatography Mass Spectrophotometer (LC-MS) instrument consist with on a Li Chrocart C18 short column (30mm × 4 mm, 3m) (Merck) at 30 ◦C, with an isocratic mobile phase of methanol–water (30:70) at a flow rate of 1 ml/min. The injection volume was 10µl.

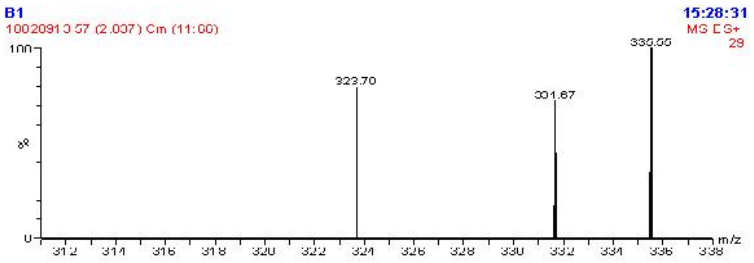

Fig: 10a LCMS profile of standard AFB_1

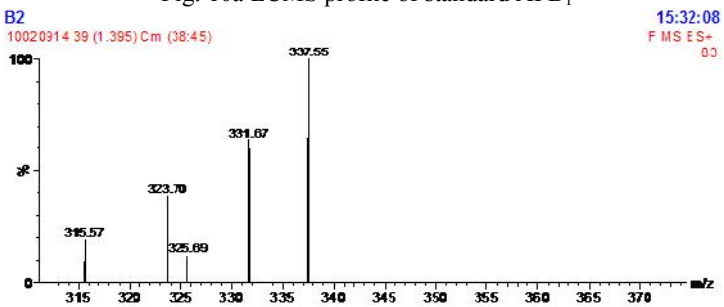

Fig: 10b LCMS profile of standard AFB_2

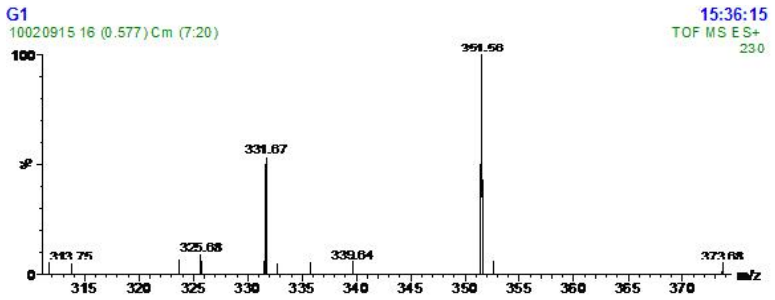

Fig: 10c LCMS profile of standard AFG_1

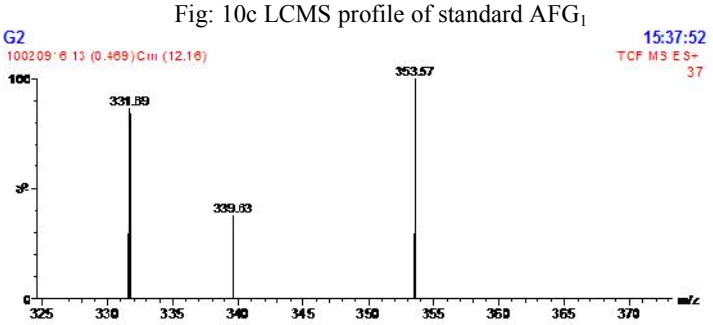

Fig: 10d LCMS profile of standard AFG_2

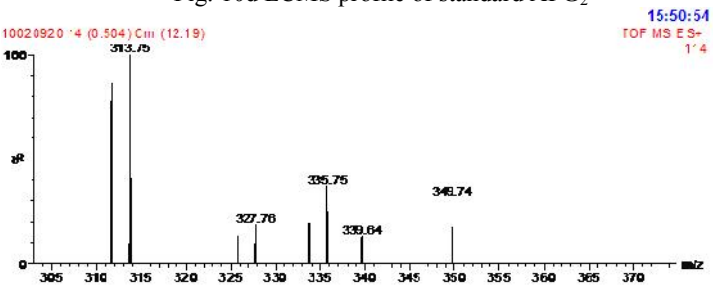

Fig: 10e LC-MS profile of Aflatoxin in soil

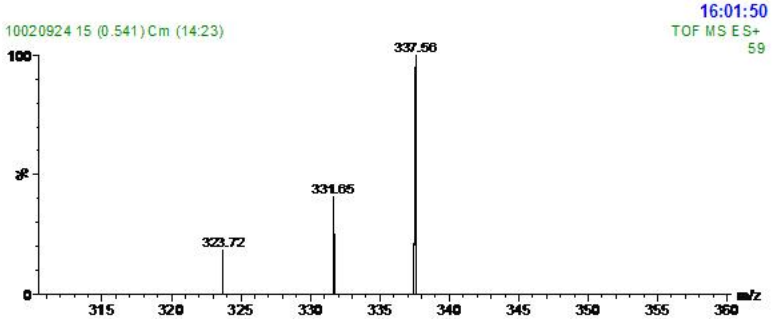

Fig: 10f LC-MS profile of Aflatoxin in Root

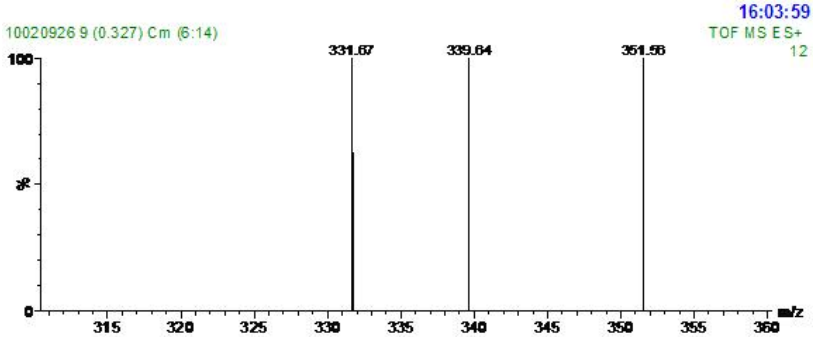

Fig: 10g LC-MS profile of Aflatoxin in stem

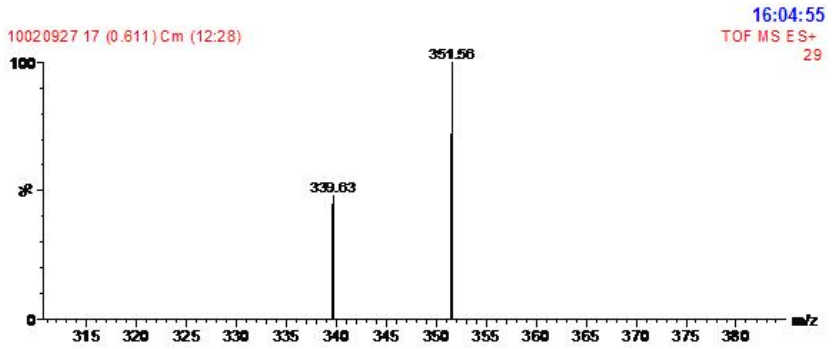

Fig: 10h LC-MS profile of Aflatoxin in leaf

LC-MS graphs showing Aflatoxin level in soil, root, stem and leaf

Mass spectrometry (ESI–MS)

Mass spectrometry was performed on a ZMD (Waters) single–quadruple equipped with an electro spray ionization (ESI) source and operating in the positive ion mode. The parameters used for the mass spectrometer in all experiments were: capillary voltage 3.0 kV, source block temperature 100 ◦C, evaporation temperature 350 ◦C, solvent gas 475 l/h, cone gas 50 l/h, low mass resolution 15, high mass resolution 15, ion energy 0.5, extractor 7, Rf lens 0.5 and electron multiplier voltage 650. Cone voltage for aflatoxins B1, G1 and G2 was 20V and for B2 it was 40V. The ions monitored in single ion recording were the protonated molecule [*M* + H]+ at *m*/*z* 313.2 for B1, 329.2 for G1, 315.2 for B2 and 331.2 for G2. Sodium adducts [*M* + Na] + at *m*/*z* 335.2, 351.2, 337.2 and 353.2 were also monitored.

RESULTS

Moisture content

The moisture content of all the soil samples collected from different region of perambalur district in Tamil Nadu was fairly high (Table 1).Seven out of ten samples recorded moisture content of 15-27.5%. The rest of three showed 11.1-12.8%.

pH

The pH of the all the samples were found between 7.0-7.24.

