The International Journal of Plant Reproductive Biology 8 (Special Volume), pp.82-87
DOI 10.14787/ijprb.2016 Spl. 82-87

Detection of aflatoxin in isolates of *Aspergillus flavus* and effect of ultraviolet Rays on its inactivation

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Received : 12.04.2015. Received : 10.06.2015; Accepted : 06.07.2015; Published online : 01.01.2016

ABSTRACT

Aspergillus flavus is the most prominent fungi for the production of aflatoxin in food & feeds especially when storage conditions favor fungal growth. *A. flavus* sp. often produces Aflatoxin B_1 & B_2 but all the strains of the *A. flavus* are not aflatoxigenic in nature. Aflatoxins are sensitive to UV irradiation, which may lead to the formation of photo degradation products. In order to kill microorganisms, the UV rays must actually strike the cell. The present study is focused on the elimination of aflatoxin producing fungi and the effect of ultra violet rays on aflatoxin inactivation or production in the strains. Ten different strains of *A. flavus* at sporulating stages were examined for the production of aflatoxin. Selected two aflatoxigenic isolates exposed to germicidal ultra violet rays at 265 nm to reduce the aflatoxin content in the fungi. Selection of the aflatoxigenic strains and the Effects of UV rays on aflatoxin production were analyzed by Thin Layer Chromatography and determination of total aflatoxin content by ELISA.

Keywords : Aflatoxin, *Aspergillus flavus*, UV rays

Aflatoxins are carcinogenic, mutagenic and teratogenic substances that have been frequently found in several products including peanuts, corn and cottonseed. In India, peanut contamination with aflatoxins is the major problem owing to climatic conditions during harvest & storage. Several species of the *Aspergillus flavus* group not only cause food spoilage but also produce potent aflatoxins (Varga *et al.* 2003). All the strains of *A. flavus* group are not aflatoxigenic in nature. In addition, the cultivation and chemical compounds in the substrate may affect the aflatoxin biosynthesis of an aflatoxin producer. Loss of aflatoxigenecity may result from domestication. Thus, the risk exists of a nontoxigenic phenotype changed to an aflatoxin producer when cultivated with a different set of conditions of substrates (Chen *et al*. 2002).

Current control measures are aimed at controlling fungal growth and aflatoxin formation in stored grains by physical methods (aeration, drying, heating, cooling, and modified atmosphere), chemical methods (ammonization, acids, bases, and food preservatives) or biological methods. However, physical methods are best treatment to inhibit, destroy, or remove undesirable microorganisms without involving antimicrobial additives or products of microbial metabolisms as preservative factors. UV or ionizing radiation is an established physical microbicide treatment (Hitoshi *et al*. 1994). The ultraviolet portion of the light spectrum includes all radiations with wavelengths from 100 nm to 400 nm. It has low wavelength and low energy. The microcidal activity of ultraviolet (UV) light depends on the length of exposure: the longer the exposure the greater the cidal activity. It also depends on the wavelength of UV used. The most cidal wavelength of UV light lies in the 260-270 nm range where it is absorbed by nucleic acid. UV energy penetrates the outer cell membrane of the microorganisms, passes through the cell body and disrupts its DNA preventing reproduction (Rustom.1997; Levetin *et al*. 2001).

Peanut kernels are good substrate for growth and subsequent aflatoxin production by aflatoxigenic molds (Diener & Davis. 1966).). Ten strains of *A. flavus* including the one isolated from peanut kernels were cultured & subjected to aflatoxin detection and the effects of ultra violet rays on inactivation of aflatoxins using TLC were studied. TLC is a technique used commonly in developing countries, owing to its simplicity and practicability with the desired precision and analyte identification even allows the visual quantification of aflatoxins (Jaimez *et al*. 2000 ; Omurtag & Yazicioglu. 2001; Stoka & Anklam. 2002).The present study focuses on the efficacy of UV radiation on aflatoxin producing ability of *A. flavus* isolate and standard strains of *A.flavus*

MATERIALS & METHODS

Fungal strains—Ten strains of *A. flavus*, including eight strains from IARI (Delhi), one strain from IMTECH (Chandigarh) and one strain isolated from peanut kernels have been used for the study.

