Tropical Agricultural Research & Extension 12(1):2009

AFLATOXIGENIC *ASPERGILLUS FLAVUS* AND AFLATOXIN FORMATION IN SELECTED SPICES DURING STORAGE

M Rajasinghe, K Abeywickrama* and R Jayasekera
Department of Botany, University of Kelaniya, Kelaniya, Sri Lanka

Accepted: 10th May 2009

ABSTRACT

Proliferation of aflatoxigenic fungi and accumulation of aflatoxins take place during storage of spices at various environmental conditions. The objective of this research was to identify the aflatoxigenic fungal species and their aflatoxin forming potential in selected spices during storage in the laboratory and refrigerator. Dilution series of spice samples namely chilli (*Capsicum annum*) powder, curry powder, turmeric (*Curcurma longa*) powder and pepper (*Piper nigrum*) powder (purchased from two producers) were prepared separately and the frequency of occurrence of aflatoxigenic and other fungi were assessed after plating each on Potato Dextrose Agar and incubating at ambient temperature. Aflatoxin formation of *A. flavus* was confirmed by growing a pure culture on a SMKY medium and identifying toxins on thin layer chromatographic plates. The presence of aflatoxins in spice samples stored at 30 ± 2^0C and 4^0C was also confirmed. Long storage of spices leads to aflatoxin production irrespective of storage temperature hence, it is advisable to consume spices such as chilli powerder, tumeric *etc.* within a relatively short period to avoid build up of aflatoxins.

***Key words**:* Afltoxins, *Aspergillus flavus*, Spices, Storage

INTRODUCTION

Spices are widely used as natural food preservatives (Jayasekera *et al.* 2004). Mustard, ginger may promote the growth of aflatoxigenic fungi, whereas certain other spices such as cinnamon, pepper act as antifungal or antiaflatoxigenic (Paster 1994).

Aflatoxin which is a potent carcinogen produced mainly by secondary metabolism of *Aspergillus flavus, Aspergillus parasiticus or A. nomius*. Aflatoxin producing *Aspergillus* spp. may proliferate on spice samples if favorable environments for growth are provided resulting in production of aflatoxins (Grybauskas *et al.,* 1988). Changing the ingredient composition of spices such as curry powder could provide a solution to overcome the storage problem of spices. The objective of this research were to identify the aflatoxigenic fungal species and their aflatoxin forming potential in selected spices during storage at 30 ± 2^0C (laboratory) and 4^0C (refrigerator).

METHODOLOGY

Storage of spices

The spices (raw chilli (*Capsicum annum*), turmeric (*Cucurma longa*), pepper (*Piper nigrum*) and raw curry powder: cumin *Cumin cyminum*, fennel – *Foeniculum vulgare*, coriander – *Coriandrum sativum*, pepper – *Piper nigrum*, rampeh – *Pandanus amaryllifolius*, curry leaves – *Murraya koenigii* and cardamom – *Amomum cardamomum*, tumeric – *Cucurma longa* at a rough proportion of 50:20:20:10:2:2:1:1) were purchased from two producers, *i.e.* William grinding mills in Dehiwala (Producer 1) and National grinding mills in Kiribathgoda (Producer 2), Sri Lanka. Spices from producer 1 were designated as curry powder 1, pepper powder 1, whereas ones from producer 2 were designated as curry powder 2, pepper powder 2 *etc*. spice samples were stored in the laboratory and at refrigeration temperature (4^oC) in sterile glass containers soon after purchase (Jayasekera *et al*. 2004).

Temperature and relative humidity in the research laboratory and in the refrigerator where spices were stored were measured three times per day using a thermometer and a thermo hygrometer ("ON" Gallenkamp UK).

Preparation of the dilution series

Using all spice samples (1g x 5 each stored at ambient/refrigeration temperature) from producer 1 and 2, a dilution series up to 10^{-6} was prepared on day zero (0), thirty (30), sixty (60), ninety (90) and one hundred and twenty (120) days after storage of spice samples. One (1) ml of each serial dilution was introduced into five replicate sterile petri dishes and molten potato dextrose agar (PDA) was poured over inoculum. Plates were manually rotated, arranged as a completely randomized design (CRD) on the laboratory table and incubated for one week at 30 ± 20C (Elliot 1988). This experiment was repeated once.

