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The deterioration of spice crops caused by the contaminating fungi

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Abstract

Fungal contamination of various foodstuffs and agricultural commodities is a major problem in the tropics and subtropics, where climatic conditions and agricultural and storage practices are conducive to fungal growth and toxin production. Contamination of food and agricultural commodities by various types of toxigenic (fungi) is a serious and a widely neglected problem. Regardless of decades of extensive research, mould infection still remains a challenging problem (Munkvold, 2003).

Keywords: Spice, crops, fungi, mycotoxins, Microbiology

Introduction

Aflatoxins are produced by different fungal species that belong to the genus Aspergillus and more specifically to the Flavi section. For years, three main aflatoxigenic species were commonly considered in the section Flavi: *A. flavus*, *A. parasiticus* and *A. nomius*. In the last decade, the use of molecular tools enabled the identification of new species belonging to the section Flavi, comprising 34 different species, of which 17 are aflatoxigenic. These species can be distinguished by subtle morphological specificities, molecular changes in some gene sequences and, most importantly, through their ability to produce different mycotoxins. Indeed, some species, including *A. flavus*, *A. pseudotamarii* and *A. togoensis*, produce aflatoxins of B type, whereas others, including *A. parasiticus*, *A. minisclerotigenes*, *A. mottae*, *A. nomius*, *A. novoparasiticus*, *A. arachidicola* and *A. korhogoensis* produce both B and G type aflatoxins. Some species may also produce other toxic secondary metabolites such as cyclopiazonic acid (Kumar *et al*., 2017) [2].

Most of the mycotoxins are easily absorbed from the site of exposure, such as the gastrointestinal (i.e., dietary consumption) or respiratory tract (i.e., inhalation dust), to the circulatory system reaching vital, as the toxin is distributed throughout the body. Mycotoxins can enter human and animal cells and exert a spectrum of effects, including permanent damage. Through natural cellular processes of transcription and translation, these mutations may manifest or even exacerbate deregulation of cell growth (Adam *et al*., 2017) [2]. Several cellular processes, including DNA replication and protein synthesis, are affected by ochratoxin A (OTA) and deoxynivalenol (DON). Moreover, aflatoxin B1 (AFB1) has been recognized for its carcinogenicity, mostly through genotoxic effects, by the World Health Organization's (WHO) International Agency for Research on Cancer (IARC) Monographs Program (De Ruyck *et al*., 2015) [3].

Aflatoxins B1, B2, G1, G2 Among the naturally occurring aflatoxins, the toxic properties of aflatoxin B1 are the most widely studied. Aflatoxins have acute toxic effects (hepatotoxic in humans and animals and immunosuppressive in animals). The major concern, however, is that the aflatoxins are among the most potent mutagenic and carcinogenic substances known. The aflatoxins appeared to be extremely potent carcinogens in animal experiments. They are potent in all species investigated, i.e. mice, rats, hamsters, fish, duck, tree shrews and monkeys, and in several organs, of which the liver is the primary target (CEC, 1996).

Mycotoxins are unavoidable contaminants of spices, posing as health threat to animals and humans. Various studies were conducted to screen various spices for fungi and mycotoxin contamination and evaluate their safety. Aiko and Mehta (2016) [14] studies 63 samples for fungal

contamination and fungal load determined using standard microbiological method. Aflatoxin and citrinin were detected using thin layer chromatography

Chromatography technique. Fifty-eight out of the 63 samples were contaminated, while five were free from fungal contamination. Analysis revealed that 47% of the samples had a fungal load above 1 9 103 cfu/g which is the permissible limit set by World Health Organization. The samples *Mesua ferrea*-II and *Terminalia chebula*-III had the highest fungal load, i.e., 5.0 9 104 cfu/g. A total of 187 fungi were isolated, out of which 28 were toxigenic which included 19 aflatoxin-producing *Aspergillus flavus* and 9 citrinin-producing *Penicillium citrinum*. The natural contamination with aflatoxin B1 was detected only in one sample, i.e., *Arachis hypogaea* (groundnut) which was present beyond the permissible limit. Though toxigenic fungi were isolated, mycotoxins were not detected from any of the medicinal herbs and spices. Medicinal herbs and spices are susceptible to toxigenic fungi; however, they also possess intrinsic factors that inhibit mycotoxin contamination.

