

# Isolation and Characterization of Contaminant Mycoflora from Stored Red Peppers

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## Abstract

Red pepper is a spice vulnerable to fungal growth and mycotoxin contamination while in the field and post-harvest during storage. Storage fungi are omnipresent and colonize fast when moisture levels go up, ultimately leading to mycotoxin production. Stored samples of the whole, crushed and ground red pepper in the Southern Indian States and Maharashtra, India were examined to isolate and characterize the prevalent contaminant fungal species. The average moisture content was below 10% for all the red pepper samples and the average total fungal load was less than  $1 \times 10^4$  CFU/g. Crushed red pepper showed higher fungal counts than ground and whole red pepper. The identified contaminant mycoflora belonged to the group; *Aspergillus*, *Eurotium* sp., *Cladosporium* sp., *Eupenicillium* sp., *Penicillium* sp., *Miscellaneous* fungi, *Neosartorya* sp., *Curvularia* sp., and *Hyphopichia* sp. Aflatoxigenic *Aspergillus flavus* was present only in 8.3% of the crushed and 2.54% of whole red pepper samples stored for more than two months.

**Keywords:** Red pepper, fungal contaminants, *Aspergillus flavus*, aflatoxin

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## INTRODUCTION

Red pepper is an important tropical spice. The brilliant red colour, aroma and pungency of red pepper make it a desired adjunct in foods. The carotenoids and capsaicinoids are considered to be the most imperative chemical groups in red pepper. The chilli oleoresins have wide use ranging from food, drugs to cosmetics. Anti-oxidant and anti-cancerous activities have also been demonstrated for red peppers.<sup>1</sup> Red pepper is therefore an important spice both in the international and domestic market<sup>2</sup>.

Red peppers are exposed to microbial contamination during pre and post-harvest.<sup>3</sup> The storage fungi; *Aspergillus* sp. and *Penicillium* sp. are omnipresent and are the dominant species causing contamination in stored red peppers resulting in mycotoxin production<sup>4</sup>. Aflatoxin is the most studied among mycotoxins. Aflatoxin production has been reported in; *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. ochraceoseus*, and *A. australis*. *A. flavus*, known to have a worldwide distribution is an important and predominant fungus responsible for aflatoxin contamination in red peppers. Aflatoxicosis can be acute to chronic with mild to severe illness. Among aflatoxins, B1 is the most toxic and potent hepatocarcinogenic compound<sup>5</sup>.

Red peppers, among the spices, are more predisposed to contamination by mycotoxigenic species<sup>6</sup>. The post-harvest practises such as drying, grading, packaging, transportation and storage are important factors as at each stage if proper care not taken, it would contribute to fungal proliferation and aflatoxin accumulation. The aim of the present study was to determine the fungal load in stored red peppers available in domestic markets of producing regions in the southern Indian states and Maharashtra, to identify the fungal contaminants and thereby evaluate the incidence of aflatoxigenic *A. flavus*.

## MATERIALS AND METHODS

### Collection of Samples

Red pepper samples (whole red pepper n=118, crushed red pepper n=24 and ground red pepper n=28) stored in polythene bags and with a storage period greater than two months were collected from Kerala (KL), Karnataka (KR), Tamil Nadu (TN), Andhra Pradesh (AN), Telangana (TG)

and Mumbai (MB). Samples of whole (W) red pepper (n= 128), crushed (C) red pepper (n= 43) and ground (Gr) red pepper (n= 51) with a storage time of less than two months were also collected from the areas. Sterile scissors, spatula, forceps and spoon (stainless steel) were used for collection. Samples were collected in sterile large plastic covers and plastic containers with proper seal. A 3-Class attribute plan was followed. A 375g incremental sample was collected from each bag. Minimum five such incremental samples collected from a lot were mixed together into a composite sample and the sample size was reduced by conning and quartering. The samples were stored at 4°C in the refrigerator until use and processed at the earliest possible maximum in a week's time.