Levels of aflatoxin:

All samples were quantitatively analyzed using TLC and quantified using HPLC. Out of 10 soil samples, 3 showed the presence of aflatoxins (B_1, B_2, G_1, and G_2). 7 out of 10 samples showed both aflatoxin G_1, G_2. Out of which G_2 has a highest range (0.06-1.45 µg/g.) in soil level.

TABLE 1 Determination of Moisture content

S.No	Place	Moisture content (%)
1	Kaikalathur	19.79
2	Sirunilla	26.64
3	Noothappur	27.55
4	Nerkunam	18.89
5	Perunilla	12.80
6	AyyanarPallayam	18.66
7	Pathangi	21.43
8	Pillangulam	21.62
9	Pasumbalur	12.5
10	V.Kalathur	11.14

TABLE 2: Aflatoxin concentration in maize soil samples collected from different places in Perambalur District

S.No	Place	Aflatoxin concentration(µg/g)			
		B_1	B_2	G_1	G_2
1	Kaikalathur	-	0.11	0.92	-
2	Sirunilla	-	-	-	0.11
3	Noothappur	-	-	-	1.45
4	Nerkunam	-	-	-	0.37
5	Perunilla	-	-	0.01	0.23
6	AyyanarPallayam	0.01	-	0.03	-
7	Pathangi	0.01	0.02	0.12	0.31
8	Pillangulam	0.1	-	-	-
9	Pasumbalur	0.04	0.13	0.01	0.01
10	V.Kalathur	0.01	0.04	0.01	0.06

TABLE 3: Aflatoxin concentration of Root samples collected from different places in Perambalur District

S.No	Place	Moisture content in percentage (%)			
		B_1	B_2	G_1	G_2
1	Kaikalathur	-	0.27	1.08	-
2	Sirunilla	0.89	0.94	-	-
3	Noothappur	-	-	1.68	2.11
4	Nerkunam	-	0.64	-	0.89
5	Perunilla	4.12	-	2.46	4.11
6	AyyanarPallayam	-	-	3.05	6.00
7	Pathangi	-	-	0.32	10.66
8	Pillangulam	0.03	-	-	-
9	Pasumbalur	0.06	0.04	0.09	0.86
10	V.Kalathur	0.01	0.05	0.10	0.13

TABLE 4: Aflatoxin concentration of stem samples collected from different places in Perambalur District

S.No	Place	Aflatoxin concentration(µg/g)			
		B_1	B_2	G_1	G_2
1	Kaikalathur	0.68	0.07	0.33	0.1
2	Sirunilla	0.38	-	-	1.30
3	Noothappur	0.16	0.10	0.05	0.15
4	Nerkunam	-	-	0.18	0.08
5	Perunilla	-	-	0.54	0.52
6	Pathangi	1.0	0.02	0.02	0.03
7	Pillangulam	0.01	-	0.03	0.05
8	Pasumbalur	0.01	0.05	0.01	14.90
9	V.Kalathur	0.01	-	-	0.03

The results are shown in Table 2. All maize plants were checked for aflatoxin. Root, stem, leaf and cob were analyzed separately. All 10 root samples showed positive result for aflatoxin. Out of 10 samples 2 contains all four aflatoxin (B_1, B_2, G_1, and G_2). 7 out of 10 samples showed B_1, and B2. Among the 4 samples G_2 was predominant (0.01-10.66µg/g.). The results are shown in Table 3. In case of stem, all samples showed positive results for aflatoxin, but decreased level in the concentration of toxin was observed. 3 out of 10 samples showed all the 4 aflatoxin (B_1, B_2, G_1, and G_2). 7 out of 10 showed B_1, G_1, and G_2. Here also G2 was highest at a range (0.1-14.90µg/g.). The results are shown in Table 4. All leaves and comb samples showed the presence of aflatoxins. Among them aflatoxin B_1 was highest, showing a range between 0.05-1.7 µg/g, The results are shown in Table 5 in case of leaves and,0.01- 0.1µg/g in case of cobs. The results are shown in Table 6.

TABLE 5: Aflatoxin concentration of leaf samples collected from different places in Perambalur District

S.No	Place	Aflatoxin concentration(µg/g)			
		B_1	B_2	G_1	G_2
1	Perunilla	-	0.01	0.03	-
2	Pathangi	1.7	-	-	-
3	V.Kalathur	0.1	0.01	0.03	0.06

TABLE 6: Aflatoxin concentration of cob samples collected from different places in Perambalur District

S.No	Place	Aflatoxin concentration(µg/g)			
		B_1	B_2	G_1	G_2
1	Sirunilla	-	-	-	0.02
2	Perunilla	-	0.02	0.01	-
3	V.Kalathur	0.03	0.01	0.05	0.01

Confirmation of aflatoxins

All extracted aflatoxins were confirmed using Liquid Chromatography Mass Spectrophotometer (LC-MS) under standard conditions.

