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RESEARCH PAPER

Estimation of aflatoxin production in the seeds of *Beta vulgaris* var. *Cicla, Amaranthus tricolor* and *Portulaca oleracea* at different relative humidity levels during storage

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Abstract : The experiment was conducted to study the effect of relative humidity and moisture content on seed germination and aflatoxin production of three vegetable seeds during storage. Six *Aspergillus flavus* strains were isolated from the three leafy vegetable seeds and screened for aflatoxin production. The evaluation of toxigenic potential of the isolates showed that 73.33 per cent of the *A. flavus* strains isolated from the seeds of three different leafy vegetables collected from Mbnr and RR districts presented a toxigenic potential. Among the *A. flavus* 66.67 per cent isolates were producers of aflatoxin B_1 and 33.33 per cent of the isolates produced aflatoxin G_1. Aflatoxin B_1 was detected in the *A. flavus* treated seeds of Bv and At collected from both Mbnr and RR dist. While, aflatoxin G_1 was detected only in Bv samples of Mbnr and RR dist. The results showed that the storage period of vegetable seeds is a critical phase, as indicated by the high percentage of aflatoxigenic isolates.

Key Words : Aflatoxin, *Aspergillus flavus*, Relative humidity, Seed germination

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INTRODUCTION

Deterioration of stored seeds and grains by toxigenic fungi is a chronic problem in the Indian storage system because of the tropical hot and humid climate. Harvested grains are infested by various species of *Aspergillus*, leading to deterioration and mycotoxin production (Reddy *et al.*, 2008). The aflatoxin producing fungi are widely distributed in nature and can grow over a wide range of environmental conditions (Holmquist *et al.*, 1983). Aflatoxins are well known to be potent toxic, carcinogenic, mutagenic, immunosuppressive, teratogenic agents, also inhibit several metabolic systems. Aflatoxins are biologically active secondary metabolites produced by *A. flavus* and *A. parasiticus* (Olsen *et al.*, 2008; Reddy *et al.*, 2009 and Shapira *et al.*, 1997). Among 18 different types of aflatoxins identified, major members are aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2. Aflatoxin B_1 is a potent carcinogen classified by the International Agency for Research on Cancer as a group I carcinogen, *i.e.*, a potent hepatocarcinogen for animals and humans (IARC, 2002). Besides their effect on the health of humans and animals, aflatoxin also has an impact on the agricultural economy through the loss of crop production. Aflatoxins affect certain plants by inhibition of seed germination (Schoental and White, 1965). Aflatoxin B_1 alters the physiology

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of seeds and seedlings (YaqubBhat and Fazal, 2011), also restricts plant growth by inhibiting seed germination, seedling growth and other physiological processes of plants (Sinha and Kumari, 1990).

Various reports have established the natural occurrence of aflatoxins in many crops and agricultural commodities, fruits and vegetables which indicate the ubiquitous nature or wide spread distribution of the fungus (Anjana and Sinha, 1983 and Hesseltine, 1974). Aflatoxin contamination is now well recognized as a public health hazard in agricultural commodities (Reddy *et al.*, 2010). Moisture and temperature are the two factors which affect seed germination, besides affecting mold growth and mycotoxin production (Bullerman *et al.*, 1984). Tariq *et al*. (2005) also reported that high moisture and temperature increase the infection of *A. flavus* and decrease the germination of soy bean seed. High moisture increased the incidence of *A. flavus* and aflatoxin B_1 production on sunflower seeds (Dawar and Ghaffar, 1992). During twelve months storage of three vegetable seeds *viz.*, *B. vulgaris* var. *cicla*, *A. tricolor* and *P. oleracea* decrease in field fungi and increase in storage fungi was noticed in our earlier study results (Rao *et al.*, 2011). *Alternaria, Fusarium* and *Cladosporium* are major field fungi, *Aspergillus* and *Penicillium* are storage fungi. *A. flavus* and *Penicillium* species were found to be the most predominant fungi on all the three vegetable seeds (Rao *et al.*, 2011) which are known to produce aflatoxin B_1, B_2, G_1 and G_2 in food and feed stuff (Peskta and Bonday, 1990). Many workers have found that poor harvesting practices, improper storage and drying leads to fungal growth and toxin proliferation (Reddy *et al.*, 2001; Bankole and Adebanjo, 2003 and Ravikiran *et al.*, 2005).