Isolation of *A. flavus* from peanuts—Peanut sample (10 gm.) was aseptically destined and weighed into stomacher bags. The sample was transferred to 90 ml of sterile normal saline of for maximum recovery and contents were homogenized by vortexing. Tenfold serial dilutions were prepared in sterile water, and 1 ml of aliquots were plated directly on Aspergillus Differential Media (HiMedia) for identification and enumeration of *A. flavus* & *A. parasiticus* (Indian Standard. 1999). The plates were incubated at 28º C for 5 days. The identification of the fungus as *A. flavus* by Lactophenol-cotton blue (HiMedia) staining and then it was confirmed by Indian Type Culture Collection, IARI, New Delhi. All strains were stored as slant cultures using Potato dextrose agar (HiMedia) at 4º C till used for further study.

Preparation of mycelia clumps and Aflatoxin production—Yeast Malt Broth was used as growth media for mycelia growth. 10 ml of reconstitute media in 50 ml centrifuge tube were autoclaved. Each tube was inoculated with a loopful of conidia from all fungal strain cultures and incubated at 25º C for 9 days to obtain aflatoxin in detectable yield and the mycelium had fully grown on the broth surface (Diener & Davis.1966)). The mycelia clumps were harvested by centrifugation (8,000x g for 1 min at 20º C) and supernatant broth was subjected to aflatoxin detection.

Detection of Aflatoxin—5 ml of broth was withdrawn into another 20 ml centrifuge tube, mixed with 6 ml of chloroform, and vortex for 5 min. Then 5 ml of chloroform was removed to glass tube and flushed with nitrogen to dryness under a hood. The residue was dissolved in 100 ml of methanol out of which 20 ml was transferred for loading onto a thin layer chromatography plate (DC-Alufolien Kieselgel 60 F254, E Merck, Darmstadt, Germany). A Standard Aflatoxin Solution prepared in methanol containing aflatoxin B1 (Sigma, Israel), G1 and G2 (Acros, New Jersey, USA) (20 ppb for all three aflatoxins) and concurrently 5ml was loaded on the TLC plate. The unique fluorescent spots were viewed under a UV box at 365 nm after separation of solute from solvent (Acetone- chloroform, 85:15 (v/v) in thin layer chromatography (Chen *et al*. 2002, Kumar *et al*. 2002).

Ultra-violet treatment—Ten strains of the *A. flavus* were selected for the production of aflatoxin, the results of which are presented in Table1. The aflatoxigenic strains were further treated with UV rays for the effects of radiations on aflatoxin inactivation. Fresh cultures of the aflatoxigenic strains on Potato Dextrose media plates were exposed directly to UV-C radiation (265 nm, germicidal) (Rustom 1997, Eccleston 1998) at 12 cm distance for different duration i.e. 2h, 5h, 8h, & 12hrs) in triplicates. The loopful of conidia from the exposed fungi were taken in sterile Yeast Malt broth for preparation of mycelia clumps and production of aflatoxin in centrifuge tubes. The above experiments were repeated for detection of aflatoxin.

Quantification of Aflatoxin by ELISA—50µL of standard solution of aflatoxin and cleaned eluted sample in duplicate added to the wells of microtitre plate. After that 50 µL of peroxidase enzyme conjugate and 50µLof mouse monoclonal anti-aflatoxin antibodies were added to each well and incubated at room temperature in the dark for 30 minutes. After washing thoroughly with 250 µL distilled water three times, 50µL of urea peroxidase (substrate) and 50µL of tetramethylbenzidine (chromogen) were added to each well, mixed thoroughly and incubated for 30 minute at room temperature in the dark. Reaction was stopped by adding 100µL 1M Sulphuric acid (Stop reagent) and the absorbance were

measured at 450nm using ELISA microplate reader Model 680 (Bio-Rad).A calibration curve was drawn using a wide range of total aflatoxin standards with concentration of 0 ppt to 4050 ppt. A plot in between the percentage absorbance and concentration of aflatoxin for a set of standard indicated a linear relationship (Fig1).

