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ISOLATION OF TOXIGENIC MYCOFLORA FROM POTENTIAL EDIBLE SOURCES AND STUDY OF THE SUSCEPTIBILITY TO PRODUCE AFLATOXIN.

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ABSTRACT

In the current study mycoflora have been isolated from potential edible sources; Cauliflower, Green peas, Raisins, Groundnut and Rice collected from different retailers in Vellore, TN, India and were further tested for producing mycotoxins. A total of 15 isolates belonging to 8 different species were isolated. Among them *Aspergillus* was predominant. The *Aspergillus* isolates were further screened for their toxigenicity. Out of 9 isolates *2* were found positive producing aflatoxins. Maximum contaminants were obtained from Cauliflower but none of them was toxigenic. Further, to find if Cauliflower is resistant to aflatoxin production, studies were carried out by inoculating toxigenic strain on it. Aflatoxin production was negligible in Cauliflower in comparison to Rice. None of the edibles showed natural contamination of aflatoxin. This study shows that it is not necessary if toxigenic fungi are present, there will be toxic level of mycotoxin in naturally occurring food materials.

KEYWORDS: **Mycotoxin, Aflatoxin, *Aspergillus flavus*, Fungal contamination, Thin layer chromatography**

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INTRODUCTION

Contamination of various foodstuffs and agricultural commodities is a major concern in the tropical and subtropical area, where the environmental conditions are favourable for the growth of fungi and toxin production. Mycotoxins are low molecular weight compounds that do not produce immediate symptoms unlike the bacterial toxins which are instant in action since the immune system recognises them as antigen and produce antibody mediated reaction [1]. Mycotoxins are secondary metabolites of fungal origin. The disease or effect caused by ingestion of food containing toxin is known as mycotoxicosis [2]. Though its impact on mankind are known since the origin of organised crop cultivation but the scientific study started only in 1960 when a large number of turkey poults died due to consumption of contaminated groundnut meal imported from Brazil [3,4]. The common mycotoxins known are aflatoxin, ochratoxin and fumonisins [5,6,7,8]. They are most commonly produced by *Aspergillus*, *Penicllium* and *Fusarium* [9, 10]. The fungi *A. flavus*, *A. parasiticus* and *A. nominus* are the common producers of aflatoxin. B_1, B2, G_1 and G_2. These are the four major aflatoxins whereas aflatoxin M_1 and M_2 are the hydroxylated metabolites of aflatoxin B_1 and B_2 [12]. Ochratoxin is a mycotoxin that exists in three secondary metabolite forms A, B and C and is produced by *Aspergillus ochraceus and Aspergillus wentii* [13]. Citrinin is a toxin that was first isolated from *Penicillium citrinum* and several species of *Aspergillus*. Aflatoxin B_2 is clinically more significant among all other fungal toxins since it is most potent mutagenic and carcinogenic [14, 15]. The metabolism of aflatoxin also plays a pivotal role in determining the toxicity. Aflatoxin B_1 might be oxidised by liver microsomal oxygenases to aflatoxin M_1, aflatoxin Q_1, aflatoxin P_1, aflatoxin B_{2a} and may be reduced by cytoplasmic reductases to aflatoxicol H_1. The health hazard of the toxin depends mainly on the geographical location as well the environmental factors prevailing in the area [16]. There are possible effects of aflatoxin on Kwashiorkor and Reye's syndrome. Chronic aflatoxicosis is seen in case of liver cancer which might be due to mutation of the tumour suppressor gene, p53 [17].

Plant based edible stuffs are very susceptible to fungal contamination[9]. The formation of aflatoxin on a given substrate or food crop involves basically an aflatoxigenic mould strain and suitable intrinsic as well as extrinsic factors. Relative humidity and moisture, growth and maturity of crop, crop variety, effect of pH and nutrients might be some of the intrinsic factors. The extrinsic factors may be temperature and time, aeration and usage of some chemical pesticides and plant extracts [18, 19]. Food based mycotoxins were studied extensively worldwide. Environmental conditions like high temperature; humidity promotes fungal proliferation and cause contamination of food [20]. Mycotoxins greatly resist thermal decomposition or being broken down during digestion so they can remain in the food [21]. Even temperature treatment such as cooking and freezing do not destroy mycotoxins [22]. In the current study different fungal species have been isolated from edible sources and tested for mycotoxins production. The Cauliflower and Rice have been used as substrate to test whether they support the growth of mycotoxin producing fungi. Also the different edibles have been directly tested for the presence of mycotoxin.