### Moisture analysis

Moisture determination in the red pepper samples was performed by azeotropic distillation. 25g sample was transferred to distilling flask and sufficient volume of hexane was added to cover the sample completely and boiling chips were added. After assembling the apparatus, the trap was filled with hexane. A loose non-absorbing cotton plug was inserted into the top of the condenser, to prevent condensation of atmospheric moisture to the condenser. After bringing to boil, it was reflux at about two drops per second until most of the water was collected in the trap, then the reflux rate was increased to 4 drops per second. After two hours the reading was noted and refluxing continued until two consecutive readings 15 min apart showed no change so that the total extraction was 3 hours. Any water held up in the condenser was dislodged with a brush/wire loop. The condenser was carefully rinsed with 5ml hexane. The volume of water in the trap was read. The moisture content was reported to an accuracy of 0.0% (by weight)<sup>7</sup>.

### Isolation and enumeration of total fungal colonies in the samples

Total fungal load was determined using the dilution plating method<sup>8</sup>. 25g sample was weighed into a sterile filter bag containing 225ml of sterile 0.1% peptone water. The filter bag containing the sample was homogenized in a stomacher for 2 minutes. The sterile filter bag formed the first dilution i.e., 10<sup>-1</sup>. 1ml from 10<sup>-1</sup> dilution was transferred into a tube containing 9ml of sterile 0.1% peptone diluent. Likewise sample

dilutions of up to  $10^{-6}$  were prepared (Dilution rate; 1:10 = 1+9). 1ml of the dilution was poured into pre-labelled sterile plates in triplicates. Dichloran 18% Glycerol agar (DG18) was immediately poured on to it. The poured plates were gently swirled clockwise and anti-clockwise. The plates were incubated upright in dark at 25°C for 5 days without disturbing. The plates were counted after five days of incubation. The plates were re-incubated for another 48 hours if no growth was observed. Plates containing 10-150 colonies were counted. The results were expressed as Colony forming units per gram (CFU/g) based on average count of the triplicates.

#### Identification of fungal isolates and Qualitative detection of aflatoxigenic fungi

The fungal growth obtained from dilution plating method was carefully observed and sub-culturing of the selected fungi was done to obtain pure cultures. For identification of fungi, the isolates were grown on standard media.<sup>9</sup> Lactophenol cotton blue staining was done for the fungal isolates and the microscopic features were noted. The colony colour, diameter and

characteristics of seven day old culture on Czapek Yeast agar (CYA) at 25°C, Glycerol 25% Nitrate (G25N) agar at 25°C, CYA at 37°C and Czapek Yeast Agar with 20% sucrose (CY20S) incubated at 25°C were noted. The identification keys were used to identify the fungal isolate<sup>9</sup>.

Further all the fungal cultures were grown on *Aspergillus* differentiation medium (ADM) and thereafter inoculated into Coconut agar medium (CAM) for qualitatively detecting the aflatoxin producing capability of the isolates. For the preparation of coconut agar medium, 100 g of fresh shredded coconut was taken and mixed with 300ml of hot distilled water and homogenized using a blender for five minutes followed by filtration using cheesecloth (two-layered). 2 N NaOH was used to adjust the pH of the medium to 7. Agar at 1.5 % was added and the media was autoclaved.<sup>10,11</sup> The fungal colonies were single point inoculated into the Coconut agar plates and incubated for 5-7 days at 30°C. Upon incubation, the plates were kept inverted and were examined under Ultra violet (UV) light at 365 nm in a dark room for fluorescence. Aflatoxin producing

**Table 1.** Total fungal load and moisture content of red peppers with more than two months storage

Region	Whole red pepper		Crushed red pepper		Ground red pepper	
	Moisture (%)	CFUs/g	Moisture (%)	CFUs/g	Moisture (%)	CFUs/g
KL	9.6	$1.1 \times 10^3$	8.1	$4.2 \times 10^3$	8.0	$2.7 \times 10^3$
TN	9.5	$1.1 \times 10^3$	9.3	$4.9 \times 10^3$	7.3	$1.3 \times 10^3$
KR	8.4	$6.5 \times 10^2$	9.5	$4.3 \times 10^3$	6.3	$8.5 \times 10^2$
TG	9	$1.1 \times 10^3$	9.2	$9 \times 10^2$	6.0	$1 \times 10^1$
AN	9.5	$1.1 \times 10^3$	7.1	$3.9 \times 10^2$	6.4	$8.8 \times 10^2$
MB	9.4	$1.2 \times 10^3$	7.9	$4.2 \times 10^3$	5.3	$8.7 \times 10^2$