*Corresponding author

Identification of aflatoxigenic and non-aflatoxigenic fungal species

Fungal colonies that grew out were tentatively identified (Barnett and Hunter 1987; Malloch 1986) after incubation. Isolated *Aspergillus* spp. and other fungal species were transferred to PDA slants for storage. Pure cultures were obtained by transferring the isolated fungal colonies to fresh PDA plates under sterile conditions. The plates were incubated for 7-10 days at room temperature and their morphological features were studied and recorded.

Vegetative and reproductive characters as seen under the microscope were used to identify the different forms of fungi to the genus level or if possible up to the species level. Slide cultures, freshly prepared slides, cultures on PDA and color photographs were used in identifying fungi. Identification was confirmed by comparing with published data/ descriptive keys (Barnett & Hunter 1987; Malloch 1986) and cultures previously isolated and stored in the Department of Botany, University of Kelaniya culture collection.

Frequency of occurrence of *A. flavus*

Fungal colonies belonging to one fungal species, for example *A. flavus* or *A. niger* grown on replicate dilution plates were counted and recorded. Similarly, total number of fungal colonies grown on the same set of plates was recorded. Frequency of occurrence of each fungal species was calculated per replicate dilution plate using the following equation (Parkinson 1994).

$$\text{Frequency of occurrence of a fungal species} = \frac{\text{Number of colonies of a fungal species} * 100}{\text{Total number of fungal colonies}}$$

Analysis of aflatoxins present in spices during storage

5g x 3 of each of spice samples namely curry powder, pepper powder, chilli powder and turmeric powder were weighed and transferred to 100ml conical flasks and 25ml of water and 25ml of chloroform were added. Then the samples were placed on a shaker (No- Y 144244/0185 Baird and Tatlock (London) Limited) at 200rpm for 30min. The chloroform / aqueous mixture was transferred to a separatory funnel and shaken gently for 5min and allowed to settle for 30min. Chloroform fraction was collected and dried with 3g of anhydrous sodium sulphate and solvent was evaporated to dryness on a rotary evaporator (R-114 & B- 482; Buchi Labortechnik AG, Switzerland). Extracts of all replicate samples were combined and the resulting extract was concentrated using a nitrogen steam (Dayananda, 1990). Residue was redissolved in 1 ml chloroform and 10ml was spotted on a precoated thin layer chromatographic (silica gel 60-F-254) plates (0.5mm thickness, 5´10cm, Merk, Germany) in triplicate. Aflatoxin B_1 standard (3.25mg/ml) and aflatoxin standard containing B_1, B_2, G_1 and G_2 (3.25mg/ml, 0.3mg/ml, 1.03mg/ml, 0.325mg/ml) were also spotted along with the extracts. TLC plates were developed in the solvent system chloroform: acetone (88: 12v/v) for 15min and observed under long wave ultra violet (UV) light at 254nm (Camag U.V. lamp Switzerland). Blue and green fluorescing spots in sample plates were identified and compared with the aflatoxin standards (Bean & Fernando 1986). The plates were then placed in an iodine chamber at which point, the four aflatoxins if present, changed to a brown color (Paranagama *et al.* 2003). Six replicate spots per spice sample (or residue) were used to confirm aflatoxins.

Aflatoxin production capability of *A. flavus*

A. flavus isolated from spice samples was maintained on PDA as a pure culture. *A. flavus* was inoculated in to slants incubated at 30 ± 2^0C on a laboratory bench for 7 days. Using sterile distilled water containing a drop of Tween - 80, conidial suspensions of *A. flavus* were prepared and spore concentration was adjusted to 10^{-6} conidia ml^{-1} using a haemocytometer (Paranagama *et al.* 2003).