Quantitative estimation of aflatoxins by HPLC (FSSAI, 2016)

The samples found positive upon thin layer chromatography were further quantified using the High Performance Liquid Chromatography (HPLC) using ultraviolet detection at 365 nm. The method used a Shimadzu LC-20AD pump with DGU-20A5 degasser, an automated sample injector system SIL 20A communication module-20A, and UV detector SPD-20A. The HPLC was set at 365 nm with a reverse-phase ODS C18 column (Shim-pack MAqC-ODS; 4.6 x 250 mm, 5 µm) under a 20 °C controlled column chamber. The samples were kept at 4 °C before injecting

Molecular identification of key species

Based on the present study, the key fungal species, identified either as toxigenic or as antagonistic were further sent for the identification using molecular techniques. The pure cultures of the fungal isolates were sent to the National Centre for Microbial Resource (NCMR), National Centre for Cell Science (NCCS), Pune, India. Briefly, the fungal sample(s) were identified based on Internal Transcribed Spacer (ITS). The ITS region is the most widely sequenced DNA region in molecular ecology of fungi and has been recommended as the universal fungal barcode sequence. It has typically been most useful for molecular systematics at the species to genus level, and even within species (e.g., to identify geographic races). The ITS code was matched with National Centre for Biotechnology Information (NCBI) database. The confidence in identification came with the availability of sequence and the extent of homology shown by the ~550 bp sequence of fungal sample with its closest neighbour in the database. Shown by the ~550 bp sequence of fungal sample with its closest neighbour in the database.

Results

During the study, various spice samples were collected for the fungal colonization. Various spice samples were procured from the local market of Jabalpur (MP) and the sources included various supermarkets, Grocery stores, as well as spice packing units and warehouses. The samples contained both branded and unbranded products, but the priority was given to the local packers of these spices.

Table 1: Frequency of fungal infestation on different spices collected during the study.

S. No.	Spice	Infestation	Rainy		Winter		Summer		Total	Frequency %
			L	P	L	P	L	P		
1	Coriander	Infested	5	3	4	1	2	3	18	94.74
		Total	5	3	4	2	2	3	19	
2	Red Chilli	Infested	1	3	2	1	3	3	13	86.67
		Total	3	3	2	1	3	3	15	
3	Turmeric	Infested	2	0	3	1	5	0	11	91.67
		Total	3	0	3	1	5	0	12	
4	Cinnamon	Infested	2	2	2	0	1	3	10	100.00
		Total	2	2	2	0	1	3	10	
5	Fenugreek	Infested	1	0	1	0	0	0	2	66.67
		Total	1	0	1	1	0	0	3	
6	Black Pepper	Infested	1	0	0	1	1	1	4	100.00
		Total	1	0	0	1	1	1	4	
7	Cumin	Infested	0	0	0	3	1	1	5	100.00
		Total	0	0	0	3	1	1	5	
8	Bay Leaves	Infested	2	1	1	1	0	2	7	100.00
		Total	2	1	1	1	0	2	7	
9	Clove	Infested	1	1	3	0	4	0	9	90.00
		Total	1	2	3	0	4	0	10	
10	Black Cumin	Infested	2	1	1	0	0	0	4	100.00
		Total	2	1	1	0	0	0	4	
11	Curry Powder	Infested	2	2	2	1	3	1	11	100.00
		Total	2	2	2	1	3	1	11	

Quantification of aflatoxins in spice samples using High Performance Liquid Chromatography

Positive TLC samples were subjected to High Performance Liquid Chromatography to quantify the aflatoxins using HPLC. The quantification was done using the peak area of standard aflatoxins run under the same chromatographic conditions. shows the HPLC chromatogram of four standard aflatoxins. AFG2 was detected at retention time of 10.02 min, followed by AFG1(11.23 min), AFB2 (15.87 min) and AFB1 (16.21 min). The concentration of standard AFG2 and AFB2 were 10 µg while AFG1 and AFB1 were 25 µg.

Table 2: Diameter of Aspergillus flavus mycelium growth (in mm) when co- cultured with other non-toxigenic fungi in a dual culture experiment. Control has the target fungi alone.