KL – Kerala, TN – Tamil Nadu, KR – Karnataka, TG – Telangana, AN – Andhra Pradesh, MB - Mumbai

**Table 2.** Total fungal load and moisture content of red peppers with less than two months storage

Region	Whole red pepper		Crushed red pepper		Ground red pepper	
	Moisture (%)	CFUs/g	Moisture (%)	CFUs/g	Moisture (%)	CFUs/g
KL	6.9	$1.6 \times 10^2$	5.8	$4.2 \times 10^2$	6.5	$2.4 \times 10^2$
TN	6.5	$2.1 \times 10^2$	6.5	$3.8 \times 10^2$	4.0	$1.9 \times 10^2$
KR	6.4	$1.9 \times 10^2$	8.2	$4.1 \times 10^2$	4.5	$2.9 \times 10^2$
TG	8	$2.4 \times 10^2$	8.2	$3.1 \times 10^2$	3.8	$1.0 \times 10^1$
AN	7.7	$2.1 \times 10^2$	5.7	$1.5 \times 10^2$	4.0	$4.0 \times 10^2$
MB	6.5	$2.4 \times 10^2$	5.8	$3.8 \times 10^2$	4.7	$2.2 \times 10^2$

KL – Kerala, TN – Tamil Nadu, KR – Karnataka, TG – Telangana, AN – Andhra Pradesh, MB - Mumbai

colonies showed fluorescence as bluish white/white ring surrounding the colony. Uninoculated CAM plates were used as control<sup>9</sup>.

## RESULTS AND DISCUSSION

In the present study, the average moisture content and total fungal load for whole, crushed and ground forms of red pepper samples with more than two months storage period were 9.2%, 8.5%, 6.6 % and  $1 \times 10^3$  CFU/g,  $3.1 \times 10^3$  CFU/g,  $1.1 \times 10^3$  CFU/g, respectively (Table 1). Similarly, for samples with lower than two months storage, the average moisture content and total fungal load in whole, crushed and ground red pepper were 7%, 6.7%, 4.6% and  $2.1 \times 10^2$  CFUs/g,  $3.4 \times 10^2$  CFU/g,  $2.2 \times 10^2$  CFU/g, respectively (Table 2). In a 3-Class attribute plan, the 'm value' i.e., the microbiological limit if  $\leq 1 \times 10^4$  CFU/g is considered satisfactory. The total fungal load in all the red pepper samples was less than  $1 \times 10^4$  CFU/g. Thus the values were within the specified microbiological limits<sup>12</sup>.

Drying and storage of red pepper post-harvest is considered critical because during these periods, the red peppers are not only subjected to changes in temperature and humidity but are also exposed to wind/dust, mechanical damage while handling and invasion by insects. Moisture content in red pepper is usually brought down to 13% during the drying stages in order to prevent fungal growth and its contamination. In the present study moisture content was found to be below 10% for all the red pepper samples. Despite the lower

moisture content and the low water activity post drying, red peppers are still susceptible to fungal growth<sup>6</sup>.

A total of 53 fungal isolates were identified from the red pepper samples: KL – 11 isolates, TN – 9 isolates, KR – 9 isolates, TG – 7 isolates, AN – 4 isolates and MB – 13 isolates (Figure 1). Majority of the isolates belonged to the genera *Aspergillus*, followed by *Eurotium* sp. (which is a teleomorph of *Aspergillus* sp. and formerly identified as the "*Aspergillus glaucus* group"), *Cladosporium* sp., *Eupenicillium* sp., *Penicillium* sp., Miscellaneous fungi, *Neosartorya* sp., *Curvularia* sp., and *Hyphopichia* sp (Figures 2 to 5).

*Aspergillus tamarii* was the fungal species prevalent in all the three forms of stored red pepper. It is a common occurrence in nuts and oil seeds. It has also been reported in spices<sup>13</sup>. *A. tamarii* are not aflatoxin producers but produce another toxin called cyclopiazonic acid and the alkaloid fumiclavine A<sup>13</sup>. *Eurotium* sp. was also a common isolate from the red pepper samples. *E. chevalieri* produces toxic compounds called echinulin and neocheinulin that causes feed refusal in swine<sup>14</sup>. No mycotoxin production reported in *E. repens*<sup>9</sup>.