A semi–defined medium (SMKY) (20g sucrose, 7g yeast extract, 3g KNO_3, 0.5g $MgSO_4$. $7H_2O$, l water) was prepared and 6 x 50ml was dispensed into six 250ml Erlenmeyer flasks and sterilized by autoclaving. After cooling, the flasks were inoculated with 5ml each spore suspensions of *A. flavus* and incubated stationary for 8 days at ambient temperature (30 ± 2^0C) (Paranagama *et al.* 2003). The liquid portion was separated from the mycelial mats by using Whatman # 1 filter paper. Subsequently, the liquid medium was transferred into 150ml conical flasks. Standard procedures were used for the extraction and identification of aflatoxins. 25ml of chloroform was added to liquid medium containing aflatoxin and gently shaken on a rotary shaker (Gyro rotary shaker-model G2 New Brunswick Co. Inc. USA) at 70rpm for 30min. The mixture was transferred to a separatory funnel and shaken gently for 5min and allowed to settle for 30min. Chloroform fraction was collected, and samples were prepared as in previous section and spotted on TLC plates as described above. Blue and green fluorescing spots in sample plates were identified and compared with the aflatoxin standards.

RESULTS

Relative humidity and temperatures in the storage areas

During the four months of storage of spices, temperature in the laboratory ranged between 28^0C and 32^0C where as relative humidity (RH) ranged between 78 - 82%. Refrigeration temperature remained constant at 4^0C with a RH of 87 - 91%.

Identification of fungal genera and frequency of occurrence

Ten fungal genera identified were *A. flavus*, *A. niger*, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Rhizopus* sp. 1, *Rhizopus* sp. 2, *Penicillium* sp. 1, *Penicillium* sp. 2, *Trichoderma* sp. and *Stachybotrys* sp. *A. flavus* from different spice samples showed similar morphological and reproductive characteristics and the identity was confirmed using a literature (Barnett & Hunter, 1987; Malloch, 1986).

The frequency of occurrence of *A. flavus* in curry powder 1 stored at ambient and refrigeration temperatures increased to maximum values and thereafter decreased to lower values. In curry powder 2, frequencies of *A. flavus* both at refrigeration and ambient temperatures showed a zero frequency up to 60 days. At 4^0C, frequency of *A. flavus* gradually reached a maximum (52%) on day 120. At ambient temperature, the frequency increased from zero to 47.5% during the last 60 days. Similarly, frequency of *A. flavus* in pepper powder 1 stored at both 4^0C and ambient temperature conditions were zero on day zero. When stored at ambient temperature, frequency increased initially and frequency of *A. flavus* increased slightly up to the 60^{th} day (maximum of 27.2%), and slightly decreased within last 60 days. Chilli powder 1 stored at ambient temperature indicated a gradual rise in frequency of *A. flavus* by day 90 (58%), and slightly decreased by day 120. The same spice sample stored at 5^0C, showed a reduction in frequency from day 30 up to day 120 (maximum of 44.7% and minimum of 25%). The growth of *A. flavus* was enhanced at ambient temperature. Chilli powder 2 stored at 5^0C showed a zero frequency of *A. flavus* up to day 60. From day 60 up to day 90, there was an increase in frequency of *A. flavus*, and then gradually decreased by day 120. When stored at ambient temperature, chilli powder initially showed 55% frequency and decreased to a value of 30% by 120 days. The patterns of change in frequency of *A. flavus* at both conditions were different from each other. When turmeric powder 1 was stored at 5^0C and at ambient temperature, the frequency of *A. flavus* was zero on day zero and 30. The maximum frequencies of *A. flavus* at ambient temperature and at 5^0C were 42% and 22%, respectively. The patterns of frequency of occurrence of *A. flavus* in tur-