Fungal contamination

The extent of fungal contamination in 10 samples, based on agar plating method in Aspergillus Differential Media (ADM), is presented in Table 7. This revealed high incidence of aflatoxin producing *Aspergillus flavus* in rhizosphere soil. But the presence of this fungus in root samples was less when compared to soil. No *Aspergillus flavus* was observed in all the stem samples carried out in this study.

TABLE 7: Determination of Fungal contamination

S.No	Place	Number of Aspergillus sps (CFU/g)		
		Soil	Root	Stem
1	Kaikalathur	$8x10^7$	$5x10^7$	-
2	Sirunilla	$4x10^8$	$1x10^8$	-
3	Noothappur	$5x10^7$	$2x10^7$	-
4	Nerkunam	$3x10^7$	$1x10^7$	-
5	Perunilla	$2x10^7$	$2x10^7$	-
6	AyyanarPallayam	$5x10^7$	$1x10^7$	-
7	Pathangi	$2x10^7$	$2x10^7$	-
8	Pillangulam	$3x10^7$	$1x10^7$	-
9	Pasumbalur	$4x10^7$	$2x10^7$	-
10	V.Kalathur	$6x10^8$	$3x10^7$	-

DISCUSSION

Aflatoxin B_1, B2, G_1 and G_2 are toxic fungal secondary metabolities produced by *Aspergillus flavus* and *A. parasiticus*. These mycotoxins cause many health disorders in humans as well as in animals. A great deal of research in this area has been continuning to find out the remedy to detoxify the various aflatoxins. The guide line level aflatoxin in foods for human consumption is 20 ppb (Bullerman, 1979). The levels of toxin found in agriculture commodities are so high, farmers usually dispose them into soil as fertilizers. Furthermore, when standing crops are severely contaminated with aflatoxin caused by *A. flavus* infection, the same crop along with their grains are incorporated back into the soil mainly as fertilizers. In the present investigation, aflatoxin levels of 10 samples of rhizosphere soil, root, stem, leaf and cob of maize plants collected from different agro climatic regions of Perambalur district Tamil Nadu were analyzed. The maize plants collected in these different ecological conditions showed high levels of toxins. This was confirmed by HPLC and LCMS method. TLC method was also carried out for qualitative determination of aflatoxin in these soil materials prior to HPLC and LCMS methods of detection. There is a serious concern as the aflatoxins level detected were much higher than the guide line levels specified. The high incidence of toxins was found in the samples where the moisture content of soil was found to be high (more than 15%). The CFU count was also found to be high in these samples. This clearly indicates that, moisture content of soil is a limiting factor in the growth of *A. flavus* in soil. Presence of toxin, despite the non prevalence of *A. flavus* in stem indicates that the toxin was absorbed by the root and transported to the stem region.

Our experiment also revealed that absence of aflatoxin from cob suggests that the toxins from soil has not reached the cob portion of the plant The absorption of toxin could have deleterious effect on the growth, development and production of plants if toxins are detected in cobs.. More harmful effects of aflatoxin contaminated soils may be through changes in rhizosphere microorganisms that are associated with plants growth (Subramanyam and Rao, 1978). The presence of these toxins in plants causes undesirable effects on grazing animals and cause acute toxicity or decreased productivity in animals and acute intoxication in humans (Dawson 1991). They are more important to humans since many

toxins are carcinogenic. Primary liver cancer is one of the leading causes of cancer mortality in Asia. Therefore it is important to keep human exposure to aflatoxin as least as possible.

CONCLUSION

The result of the preliminary study indicates a vast and through research is needed to determine the longevity of aflatoxins in different types of soil. Until more is known of the aflatoxin in the environment, some restraint should be exercised in the disposal of contaminated commodities by introducing them into agricultural soils. This study for a shorter duration could be able to find out aflatoxin levels in a small area of agricultural land in Perambalur district of Tamilnadu. Further research can be undertaken to study the mechanism of formation of aflatoxins, its surveillance in rhizosphere and finally remediation, using modern biological tools.

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