MATERIALS AND METHODS

Sample: Samples of five different edibles namely Groundnut, Raisins, Cauliflower, Greenpeas and Rice were collected from retailers in Vellore, TN, India. These are brought to the laboratory and immediately analysed.

Media: Potato dextrose agar (PDA: potato infusion-200gm/L, dextrose -20gm/L, agar -15gm/L) and Chloramphenicol powder, Yeast Extract Sucrose (YES) Broth (HiMedia Laboratories Pvt. Ltd, Mumbai) (Yeast extract-20 gm/L, Sucrose-150 gm/L), Ethyl Acetate (HiMedia Laboratories Pvt. Ltd, Mumbai). PDA

is a general media for the growth of the fungi was used for isolation and sub culturing of the fungi. YES was used to culture the pure isolates for mycotoxins production. The solvents used were Toluene (Thomas baker chemical Pvt. Ltd, Mumbai*),* Formic acid and chloroform (Rankem, RFCL Ltd, New Delhi).

(i) Isolation of mycoflora from samples
All the samples were surface sterilized by thoroughly washing with autoclaved distilled water. Cauliflower (small pieces), raisins, greenpeas and groundnuts were directly placed on PDA media with chloramphenicol in petriplates and incubated for 5-7 days at room temperature. Colonies appeared were further sub-cultured in new plates. The sub-culturing was repeated for 3-4 times until pure culture was obtained. Colony morphology and microscopic studies were carried out.

(ii) Screening of toxigenic fungi
The Fungi isolated were inoculated in the 5 ml of Yeast extract sucrose media (2%yeast extract and 20% sucrose) and incubated at (26± 2◦c) for 7 days. After incubation the solid mass of fungal culture was removed by filtration through Whatman No.1 filter paper and discarded [23]. Four ml of broth from each culture was transferred to different test tubes and extracted with equal volumes of chloroform. After vortexing vigorously the test tubes were kept on stand for 15 min as such to separate the two phases; organic and aqueous. The aqueous phase was removed by micropipette and the organic phase was used for further analysis [24].

(iii) Detection
Thin layer chromatography of mycotoxins (TLC) TLC was performed in all instances on pre-coated silica gel G glass plates. The solvent used is toluene, ethyl acetate and formic acid (6:3:1)[29]. 20µl of each of the analyte was spotted on TLC plate and then air dried. The plate was kept into the TLC chamber saturated with solvent. TLC was performed along with standard aflatoxin B_1 and B_2 to analyse the extracts. Further, the spiking of samples with standard was done to confirm the presence of aflatoxin spots on TLC. Plates were observed under UV illuminator at 365 nm. UV spectrophotometer
The concentration of aflatoxin was measured by UVspectrophotometer (Shimadzu 1800) at 365 nm. The amount was calculated by comparing with the standard graph for aflatoxin B_1.

(iv)Aflatoxin production in Cauliflower and Rice
Twenty grams of Rice (moistened with 2 ml of water) and Cauliflower (each surface sterilized using antibiotic) were inoculated with *Aspergillus flavus*. The inoculated samples were incubated at 28°C for 0 to 10 days. Every alternate day samples were removed and extracted with chloroform. TLC of extracts was performed to detect the presence of aflatoxin and quantification was carried by UV spectrophotometer.

(v) Screening of the samples for natural aflatoxin contamination
Five grams of each sample (greenpeas, cauliflower, raisin, groundnut and rice) was crushed in mortar pestle separately and transferred to beaker containing 5 ml of chloroform with continuous shaking by wrist action for 15 minutes with the lid being covered. Chloroform was filtered with Whatmann filter paper 1. The extract was concentrated and applied on TLC plates for aflatoxin detection.