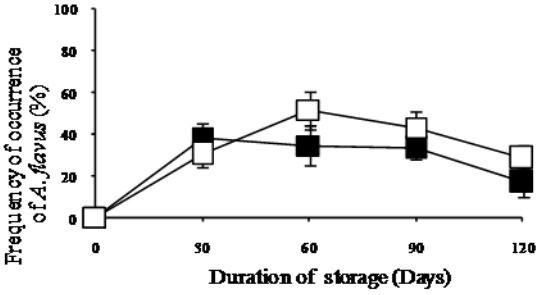

Figure 1. Frequency of occurrence of A. flavus in curry powder 1 as a function of storage period. Values represent the mean of six replicates ± standard error; ■ A. flavus at ambi-

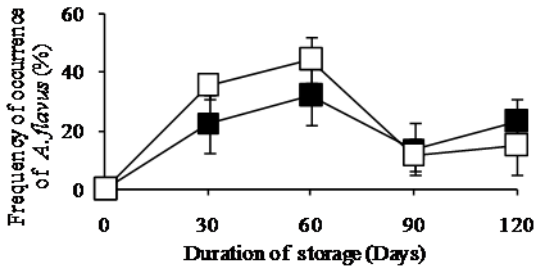

Figure 2. Frequency of occurrence of A. flavus in pepper powder 1 as a function of storage period. Values represent the mean of six replicates ± standard error; ■ A. flavus at

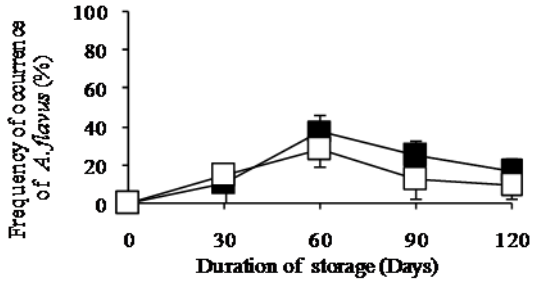

Figure 3. Frequency of occurrence of *A. flavus* in pepper powder 2 as a function of storage period. Values represent the mean of six replicates ± standard error; ■ *A. flavus* at ambient temperature; □ *A. flavus* at refrigeration temperature

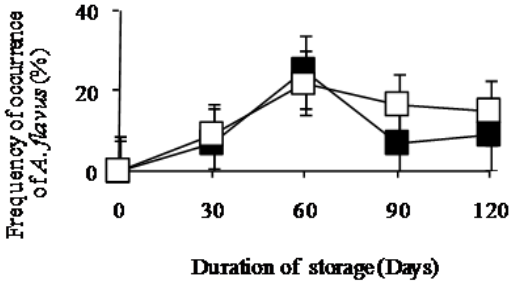

Figure 4. Frequency of occurrence of A. flavus in turmeric powder 2 as a function of storage period. Values represent the mean of six replicates ± standard error; ■ A. flavus at ambient temperature; □ A. flavus at refrigeration temperature.

Table 1: Aflatoxins in spice samples by day 90 and 120 visualized by TLC

Type of spice	Environment of storage	Presence/ absence of aflatoxinsa				Presence/ absence of aflatoxinsb			
		B1	B2	G1	G2	B1	B2	G1	G2
Curry powder 1	laboratory	+	+	-	-	+	+	-	-
Curry powder 1	refrigerator	+	+	-	-	+	+	-	-
Curry powder 2	laboratory	-	-	-	-	+	+	-	-
Curry powder 2	refrigerator	+	+	-	-	+	+	-	-
Pepper powder 1	laboratory	+	+	-	-	+	+	-	-
Pepper powder 1	refrigerator	+	-	-	-	+	-	-	-
Pepper powder 2	laboratory	+	-	-	-	+	-	-	-
Pepper powder 2	refrigerator	+	-	-	-	+	-	-	-
Chilli powder 1	laboratory	+	-	-	-	+	+	-	-
Chilli powder 1	refrigerator	+	+	-	-	+	+	-	-
Chilli powder 2	laboratory	-	-	-	-	+	+	-	-
Chilli powder 2	refrigerator	-	-	-	-	-	-	-	-
Turmeric powder 1	laboratory	+	+	-	-	+	+	-	-
Turmeric powder 1	refrigerator	+	+	-	-	+	+	-	-
Turmeric powder 2	laboratory	+	-	-	-	+	-	-	-
Turmeric powder 2	refrigerator	+	+	-	-	+	+	-	-