S. No	Fungi co-cultured with *Aspergillus flavus*	Radial growth of A. *flavus* (in mm)						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	Control (*Aspergillus flavus*)	6	9	11	20	33	52	80
2	*Mucor racemosus*	6	12	24	28	26	25	25
3	*Aspergillus niger*	6	10	14	18	21	24	24
4	*Aspergillus terreus*	6	10	14	15	18	22	22
5	*Rhizopus sp.*	6	18	31	32	26	25	25
6	*Alternaria sp.*	6	11	15	19	22	26	26
7	*Cladosporium sp.*	6	9	11	14	21	24	24
8	*Curvularia sp.*	6	10	13	17	21	24	24

The results were provided by NCCS using BLAST method based on the nucleotide sequence and NCBI database to the extent of homology by ~550 bp sequence. The nucleotide sequence and identification of fungal strains is given below.

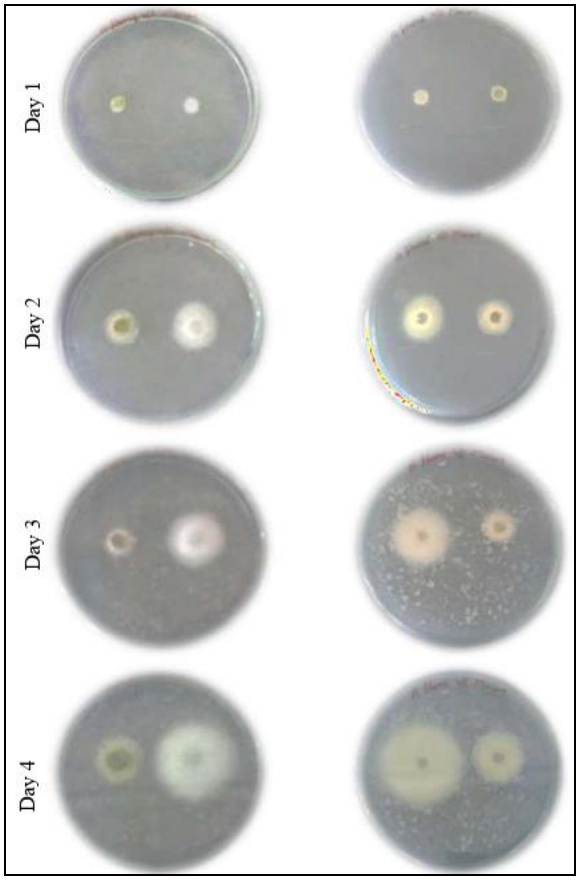

Fig 1: Antagonistic activity of *A. flavus* against *Mucor racemosus.*

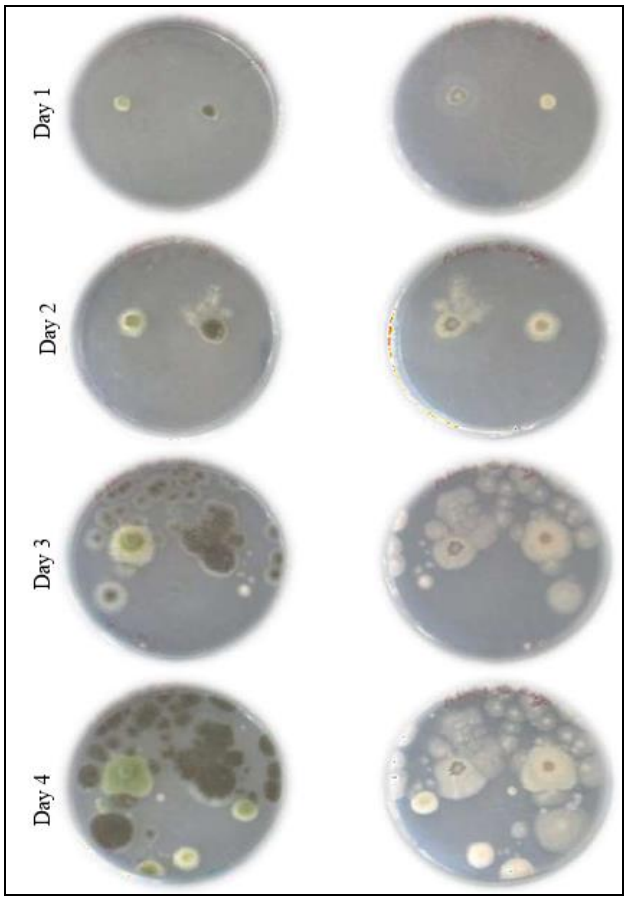

Fig 2: Antagonistic activity of A. *flavus* against *Aspergillus niger*

Penicillium polonicum

TCACCTCCACCCGTGTTTATTTTACCTTGTTGCTTCG
GCGGGCCCGCCTTTACTGGCCGCCGGGGGGCTCAC
GCCCCCGGGCCCGCGCCCGCCGAAGACACCCCCGA
ACTCTGTCTGAAGATTGAAGTCTGAGTGAAAATAT
AAATTATTTAAAACTTTCAACAACGGATCTCTTGGT
TCCGGCATCGATGAAGAACGCAGCGAAATGCGATA
CGTAATGTGAATTGCAAATTCAGTGAATCATCGAG
TCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGG
GGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAA
GCCCGGCTTGTGTGTTGGGCCCCGTCCTCCGATTCC
GGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGC
GTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCG
CTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCC
AAATTTTTATCCAGGTTGACCTCGGATCAGTAGGG
ATACCCGCTGAACTTAAGCA.

Penicillium cuddlyae

GGGGTCGGCTGGCGCCGGCCGGGCCTGCAAAGCG
GGTGACAAAGCCCCATACGCTCGAGGACCGGACGC
GGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCGGGG
GGACGGCGCCCAACACACAAGCCGGGCTTGAGGG
CAGCAATGACGCTCGGACAGGCATGCCCCCCGGAA
TACCAGGGGGCGCAATGTGCGTTCAAAGACTCGAT
GATTCACTGAATTCTGCAATTCACATTACTTATCGC
ATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAG
AGATCCGTTGTTGAAAGTTTTAACTAATTTGTGCTT
ATCGCTCAGACTGCAATCTTCAGACAGCGTTCAAT
GATGTCTCCGGCGGGCGCGGGCCCGGGGGCAGGTG
CCCCCCGGCGGCCAGACTGGCGGGCCCGCCGAAGC
AACAAGGTACGGTATACAC.

Fusarium chlamydosporum

TGTGACATACCTATACGTTGCCTCGGCGGATCAGC
CCGCGCCCCGTAAAACGGGACGGCCCGCCGCAGG
ACCCACAAACCCTGAATTTTATTGTAACTTCTGAGT