The leafy vegetables *i.e. Beta vulgaris* var. *cicla*; *Amaranthus tricolor* and *Portulaca oleracea*, are the important crops used by human beings. They occupy a large area under cultivation throughout Andhra Pradesh. The seeds are stored mainly for the propagation of these leafy vegetables. They constitute an important component in human diet. Similar information on vegetable crop seeds is very scanty. Hence, it was considered to study the effect of relative humidity and moisture content on seed germination and aflatoxin production of three vegetable seeds during storage.

MATERIAL AND METHODS

Collection of seed samples :

Seeds of three leafy vegetable crops, *Beta vulgaris* var. *cicla*, L., *Amaranthus tricolor*, L. and *Portulaca oleracea*, L. were collected, from Mahbubnagar and Rangareddy districts of Telangana state, India. These crops are cultivated throughout the year. Random seed samples were obtained from 4 geographical locations throughout the major fields.

Isolation of *A. flavus* :

Seed samples of three leafy vegetables were surface sterilized in 1 per cent sodium hypochlorite solution for 10 minutes and then rinsed thrice with sterile distilled water (SDW). The surface sterilized seeds were aseptically plated on Petri plates containing modified Czapek Dox agar medium with Rose Bengal (30 mg/L) and streptomycin sulphate (0.2 g/L) and incubated at 28 ± 1ºC for 7 days. The colonies appearing in the Petri plates were picked up, based on cultural and morphological characteristics, the toxigenic fungus *A. flavus* was identified, and single spore isolates were maintained on potato dextrose agar (PDA) slants at 4ºC.

Relative humidity studies :

250 g seeds of each variety were surface sterilized as mentioned earlier. Excess water was decanted from the seeds and dried at 55°C in a hot air oven. They were inoculated with 5 ml of spore suspension prepared from 7-day-old culture of each *A. flavus* strain grown on PDA medium. The seeds were incubated at 75 per cent and 85 per cent relative humidity levels, maintained in the desiccators (Wink and Sear, 1950), at room temperature (28 ± 2ºC) for a period of 90 days. The relative humidity levels of 75 per cent and 85 per cent were maintained by saturated NaCl and KCl solutions and the desiccators were made air tight. The seeds of each sample were harvested periodically at 15, 45 and 90 day intervals and analyzed for moisture content, germination percentage and elaboration of aflatoxins. The untreated seeds were used as control. Three replicates were maintained for each sample.

Determination of seed moisture content (SMC) :

Each seed sample (100 g) was ground in a blender and known weight of the resultant powder was dried in an oven at 105ºC for 24 hours, cooled in a dessiccator and reweighed. The moisture content (MC) is expressed as the percentage of the wet weight.

Seed germination test :

The treated and non-treated seeds were plated (5 per plate) in Petri plates containing germination paper with thin layer of sterile cotton soaked with sterile distilled water. Germination counts were taken after incubation period of 72hrs. Germination percentage was determined as per ISTA rules (1996).

Extraction of aflatoxin from artificially infested seeds :

Aflatoxins in seeds sample were estimated following the method of Pons *et al*. (1966). 50g sample and 250ml acetone: water (85:15 v/v) were mixed thoroughly in Erlenmeyer flask by shaking for 30min. The extract was filtered through Whatman number 1 filter paper. To 100ml of this filtrate, 20ml of 20 per cent lead acetate and 60ml of water were added. Lead acetate was used for precipitation of insoluble pigments as lead derivatives. The volume was brought down to 150ml by subjecting to boiling in a steam bath, later it was cooled to

20°C in ice water. This extract was then centrifuged at 2000 rpm for 10min. The precipitate was dispersed by 60ml of acetone and re-centrifuged. The supernatant solution was taken in a 250ml separating funnel and shaken vigorously for 1min with 50ml chloroform. The extraction process was repeated twice. The lower phase (chloroform layer) was collected in a 250ml beaker and was allowed to evaporate on a steam bath until 2-3ml extract was left behind. This was then transferred to the chromatographic column with 10ml of chloroform. The column was first washed with 100ml of di-ethyl-ether. This was discarded and then 150ml of chloroform : methanol (97:3) was passed through the column. This was collected in a 250ml beaker, after passing through a layer of anhydrous Na_2SO_4. It was evaporated to near dryness and dissolved in 1ml of chloroform. The sample extract was spotted on the activated TLC plates. The plates were developed in chloroform : acetone (95:5 of 97.3 v/v) solvent system.