[a] = Presence / absence of aflatoxins in spice samples stored for 90 days
[b] = Presence / absence of aflatoxins in spice samples stored for 120 days
Values represent the observation of six replicate spots

meric powder 2 stored at room temperature and ambient temperature were quite similar. Frequencies of *A. flavus* both at refrigerator and ambient temperatures showed a zero frequency on day zero. Frequency of *A. flavus* at ambient temperature steadily increased to a maximum of 25% by day 60. Similarly, frequency of *A. flavus* at 4^0C increased to a maximum value (22.2%) by day 60 and again decreased to 7% on day 90 (Fig 1-4).

Aflatoxin in spice samples

Extracts of samples of spices from two producers, which were stored for less than 60 days, when spotted and developed on TLC, no fluorescing spots were observed. By day 90 and 120, fluorescing spots were observed in all TLC plates spotted with extracts of spice samples, except chilli powder 2 stored at 4^0C. (Table 1).

Except chilli powder 2 stored at 4^0C, all other extracts from spices stored for 120 days showed fluorescing spots on TLC, at an R_f value of 0.88. A similar R_f value was recorded for the aflatoxin B_1 sample. Fluorescing spots with R_f = 0.80 were identified from several spices. R_f values of these spots matched the R_f of the authentic standards of aflatoxin B_2 (Table 1).

Table 2: Aflatoxins in extracts of isolated *A. flavus* from spices visualized by TLC

Type of spice	Environment of storage	Presence/ absence of aflatoxinsa				Presence/ absence of aflatoxinsb			
		B1[c]	B2	G1	G2	B1	B2	G1	G2
Curry powder 1	laboratory	+	+	-	-	+	+	-	-
Curry powder 1	refrigerator	+	+	-	-	+	+	-	-
Curry powder 2	laboratory	+	+	-	-	+	+	-	-
Curry powder 2	refrigerator	+	+	-	-	+	+	-	-
Pepper powder 1	laboratory	+	+	-	-	+	+	-	-
Pepper powder 1	refrigerator	+	+	-	-	+	+	-	-
Pepper powder 2	laboratory	+	+	-	-	+	+	-	-
Pepper powder 2	refrigerator	+	+	-	-	+	+	-	-
Chilli powder 1	laboratory	+	+	-	-	+	+	-	-
Chilli powder 1	refrigerator	+	+	-	-	+	+	-	-
Chilli powder 2	laboratory	+	+	-	-	+	+	-	-
Chilli powder 2	refrigerator	+	+	-	-	+	+	-	-
Turmeric powder 1	laboratory	+	+	-	-	+	+	-	-
Turmeric powder 1	refrigerator	+	+	-	-	+	+	-	-
Turmeric powder 2	laboratory	+	+	-	-	+	+	-	-
Turmeric powder 2	refrigerator	+	+	-	-	+	+	-	-

[a] = Presence / absence of aflatoxins in extracts of *A. flavus* isolated from spices stored for 90 days
[b] = Presence / absence of aflatoxins in extracts of *A. flavus* isolated from spices stored for 120 days
Values represent the observation of six replicate spots.

Aflatoxin identification in extracts of *A. flavus* isolated originally from spice samples and grown in a liquid medium

Extracts of *A. flavus* isolated originally from spices of two producers, when spotted and developed on TLC, fluorescing spots of aflatoxin B_1 and B_2 were observed in chilli powder 1 stored at 4^0C and ambient temperature and chilli powder 2 stored at ambient temperature by day zero. Fluorescing spots of aflatoxin B_1 and B_2 were observed in extracts of *A. flavus* (originally isolated from spice samples) when subjected to TLC by day 90 and 120. However, Aflatoxin G_1 and G_2 could not be detected from any of the spice sample extracts (Table 2).