The other prevalent fungal isolates were from the Genera: *Cladosporium* and *Eupenicillium*. No mycotoxin production has been reported in *C. herbarum* and *C. macrocarpum*.

*E. hirayamae* are not mycotoxin producers whereas, *E. ochrosalmoneum* produces

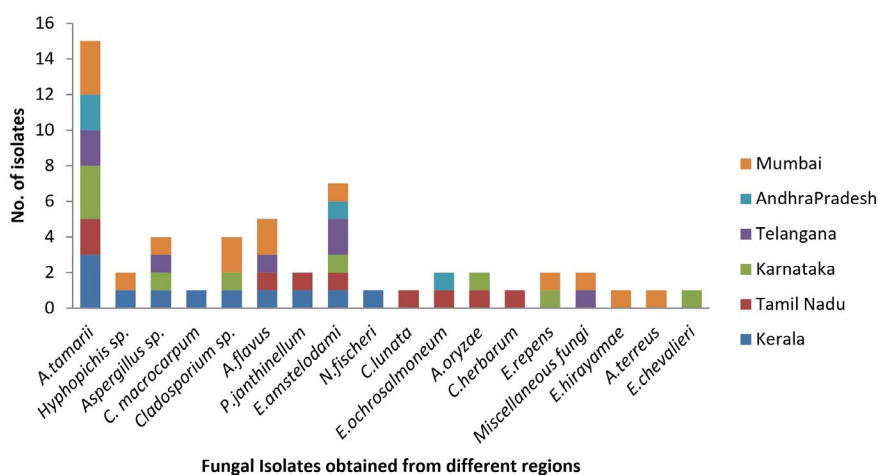
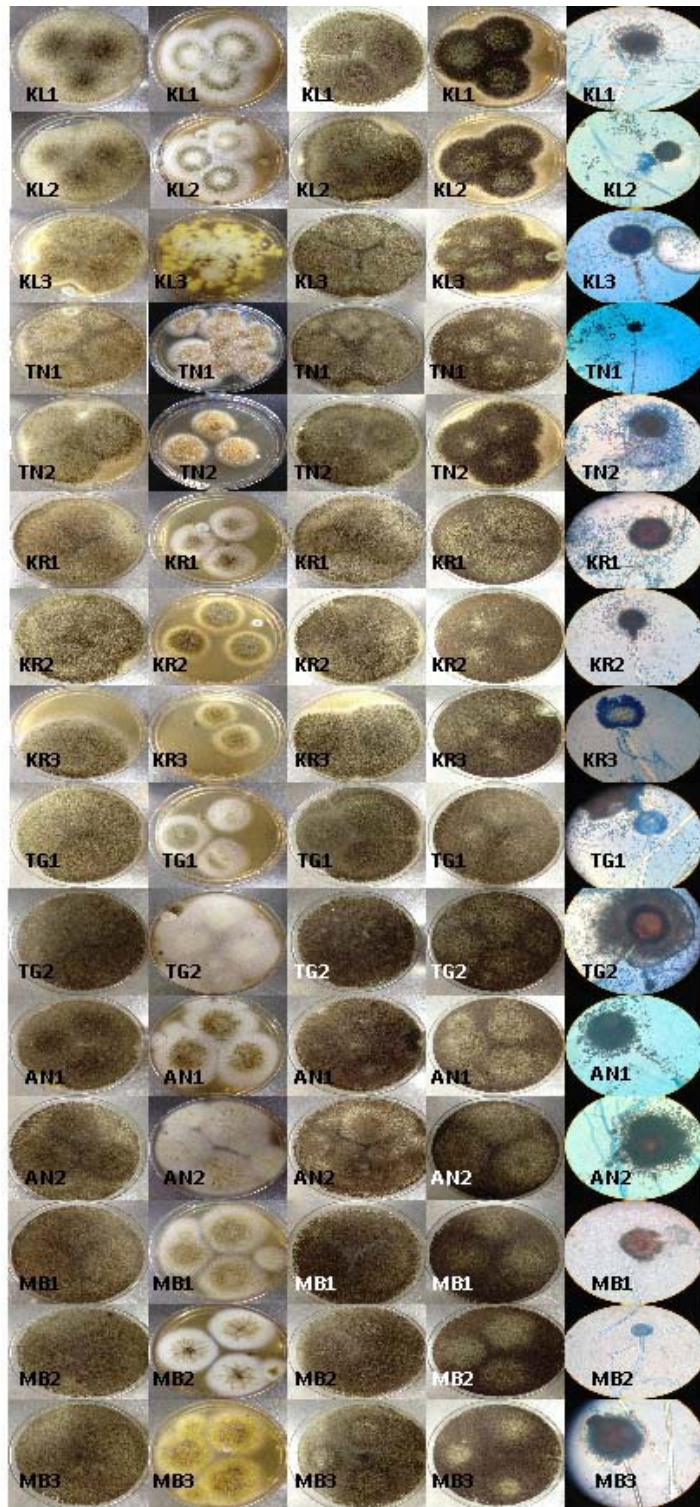
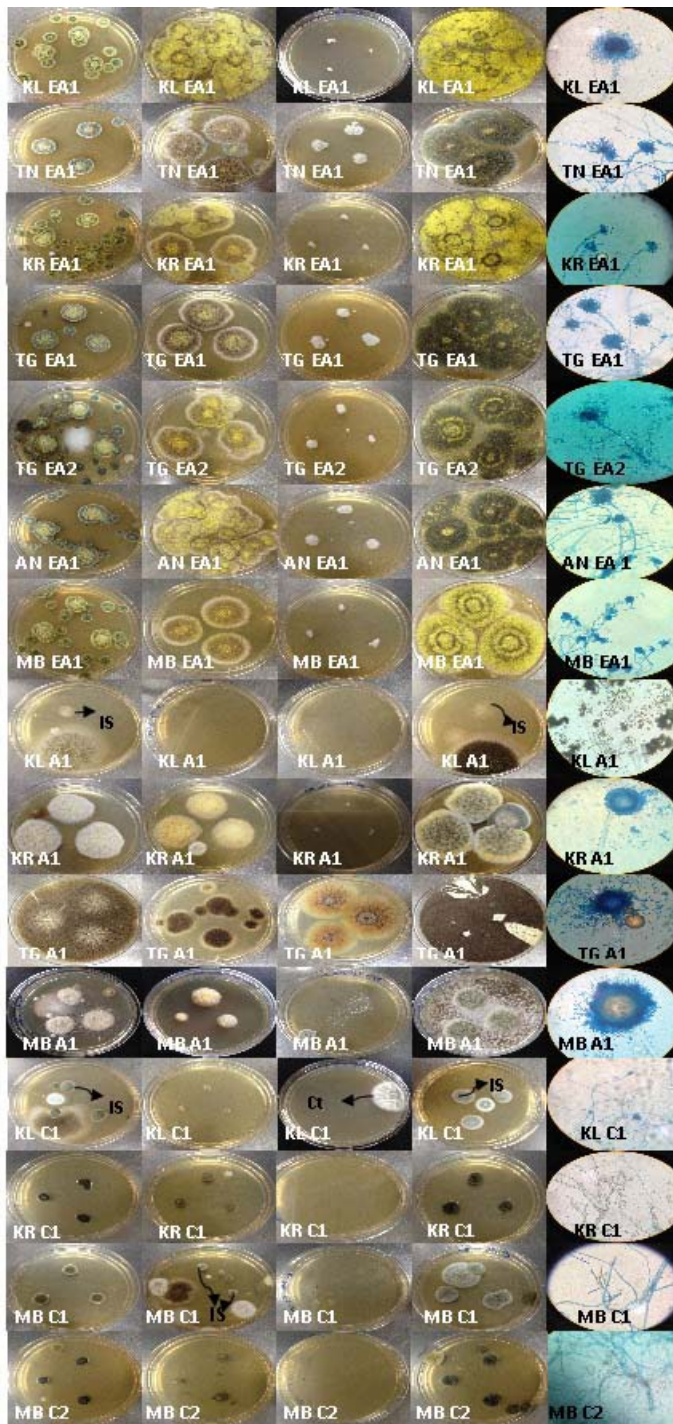