Subsequently, they were air dried at room temperature for about 10min and observed under long wave-ultra violet light (365nm) illumination. The aflatoxins were confirmed chemically by treating the fluorescent spot with tri floro acetic acid (TFA) (Stack and Pohland, 1975). Quantitative estimation of aflatoxins in the extract was carried spectrophotometrically (Nabney and Nesbitt, 1965) using standard aflatoxins (Sigma) as reference.

Screening of aflatoxins from *Aspergillus flavus* isolates :

Six *Aspergillus flavus* strains isolated from the three leafy vegetable seeds were screened for aflatoxin production.

Toxigenicity of *Aspergillus flavus* strains :

Spores from 8-day-old cultures of each *A. flavus* isolates grown on PDA were washed into SDW containing 0.01per cent Tween 80. The spore concentration was adjusted to 10^5 spores/ml using a haemocytometer, and 500µl of this spore suspension was added to 50ml of semi-synthetic liquid medium in 250ml Erlenmeyer flasks. One ltr. of the semisynthetic liquid medium contained the following: sucrose, 200g; $MgSO_4 \cdot 7H_2O$, 0.5g; KNO_3, 3.0g; and yeast extract, 7.0g (Diener and Davis, 1966). The flasks were incubated at 28 ± 1°C on a mechanical shaker at 120 rpm for 20 days. Five flasks were maintained for each *A. flavus* isolate. The cultures were then filtered through Whatman filter paper number 1. The culture filtrates of all the replicates were pooled and the experiment was repeated twice. To 40ml of culture filtrate, 20ml of chloroform was added and shaken for 5 minutes in a separating funnel. The chloroform layer was collected and passed through anhydrous sodium sulphate in a column. Finally, the chloroform was evaporated to dryness on a rotary vacuum evaporator and the residue was dissolved in 100µl of chloroform and used for analysis of aflatoxins by TLC.

Preparation of thin layer chromatograms :

The TLC (Thin Layer Chromatograms) plates for spotting the extract were developed as detailed in AOAC methods (1995). 30g of silica gel was mixed with water, shaken vigorously for 1min and poured into applicator by adjusting with H_2O to obtain best consistency of slurry for spreading. Immediately five 20×20cm glass plates were coated with 0.25nm thickness of silica gel suspension, and the plates were left undisturbed until gelled (10min). Dry coated plates activated for 2hr at 80°C and stored in desiccating cabinet with active silica gel desiccant until just before use.

Development of chromatograms :

Aflatoxin extraction and analysis :

2, 5, and two 10µl of the extracted samples were spotted on imaginary line 4 cm from bottom edge of pre coated TLC plate along with different standard aflatoxin compounds (Sigma Chemical Co., St. Louis, MO, USA) dissolved in chloroform (10µg/ml) spotted along with the test samples. The chromatoplates were developed in a tank (using only one plate per tank) containing chloroform : methanol in the ratio of 97:3 for 30min, dried at 23-25°C. After the development, the plates were viewed under long UV light at 365nm. Blue-fluorescence similar to standard Aflatoxin B_1 indicated the presence of aflatoxin B_1 and green-fluorescence indicated the presence of aflatoxin G_1 in the sample fractions.

Quantification of aflatoxin B_1 by TLC :

The TLC plates were developed in a tank containing chloroform: acetone in the ratio of 88 : 12, for 30min. Quantification of aflatoxin B_1 was done using long UV light by evaluating the plate itself. Aflatoxin content in each sample was calculated using the formula :

$$\text{Aflatoxin} = \frac{\text{Fluorescent intensity of the standard factor} \times \text{concentration of the standard} \times \text{dilution factor}}{\text{Fluorescent intensity of the sample}} \text{x100}$$

RESULTS AND DISCUSSION

Six toxigenic fungal isolates of *Aspergillus flavus* were isolated and designated as: S_1 (Strain 1) and S_2 (Strain 2) from *Beta vulgaris* var. *cicla,* of Mahbubnagar (Mbnr) and Rangareddy (RR) districts, respectively; S_3 (Strain 3) and S_4 (Strain 4) from *Amaranthus tricolor,* of Mbnr and RR dists., respectively; S_5 (Strain 5) and S_6 (Strain 6) from *Portulaca oleracea,* of Mbnr and RR dists., respectively.