TTAAAAAACAAATAAATCAAAACTTTCAACAACGG
ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCA
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCGC
CAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATT
TCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGAT
CGGGCTGCGGTTCTACCGCGTCCCGGCCCCGAAAT
CTAGTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGT
AGCTAACACCTCGCAACTGGAACGCGGCGCGGCC.

It was an important observation that needs to be mentioned that the nucleotide sequence has similar percent of homology with more than one strain of fungi, and hence it is difficult to identify these fungi. We have adopted the molecular taxonomy in conjunction of our earlier recording of macro- and micro-morphological characters.

Discussion

Food is one of the fundamental needs of life. The rapid growth in population raised the food intake requirement. The extremely safe handling, storage and processing conditions are required as the number of commodities increased day today. Microorganisms are present everywhere in our environment. Depending upon the state of occurrence, the microorganisms can either be poisonous or non-poisonous.

Fungi are ubiquitous in nature and can affect plants, animals as well as humans. Fungal contamination of food is not a new phenomenon, and methods to avoid fungal contamination of food are in practice since evolution of mankind (Nazir *et al*., 2019) [12]. Fungal toxins (mycotoxins) are one of the major threats of consuming fungi infested food items by humans and the animals, as these mycotoxins present a wide degree of acute and chronic toxicosis (Thanushree *et al*., 2019) [13].

Conclusion

Although there are more than 300 mycotoxins discovered so far, only few are known to infest food commodities. Spices are reported to have only aflatoxin contaminations, and hence FSSAI has set up the limit of 30 ppm for spices (www.fssai.gov.in). Although this limit is almost twice as the limit of European Union, it is important to note that most of the spices are cultivated in tropical conditions, including India, where knowledge about toxin contamination and technological advantage for storage and transport is negligible.

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