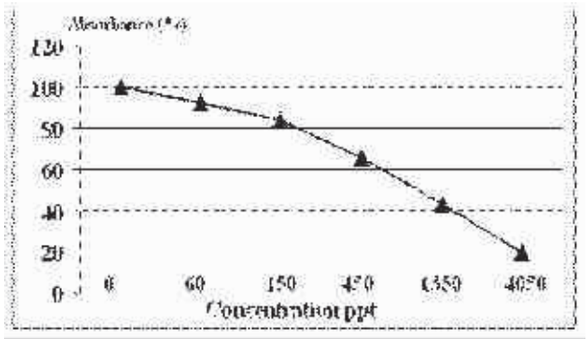

Fig. 1— Standard curve of different concentration of Total Aflatoxin.

RESULT AND DISCUSSION

Table 1 has shown ten different strains of *A. flavus*, including eight strains from IARI (Delhi), one strain from IMTECH (Chandigarh) and one strain isolated from peanut kernels have been cultured and screened for aflatoxin detection through Thin Layer Chromatography. TLC is a technique used commonly in developing countries, owing to its simplicity and practicability with the desired precision and analyte identification even allows the visual quantification of aflatoxins (Stroka & Anklam 2002). The Table 1 showed the appearance of the fungal colonies of the strains on PDA media. All the strains of *Aspergillus* species showed different culture characteristic and colors. Four strains i.e. 1717, 1419, *A. flavus* MTCC 2008 and the strain isolated from peanut, showed some similarities in morphology.

Figure 2 showed the screening of aflatoxigenic strain by TLC plate method having 4 different strains of *A. flavus* (ITCC 1717, isolated strain from peanut, ITCC1419 and MTCC 2008) with aflatoxin standard (mixture of B1,G1 & G2 of the concentration of 2ppb) under long wave ultraviolet rays (365 nm). The aflatoxin standard showed three different fluorescent spots on TLC. The upmost spot was B1, middle one was G1 and lower one was G2. Only aflatoxigenic strains showed fluorescence under UV i.e. ITCC 1717 and isolated strain of *A. flavus* from peanut. All the strains tested for aflatoxin production by thin layer chromatography only two *A. flavus* strains showed blue-green fluorescence on TLC plates under UV rays (365 nm) as shown by aflatoxin standard. *Aspergillus* section *flavi* strains isolated from peanuts, wheat and soyabean grown in Argentina were screened for aflatoxin production. *A. flavus* was the predominant species in all substrates. *A. flavus* isolated from all substrates was very high and incidence of aflatoxigenic *A. flavus* strains was higher in peanuts (69%) than wheat (13%) or soyabean (5%) (Vaamonde *et al*. 003). Sundin & Jacobs (1999) analysed ultraviolet radiation (UVR) sensitivity and UV radiation survival strategies of a bacterial community from the phyllosphere of field-grown peanut. Selected two aflatoxigenic isolates exposed to germicidal Ultra violet

S.No	Strain tested	Appearance on slant	Appearance on AD Media	Presence of Aflatoxin
1.	*A.flavus* ITCC 1670	Brownish	Light Green	ND
2.	*A.flavus* ITCC 315	Green	Grassy green	ND
3.	*A.flavus* ITCC 1654	Dark green	Grassy green	ND
4.	*A.flavus* ITCC 1623	cottony	Cottony	ND
5.	*A.flavus* ITCC 325	Brownish black	Dark green	ND
6.	*A.flavus* ITCC 1466	Cottony Brown	Whitish Green cottony	ND
7.	*A.flavus* ITCC 1717	White cottony	Whitish Green	+
8.	*A.flavus* ITCC 1419	Brown	Green cottony	ND
9.	*A.flavus* MTCC 2008(IMTECH)	Green	Green	ND
10.	*A.flavus* isolate from peanut	Green	Green	.

Table 1—Different types of *Aspergillus flavus*, their appearance and capability of aflatoxin production

rays at 265 nm to determine the detoxifying ability on tested *A.flavus* strains. Selection of the aflatoxigenic strains and the effects of UV rays on aflatoxin production were analyzed by Thin Layer Chromatography and determination of total aflatoxin content by ELISA.