Relative humidity studies :

The results of three leafy vegetable seeds treated with *A. flavus* stored at two different relative humidity (RH) levels *viz.,* 75 per cent and 85 per cent, and tested for moisture content, percentage of germination, qualitative and quantitative estimation of aflatoxins are presented in Table 1.

Moisture content :

The *A. flavus* treated seeds of three leafy vegetables,

showed high moisture content (MC) compared to untreated control samples recording maximum in 90d stored samples at 75 per cent RH. Among the selected seed samples, *A. tricolor* seed samples recorded maximum MC compared to the other seed samples. The *A. flavus* treated seeds of *A. tricolor* and *P. oleracea* stored at 75 and 85 per cent RH levels showed increase in MC compared to untreated controls. However, seeds of *B. vulgaris* (Bv) stored at both RH levels recorded decrease in MC over non treated controls. The *A. flavus* treated seeds of three leafy vegetables, showed increase in MC with increase in storage period. However, showed gradual increase up to 45d storage period thereby, decreased in 90 days stored samples incubated at 85 per cent RH. Similar trend was observed in non treated control samples. It is a well known fact that moisture content of the seed increases depending on the humidity prevailing in the seed environment. In the present investigation, the MC increased during storage except in *A. flavus* treated seeds at 85 per cent RH. However, *A. flavus* treated and control seeds showed higher MC levels at 85 per cent RH than at 75 per cent RH. These results are in confirmation with the earlier reported results (Waghray and Vaidehi, 1988). Increase in MC could be due to the fungal activity which was more at higher relative humidity as reported earlier (Singh, 1987 and Bhushan *et al.*, 2013).

Germination percentage :

In both treated and non-treated seed samples it was observed that percentage of germination decreased correspondingly with increase in MC of seed, RH and storage period (Table 1). Further, it was observed that percentage of seed germination reduced in *A. flavus* treated seed samples over non treated control samples. The initial germination of the non treated control seeds was 80 to 90 per cent and it decreased as the storage period increased.

The treated and untreated seeds of Bv stored at both 75 per cent and 85 per cent RH levels showed poor germination

Table 1 : Percentage of moisture content (MC), germination percentage (GP), Qualitative estimation of aflatoxin (QeAf) and amount of aflatoxin (AmAf) of seeds treated with *Aspergillus flavus* and untreated seeds of three leafy vegetables stored at different relative humidity levels for 90 days

Fungal treatments	RH (%)		Storage period in days											
			0	15	45	90	0	15	45	90	0	15	45	90
			Beta vulgaris var. *Cicla*				*Amaranthus tricolor*				*Portulaca oleracea*			
Aspergillus flavus	75	MC	–	9.06	10.1	10.33	–	11.94	12.48	12.91	–	9.2	9.52	9.77
		GP	–	35	20	15	–	30	40	15	–	90	80	65
		QeAf	–	–	B_1	B_1	–	–	B_1	B_1	–	–	–	B_1
		AmAf	–	–	171	154	–	–	135	135	–	–	–	104
	85	MC	–	11.54	10.92	10.3	–	13.38	13.74	12.17	–	10.2	11.06	9.81
		GP	–	35	20	30	–	45	35	15	–	90	70	40
		QeAf	–	–	B_1	B_1	–	–	B_1	–	–	–	–	–
		AmAf	–	–	178	178	–	–	85	–	–	–	–	–
Control	75	MC	14.5	9.42	10.1	10.56	10.02	10.02	11.02	12.11	10.6	6.96	5.72	9.11
		GP	85	20	20	10	90	70	55	30	80	85	85	70
		QeAf	–	–	–	–	–	–	–	–	–	–	–	–
		AmAf	–	–	–	–	–	–	–	–	–	–	–	–
	85	MC	14	9.9	11.7	11.32	10	12	13.14	12.15	10	7.42	8.35	9.49
		GP	90	15	10	50	90	65	45	40	85	95	95	90
		QeAf	–	–	–	–	–	–	–	–	–	–	–	–
		AmAf	–	–	–	–	–	–	–	–	–	–	–	–

Table 2 : Aflatoxin production by six isolates of *Aspergillus flavus*

Seed samples	Areas	Aflatoxin production			
		B_1	B_2	G_1	G_2
Beta vulgaris var. *Cicla*	Mahbubnagar	+	-	+	-
	Rangareddy	+	-	+	-
Amaranthus tricolor	Mahbubnagar	+	-	-	-
	Rangareddy	+	-	-	-
Portulaca oleracea	Mahbubnagar	-	-	-	-
	Rangareddy	-	-	-	-