RESULTS AND DISCUSSION

All the samples of edibles were surface sterilized and incubated on the PDA medium showed heavy fungal growth (Fig. 1) Based on the morphological characteristics and colony appearance a total of 15 isolates belonging to 8 different species were isolated (Table1). Among these the *Aspergillus* is predominant followed by *Fusarium*. Out of 15 isolates, 6 were isolated from Cauliflower. Cauliflower seems to be most contaminated and supporting growth of various fungi. Raisins are least contaminated as only two species were isolated from them. As the water activity is very low in Raisins due to high sugar content, it may not be very suitable substrate

for fungal growth [25]. However, there are reports of aflatoxin contamination in Raisins [26]. Five isolates were obtained from Greenpeas and 2 from Groundnuts.

Figure 1
PDA plates showing fungal growth from different edible samples after incubation for 5-7days

Table 1
Fungal contaminants isolated from edible samples collected from retail market.

SAMPLE	NO. OF ISOLATED FUNGI	NAME OF THE ISOLATED FUNGI
Cauliflower	6 species	*Aspergillus niger, A. flavus, Alternaria sps., Rhizopus, Fusarium sps, Unidentified,*
Groundnut	2 species	*Aspergillus niger, A. flavus,*
Greenpeas	5 species	*Aspergillus niger, A. flavus, A. ochraceus, Fusarium, Mucor sp.*
Raisins	2 species	*Aspergillus niger, A. flavus*

The isolated *Aspergillus* species were screened for their toxigenicity in terms of aflatoxin production. These isolates were inoculated in YES medium which is suitable medium for the aflatoxin production. Out of 9 *Aspergillus* isolates screened, 2 were toxigenic, obtained from Greenpeas showed high aflatoxin production (Fig. 2). These isolates were identified as *Aspergillus flavus.* The identification was further confirmed by the Agharkar Institute, Pune, India. The toxigenic strain X2 produced both aflatoxin B_1 and B_2 while the X1 produced only B_1. The quantity of B_1 produced was much lesser in comparison to X2. None of the *Aspergillus* samples isolated from Cauliflower were found toxigenic. Though the Cauliflower was highly contaminated and maximum fungal isolates were obtained from this sample but the isolates were not toxigenic. To find if the Cauliflower is resistant to the aflatoxin production, toxigenic strain of *Aspergillus* was inoculated on the Cauliflower and incubated in suitable condition. Rice is known substrate for aflatoxin production [27]; hence it is used as control and incubated in same conditions. After each incubation period samples were analysed. As reported [27] the rice shows aflatoxin production from 2nd day of incubation and it gradually increased up to 10th day. In the Cauliflower though fungi grew but the aflatoxin production was below the detection limit of TLC as the fluorescent spot corresponding to aflatoxin B_1 could not be observed (Fig. 3 and 4). The UV spectrophotometer data showed that Rice supported the aflatoxin production up to 9 µg/g of Rice by 10th day of incubation. However, in Cauliflower only 0.6 µg/g toxin was noted (Fig. 5). These results show that cauliflower naturally prevents the aflatoxin production. The contamination of toxigenic fungi may occur from the soil during growth or harvesting or from environment during storage

and sale [28], but toxin production is inhibited. To find whether the edibles used in the present experiment are naturally contaminated with aflatoxin, samples were analysed. Randomly, 5 g of each sample (in triplicate) was taken after nicely mixing, extracted with chloroform and analysed by TLC. None of the sample showed the aflatoxin spot on TLC.