DISCUSSION

Temperature between 27°C – 38°C and a relative humidity of 85% is optimum for the growth of *Aspergillus flavus* (Grybauskas *et al.* 1988). During the current research, we assumed that the humidity and temperature inside the storage containers remained in equilibrium with the ambient humidity and temperature levels, as the containers were not 100% airtight. In the laboratory, the temperature and humidity conditions that favor the growth of *A. flavus* was satisfied to a certain extent. Therefore, a higher frequency of occurrence of *A. flavus* was

observed in most of the spice samples, stored at ambient temperature.

In this study, both pepper powder 1 and 2 supported the growth of several fungal species including aflatoxigenic *A. flavus* and *A. niger*, however the frequency of occurrence of *A. flavus*, was low to moderate (27.7% to 44.2%). Pepper oil, has previously been reported as having antimicrobial and antiaflatoxigenic properties (Patkar *et al.* 1993). Thus, the lower frequency of occurrence of *A. flavus* may be due to the antifungal property of pepper in ground spice samples.

Although the patterns of frequencies of occurrence were somewhat similar in curry powder 1 and 2, the maximum values were dissimilar. The discrepancy may be due to the variations in compositions and proportions of curry powder ingredients used by the two producers. According to the producer 2, curry powder 2 is prepared by grinding and mixing of cumin, fennel, coriander, black pepper, *Pandanus* leaves, curry leaves, cardamom and turmeric in an approximate proportion of 50:10:10: 5:2:2:1:1. The relative antimicrobial activity in the mixture of spices could well be due to the action of various essential oils present (Patkar *et al.* 1993). According to Chowdhury *et al.* (2008), carvacrol, citral, geraniol and menthol present in tumeric essential oil exhibit antifungal/antibacterial properties.

The pattern of frequencies of occurrence and the maximum values also were different in chilli powder 1 and 2. These differences could be due to the disparities in pre and post-harvest practices. Lower frequency of occurrence of *A. flavus* could be due to the presence of potent alkaloids in chilli powder such as capsaicin (www. http:// unitproj.library.ucla.edu/biomed/spice/index.cfm, 2002).

During the entire period of storage of spices, 13 fungal species were detected including *Aspergillus flavus*. Extracts of *A. flavus* (isolated originally) from stored spice samples showed fluorescing spots indicating the presence of aflatoxin B_1 and (some times B_2) during TLC analysis. Around 90^{th} and 120^{th} days of storage, frequency of *A. flavus* declined. It has been previously reported that mycelial growth of *A. flavus* was proportional to the production of aflatoxins; growth can also occur without toxin production and *vise versa* (Jay 1992). During a previous research conducted in Sri Lanka, among the rice samples tested, parboiled rice indicated a higher frequency of occurrence (83.5%) of *A. flavus* after three months of storage with a low level of aflatoxin of 7.3mgkg^{-1} (Jayaratne 2003).

The potential for growth of *A. flavus* on spices should be high in tropical countries including Sri Lanka, due to the favorable temperature, moisture and humidity conditions prevailing during the storage. According to the preliminary study conducted, the storage of ground spices namely, curry, chilli, turmeric and pepper powders lead to the growth of *A. flavus* and production of aflatoxin. Storage under refrigeration conditions does not seem to be a solution to avoid the proliferation of toxigenic fungi in spices. However, the observations of this study could be useful, as many spices are consumed in substantial amounts daily in Asia and many other continents in the world.

CONCLUSIONS

During this survey *A. flavus* was isolated from all spice samples tested. A higher frequency of occurrence of *A. flavus* was observed in most of the spice samples, stored at ambient temperature. Aflatoxin producing fungi are prevalent on spices used for culinary purposes. It is advisable to store and consume spices such as chilli powerder, tumeric, pepper powder *etc.* within a relatively short period to avoid build up of aflatoxins.

ACKNOWLEDGMENTS

We acknowledge Prof. Priyani Paranagama, University of Kelaniya, Sri Lanka for providing authentic standards of aflatoxins.

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