+ Present, - absent

compared to the other two seed samples. Interestingly, *A. flavus* treated seeds of Bv showed higher germination percentage than untreated seed samples at both 75 and 85 per cent RH levels. *A. flavus* treated seed samples of *A. tricolor* and *P. oleracea* showed decrease in germination percentage at 85 per cent RH compared to 75 per cent RH. However, showed increase in germination percentage at 85 per cent RH as compared to 75 per cent in non-treated samples. It is clear from the studies that *A. flavus* treated seeds recorded less germination percentage over control seeds except with the seeds of Bv. There are reports that infection by storage fungi is the primary cause for loss of germination (Barton, 1961; Harrington, 1963; Mills and Frydman, 1980; Reddy *et al.*, 2001; Tariq *et al.*, 2005 and Rao *et al.*, 2014).

Qualitative and quantitative estimation of aflatoxins :

The results of qualitative and quantitative determination of aflatoxins in the *A. flavus* treated seeds of three leafy vegetables stored at 75 and 85 per cent relative humidity levels are presented in Table 1. *A. flavus* treated seeds collected from Mbnr and RR dists. tested for *in vitro* toxigenecity, S_6 was observed to be non-toxigenic and S_1-S_5 produced aflatoxins (Table 2). The non treated control seeds have not produced any aflatoxin.

The evaluation of toxigenic potential of the isolates showed that 73.33 per cent of the *A. flavus* strains isolated from the seeds of three different leafy vegetables collected from Mbnr and RR districts presented a toxigenic potential. Among the *A. flavus* 66.67 per cent isolates were producers of aflatoxin B_1 and 33.33 per cent of the isolates produced aflatoxin G_1.

Aflatoxins were detected in the *A. flavus* inoculated seeds of all the three leafy vegetables stored at 75 and 85 per cent RH (except Po stored at 85% RH). Aflatoxin B_1 was detected in the *A. flavus* treated seeds of Bv and At collected from both Mbnr and RR dist. While, aflatoxin G_1 was detected only in Bv samples of Mbnr and RR dist. The toxigenic potential of the strains stored at 75 per cent RH ranged from 104 to 171 ng/kg seed. While, those stored at 85 per cent RH ranged from 85 to 178 ng/kg seed. The largest quantity of aflatoxin B_1 178 ng/kg seed was detected in the *A. flavus* treated seeds of Bv stored for 45 and 90 days at 85 per cent RH. While, those incubated at 75 per cent RH produced 171 and 154 ng/kg seed in the seeds stored at 45 and 90 days, respectively. *A. tricolor* seeds produced 135 ng/kg seed Aflatoxin B_1 in 45 and 90 days stored seeds incubated at 75 per cent Rh. However, less amount of 85 ng/kg seed aflatoxin B_1 was recorded in 45 days stored seeds incubated at 85 per cent RH.

As such no information is available on the aflatoxin production in these three leafy vegetable seeds. These results are in agreement with Rustom, 1997 and Leong *et al.*, 2010, who had reported the incidence of aflatoxins in food is relatively high in tropical and subtropical regions, where the warm and humid weather provides optimal conditions for the growth of the moulds in starch-based foods from Malaysia.

It is clear from the table that the amount of aflatoxin B_1 showed a decrease from 45th day to 90th day and it was completely absent at initial period of storage in *Beta vulgaris* treated seeds stored at 75 per cent relative humidity. During the early period of storage, no aflatoxins were detected in all these three samples. Comparatively, *Beta vulgaris* seed samples have recorded higher amounts of aflatoxin B_1 than the remaining seed samples incubated at 75 per cent and 85 per cent relative humidity levels.

This study demonstrated that the production conditions applied in selected areas contribute to the susceptibility of vegetable seeds to the colonisation with aflatoxigenic fungi during different stages of maturity in the field and storage. The results showed that the storage period of vegetable seeds is a critical phase, as indicated by the high percentage of aflatoxigenic isolates. However, no correlation was observed between the presence of the isolates and temperature, relative humidity. The results demonstrated the importance of good agricultural practices during the production, harvest and storage of vegetable seeds in the areas selected.

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