Fig. 1. Isolates of fungal genera from stored red peppers



**Fig. 2.** Isolates of *Aspergillus tamarii* grown on CYA 25°C, G25N 25°C, CYA 37°C, CY20S 25°C and microscopic view  
KL – Kerala, TN – Tamil Nadu, KR – Karnataka, TG – Telangana, AN – Andhra Pradesh, MB - Mumbai

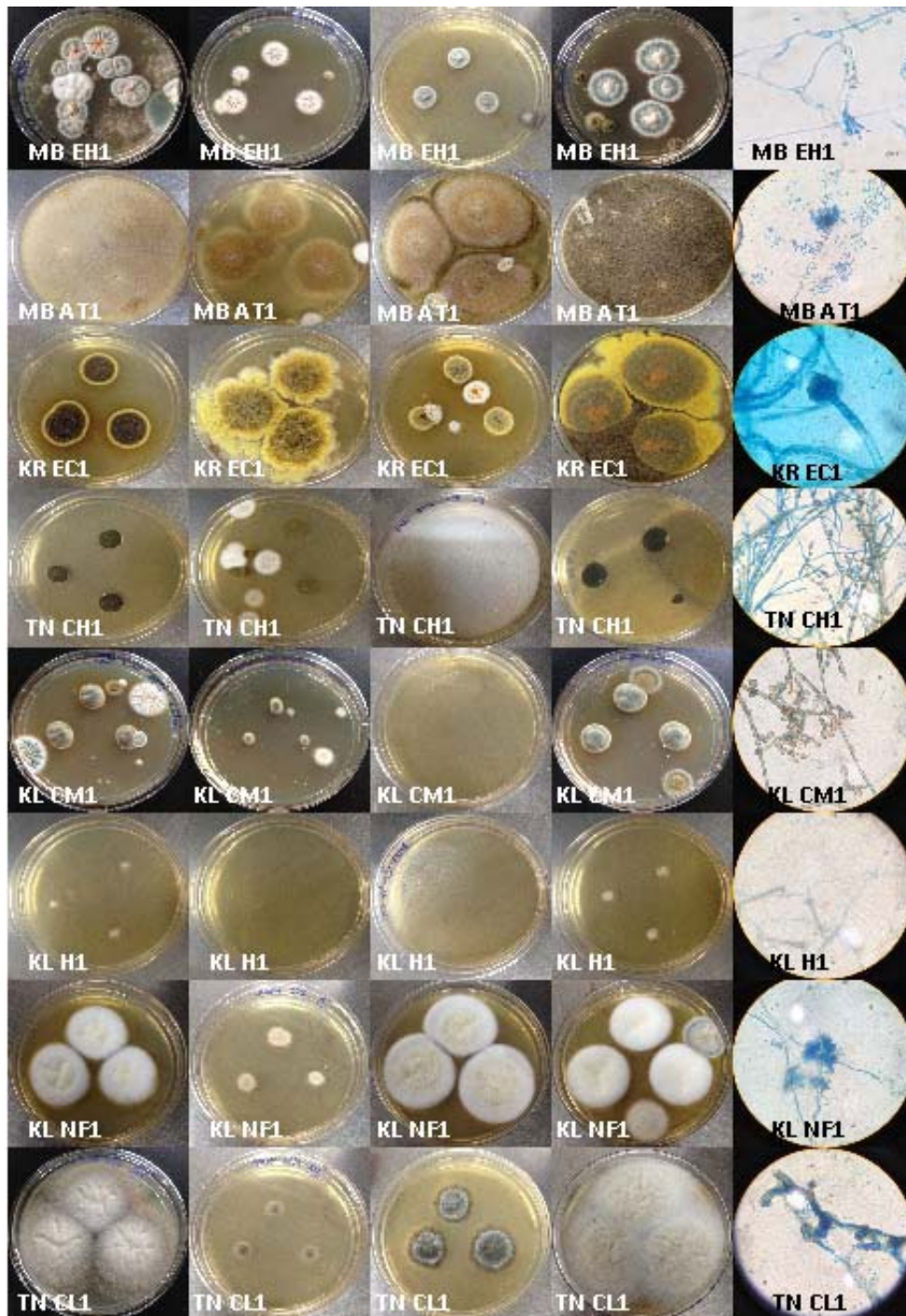


**Fig. 3.** Isolates of *Eurotium amstelodami*, *Cladosporium* sp., and *Aspergillus* sp., grown on CYA 25°C, G25N 25°C, CYA 37°C, CY20S 25°C and microscopic view