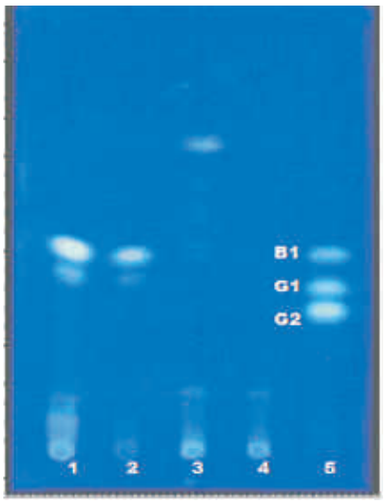

Fig. 2—TLC plate of extracted aflatoxins from different *A. flavus* with aflatoxin standards.
The spots from left to right—
1. ITCC 1717, 2. Isolates from peanuts, 3. ITCC 1419, 4. MTCC 2008, 5. Mixture of aflatoxin std. B1, G1 and G2.

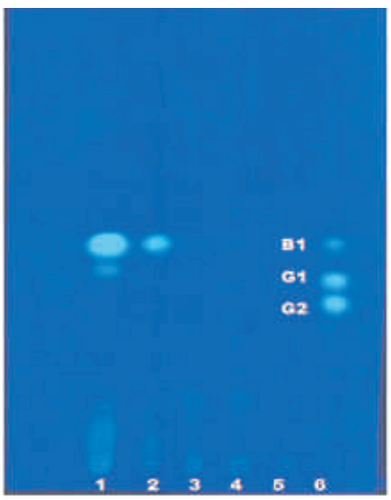

Fig. 3—Effects of different UV radiation doses on extracted aflatoxins of 1717(ITCC).
The spots from left to right—1. Control 1717 (ITCC), 2. UV irradiated 1717 (2 h), 3. UV irradiated 1717 (5 h), 4. UV irradiated 1717 (8 h), 5. UV irradiated 1717 (12 h), 6. Mixture of aflatoxin std. B1, G1 and G2.

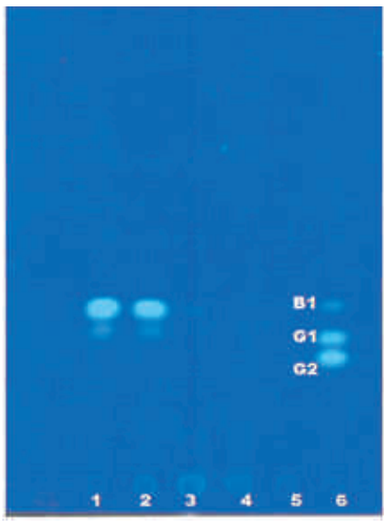

Fig. 4—Effects of different UV radiation doses on extracted aflatoxins from *A. flavus* isolate.
The spots from left to right—1. Control of isolated *A. flavus* from peanuts, 2. UV irradiated *A. flavus* strain of peanut (2h), 3. UV irradiated *A. flavus* strain of peanut (5h), 4. UV irradiated *A. flavus* strain of peanut (8h), 5. UV irradiated *A. flavus* strain of peanut (12h), 6. Mixture of aflatoxin std. B1, G1 and G2.

The both selected *A.flavus* strains were exposed to ultra violet rays at different duration such as 2 h, 5 h, 8 h and 12 h. The distance between source and the object was 12 cm and the source power was 9 watt. The results indicate that the aflatoxin was reduced as the duration of exposure was increased. It is very clear in the photographs that extracted aflatoxin matched with aflatoxin B1 of the standard solution. The extracted aflatoxin of the control showed maximum fluorescence and it goes in decreasing order in UV exposed samples (2h, 5 h), even in 8 and 12 h exposure time the fluorescence was not observed under UV light. The aflatoxigenic strains 1717 and the isolated strain of *A. flavus* from peanut have been showed almost similar pattern of aflatoxin reduction. In both cases aflatoxins were inactivated by UV rays and on longer period exposures, the aflatoxins were undetectable by TLC.