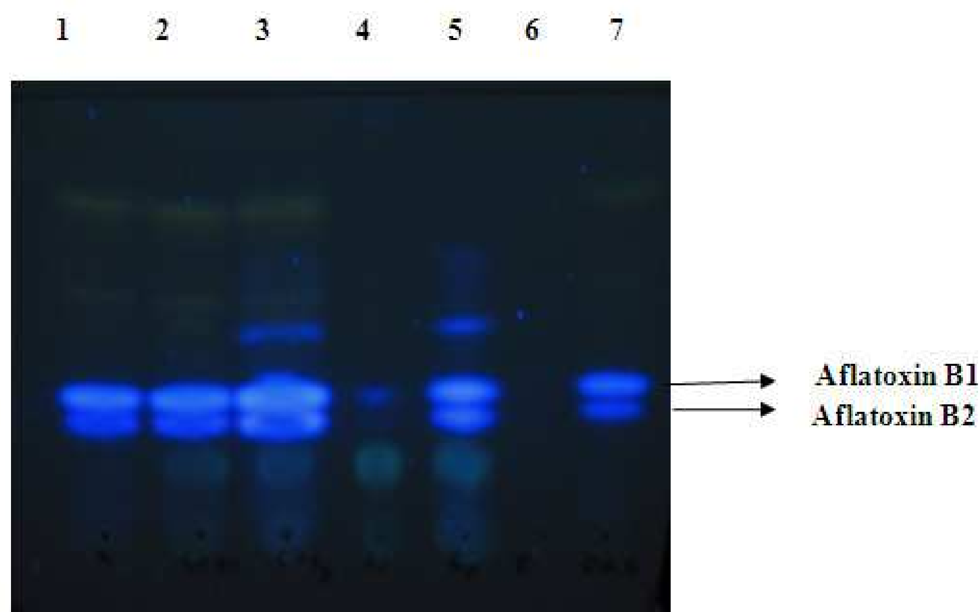
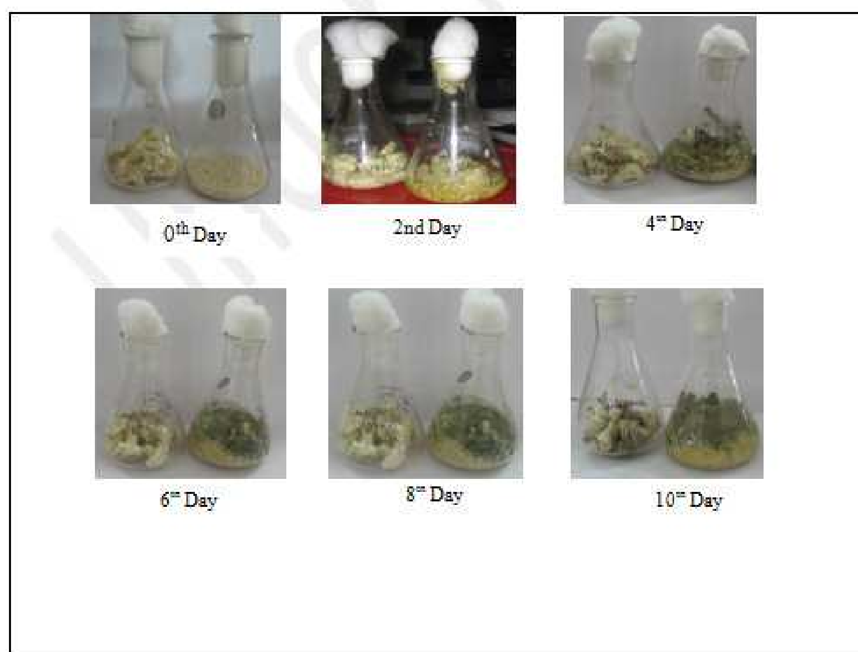


Figure 2

Thin Layer Chromatogram of choloform extracts of Aspergillus culture in YES medium along with standard aflatoxin. Lane 1- Standard aflatoxin B1 and B2, Lane 2- Standard aflatoxin + extract from X1 , Lane 3- Standard + extract from X2 , Lane 4- extract from X1 alone , Lane 5- extract from X2 alone , Lane 6- Rice extract, and Lane 7- Rice extract + Standard aflatoxin .

X1 and X2= Aspergillus spp. isolated from Greenpeas.

A)



B)