KL – Kerala, TN – Tamil Nadu, KR – Karnataka, TG – Telangana, AN – Andhra Pradesh, MB – Mumbai, EA – *E. amstelodami*, A – *Aspergillus* sp., C – *Cladosporium* sp., IS – Isolate, Ct - Contamination



**Fig. 4.** Isolates of *Aspergillus flavus*, *Penicillium janthinellum*, *Eupenicillium ochrosalmoneum*, *Aspergillus oryzae*, *Eurotium repens* and Miscellaneous fungi grown on CYA 25°C, G25N 25°C, CYA 37°C, CY20S 25°C and microscopic view KL – Kerala, TN – Tamil Nadu, KR – Karnataka, TG – Telangana, AN – Andhra Pradesh, MB – Mumbai, AF – *A. flavus*, PJ – *P. janthinellum*, EO – *E. ochrosalmoneum*, AO – *A. oryzae*, ER – *E. repens* and MF - Miscellaneous fungi, IS – Isolate



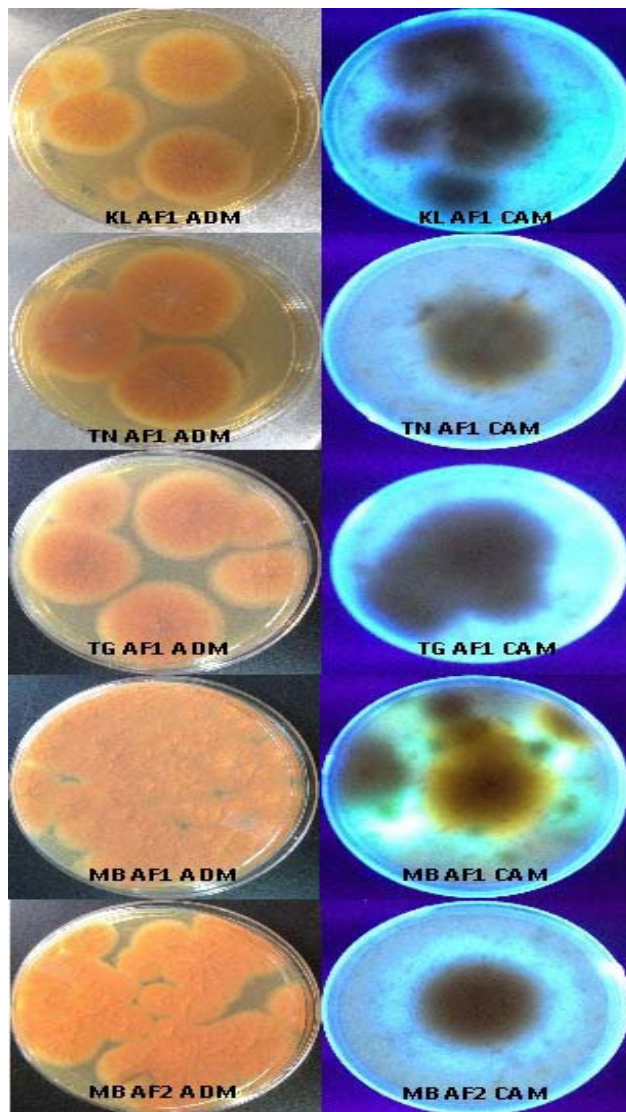
**Fig. 5.** Isolates of *Eupenicillium hirayamae*, *Aspergillus terreus*, *Eurotium chevalieri*, *Cladosporium herbarum*, *Cladosporium macrocarpum*, *Hyphopichia* sp., *Neosartorya fischeri* and *Curvularia lunata* grown on CYA 25°C, G25N 25°C, CYA 37°C, CY20S 25°C and microscopic view.