To confirm the results of TLC, both the extracted aflatoxin samples from *A. flavus* ITCC1717 and isolates from peanut strains were quantified with ELISA. Fig.5 and Fig.6 showed non- irradiated strains and the irradiated strains with different doses of UV radiation (2

h, 5 h, 8 h and 12 h) were determined by ELISA. It was found that, the total aflatoxins value, present in the fungi before and after the irradiations. These results showed that 12 h UV radiation detoxified aflatoxins up to 94% to 98% in both the cases.

The detoxification of aflatoxin in fungi by UV could be due to two mechanisms: the direct inactivation of *A. flavus* conidia by the radiation or a change in the aflatoxin biosynthesis. UV-C is germicidal and, hence can inactivate fungal spores. Aflatoxins are sensitive to UV radiation. AFB_1 absorbs UV light at 222, 265 and 362 nm, which may lead to the formation of up to 12 photo degradation products (Samarajeewa *et al*. 1990). Exposure of artificially contaminated milk to UV light inactivated 3.6-100% of AFM_1 in the milk, depending on exposure time (2-60 min). Also, addition of hydrogen peroxide (1%) to the UV- irradiated milk (10 min) completely (100%) destroyed AFM_1 (Yousef & Marth 1985). The photo-degradation products were less toxic to chick embryo than the parent toxins (Andrellos *et al.* 1967). Treatment of peanut oil with UV light for 2 h destroys 40-45% aflatoxin (Shantha & Murthy 1977). UV Radiation (30 min) of dried figs artificially contaminated with AFB_1 (250 µg/Kg) reduced the aflatoxin level by 45.7% (Altug *et al*. 1990). Green *et al* (2004) also reported that 35 mJ/cm2 ultraviolet irradiation can cause 90% inactivation of *A. flavus* on an agar surface

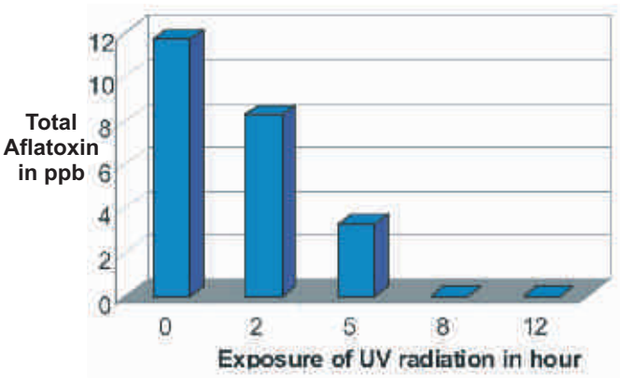

Fig. 5—Effect of UV radiation on total aflatoxin production of *A.flavus* ITCC 1717 at different interval of time.

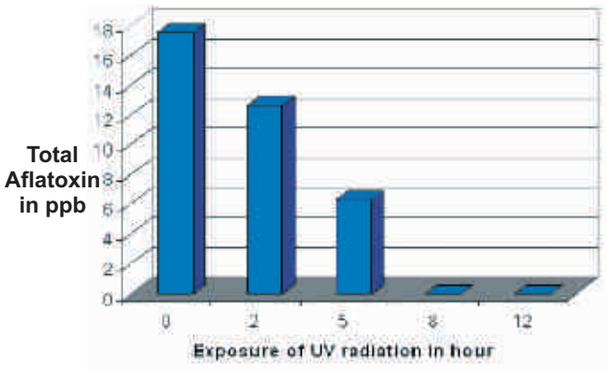

Fig. 6—Effect of UV radiation on total aflatoxin production of *A.flavus* isolate at different interval of time.

UVC radiation was reported to significantly reduce the concentrations of *P. corylophilum, Aspergillus versicolor* and *Cladosporium* spp. within air-handling units (Levetin *et al*. 2001).

In conclusion UV irradiation is quiet effective in detoxifying aflatoxin in *A. flavus* after exposing the fungi to different interval of exposure time. The most effective duration was 8 h and 12 hrs. At this exposure time aflatoxin was reduced tremendously which was available in trace amount. As per specification laid down by the Indian Government the Total aflatoxin level should be less than 30 ppb. The UV radiation could be better option for the detoxification of food commodities. However, the effect of radiation on food value should be investigated to know the loss or no effect on food properties,

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