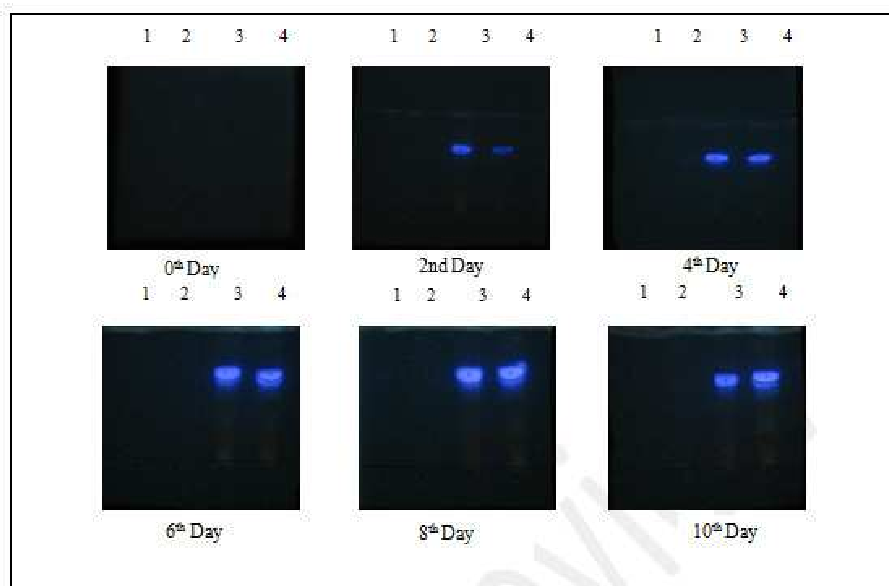


Figure 3
A. Flask containing Cauliflower and Rice inoculated with toxigenic A. flavus strain and incubated for various time periods up to 10 days.
B. Thin Layer Chromatogram of the extracts from the Cauliflower and Rice samples corresponding to o to 10 days of incubation. Lane 1, 2= aflatoxin spots obtained from Cauliflower extract, Lane 3, 4= aflatoxin spots obtained from Rice extract

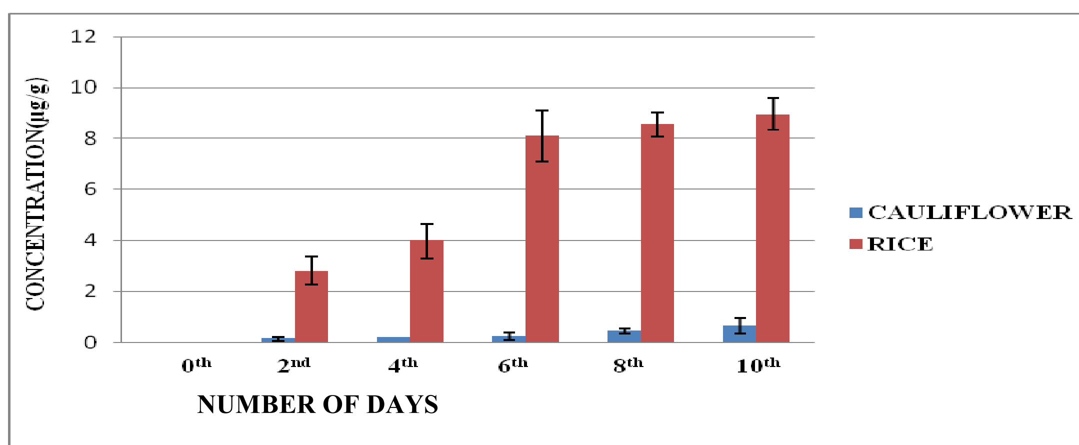


Figure 5
Comparison of Aflatoxin production on Rice and Cauliflower at different incubation period by the toxigenic strain of Aspergillus flavus.

Mycotoxins are produced by fungi on plant in field before the harvest period or during the storage, these mycotoxin are toxic to human beings and animals. When the mycotoxin enters the food chain it passes on from producer to consumers to decomposer thereby the biotic cycle is destructed. When the cattle are fed with low grade aflatoxin contaminated feed, these can be metabolised to aflatoxin M_1 and M_2 and pass on to human beings through milk[30]. According to ICMR Indian tolerance limits for aflatoxins is 30 µg per Kg of the food sample[31]. In the present study, many fungal contaminants were isolated from the edible samples. However, none of the samples were found contaminated with aflatoxin naturally. Either the edibles do not support the secondary metabolism of fungi

by which aflatoxin are produced or produced in such a small quantity that could not be detected by the TLC the method used for analysis. The presence of toxigenic fungi may not necessary be the indication of hazardous level of mycotoxin in food material. The current study suggests the ubiquitous presence of toxigenic fungi which may pose potential threat of mycotoxin in edibles. However the mycotoxin production is dependent on the substrate as the Cauliflower found to be resistant for the production of aflatoxin.

CONCLUSION

On the basis of the findings from current study, it is clear that toxigenic fungi are present in tested edibles can pose a potential problem for human health. The Cauliflower which supports the growth of different fungi did not support the production of aflatoxin. This shows that substrate plays a pivotal role in toxin production. Hence, the severity of mycotoxin problem cannot be assessed by presence of toxigenic isolates, it is necessary to check whether the substrate does really supports the growth and toxin production.

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