MB – Mumbai, KR – Karnataka, TN – Tamil Nadu, KL – Kerala, EH – *E. hirayamae*, AT – *A. terreus*, EC - *E. chevalieri*, CH – *C. herbarum*, CM – *C. macrocarpum*, H - *Hyphopichia* sp., NF – *N. fischeri* and CL – *C. lunata*



a mycotoxin; citreoviridin in maize<sup>15</sup>. Some of the fungal isolates belonged to the genera *Penicillium*, *Hyphopichia*, *Curvularia* and *Neosartorya*. *Penicillium janthinellum* produces janthitrems; a series of tremorgenic toxins<sup>16</sup>. The genus *Hyphopichia* sp., which is a yeast-like fungi do not produce any mycotoxins<sup>9</sup>. In *Curvularia* sp., mycotoxin production has not been reported. *Neosartorya* produces fumitremorgens A and C and verruculogen<sup>17,18,19</sup>.

Most of the contaminant fungi isolated in the present study are a common occurrence in stored foods. Though some of them are mycotoxin producers, aflatoxin production is mainly seen among the species of *Aspergillus* genera. In the present study, five isolates of aflatoxigenic *A. flavus* were obtained from the samples. The characteristic orange colour in reverse of the ADM was indicative of *A. flavus* isolates (Figure 6). All the other isolates showed uncoloured/pale reverse



**Fig. 6.** Characteristic appearance of aflatoxigenic *A. flavus* on CAM and ADM  
KL – Kerala, TN - Tamil Nadu, TG – Telangana, MB – Mumbai, AF – *A. flavus*, ADM – *Aspergillus* differentiation medium, CAM – Coconut agar medium

on ADM. Qualitative detection of aflatoxigenic *A. flavus* was done by growing the isolates on CAM. Aflatoxigenic *A. flavus* isolates grown on CAM, after incubation of five days showed a ring of blue fluorescence surrounding their colony growth when observed under UV light of 365nm (Figure 6).

*A. flavus* is the main producer of aflatoxins; causing acute and chronic toxicity in animals and humans; acute liver damage, induction of tumours, teratogenic effect, liver cirrhosis, immunosuppression and interference with protein uptake. Chronic aflatoxin exposure also linked with impaired hepatic metabolism<sup>20,21</sup>. Importantly aflatoxin B1 has been classified as a group 1 carcinogen<sup>22</sup>.

In the present study, aflatoxigenic *A. flavus* was present only in 8.3% of crushed and 2.54% of whole red pepper samples stored for more than two months. *A. flavus* was not present in any of the other red pepper samples. Proper handling, packaging and storage conditions are essential factors during post-harvest of red peppers. With increase in storage time, the samples are being subjected to temperature/humidity fluctuations due to the environmental conditions and the storage facilities maintained by suppliers of the respective regions. This could be the reason for incidence of *A. flavus* in a small percentage of samples stored for more than two months.

Red pepper is a spice used on a daily basis in all Indian households. In our country, red pepper is cultivated in the states of Andhra Pradesh, Gujarat, Karnataka, Maharashtra, Orissa, Rajasthan, Tamil Nadu, Telangana, Uttar Pradesh, West Bengal and Madhya Pradesh. India is not only the leading producer and exporter but also the leading consumer of red peppers<sup>23</sup>. The escalating international demands, the need for revenue build-up together with stringent regulations by the developed nations, could result in the best quality products being sold out of the country, and may leave behind a compromised lot that could enter the domestic market<sup>5</sup>. The people of the lower income group might be at risk by buying and consuming such red peppers. Continuously consuming toxin contaminated spice on a daily basis can have both acute and chronic impact on the health of the individual (young children, adult,

immunocompromized individuals and nursing mothers). Considering the gastronomic use of red peppers in India, it would be of good reference if monitoring of red pepper in domestic markets of the major producing areas is done. This would aid in eventually generating a national database of information regarding the fungal load, the prevalent fungal contaminants and especially the incidence of aflatoxigenic *A. flavus* in the different regions and thus provide a rationale to strengthen the risk analysis procedures and ultimately ensure that good quality and safe red peppers are reaching the consumers.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTIONS

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

#### FUNDING

None

#### DATA AVAILABILITY

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

#### ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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