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Screening of Millets for the Natural Occurrence of Aflatoxin B_1 and their Susceptibility in Samples Collected during Pre-harvesting, Harvesting and Post-harvesting Conditions

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ABSTRACT

Aflatoxin contamination of food and feed have global concerns because of potential threat to human and animal health as a result many countries have regulatory guidelines on its level in food and feed. Screening of minor or small millets and pearl millet showed no natural occurrence of aflatoxin B_1 in pre-harvesting, harvesting, and post-harvesting conditions. Upon Aspergillus flavus infestation under controlled conditions millets show varying response. In post-harvest (market) samples AFB_1, production was seen in finger millet, foxtail millet, pearl millet, and sorghum while little and kodo millet showing no response with respect to aflatoxin contamination in all the conditions studied indicating small millets as low-risk agriculture commodity.

Key words: *Aflatoxin B_1, Minor or small millets, Pearl millet, Aspergillus flavus, Sorghum.*

1. INTRODUCTION

Millets are traditionally consumed as a staple food by a large population of poor people in Arid and Semi-arid parts of the world, especially in Africa and Asia. They are nature's nutraceuticals which are very good sources of energy, protein, fat, minerals, and phytochemicals including phytic acid, polyphenols, and antioxidants which have several potential health benefits such as reducing the risk of developing chronic diseases such as cardiovascular disease, diabetes, and some cancers [1-8]. They are grown in extremely harsh conditions and require little input for their production and they are pest free crops which can be stored well for longer periods and helpful when there are crop failures. In an era where global food security has narrowed down to a few crops and in areas where there is food and nutritional insecurity imposed by various agronomic, socioeconomic and other factors, millets provide food and nutritional security [2-4].

Aflatoxin contamination of food and feed has very high significance due to its deleterious effects on human and animal health and its importance in international trade. According to World Health Organization dietary exposure of aflatoxins leads to health-related hazards (Aflatoxicosis). Aflatoxins produced by *Aspergillus* species are secondary metabolites which cause immunosuppression, impaired growth, various cancers, and death depending on the type, period, and amount of exposure [9-12]. Of the four aflatoxins (B_1, B_2, G_1, and G_2), aflatoxin B_1 (AFB_1) is the most potent carcinogen [13-15]. AFB_1 is an wide spread food safety issue for consumers throughout the food delivery chain as reported in several surveys. According to Food and Agricultural Organization, 25% of world's food crops are significantly contaminated with mycotoxins [16]. Aflatoxin contamination impacts disproportionately on the livelihoods of the rural people and mycotoxicosis-related economic losses are prevalent in many countries. More than hundred countries have regulatory guidelines for limit of AFB_1 indicating its importance in food, feed, and trade-related aspects. While information on aflatoxin contamination of many cereals, groundnut, maize, etc., are available, not many studies are available regarding aflatoxin contamination of small millet grains in different conditions such as pre-harvesting, harvesting, and post-harvesting; hence, the need for present study (Figure 1).

2. MATERIALS AND METHODS

2.1. Millet Grains

Pre-harvesting millet and sorghum samples were collected from the fields of Vempalle, Utkur, and Yerraguntla in Kadapa district of Andhra Pradesh, India, and harvesting samples from the farmers of the above places after harvesting. Post-harvesting (market)

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millet and sorghum samples were collected from the local market. Samples collected in polypropylene containers were cleaned and kept in a cool and dry place until further use. Aflatoxin reference standards and polyester silica gel-G thin layer chromatography (TLC) plates (thickness 250 µm) were sourced from Sigma Chemicals Co., St. Louis, USA. All other chemicals and reagents used were of analytical grade.

2.2. Strain

A fungus strain, *Aspergillus flavus* (NRRL 6513) was obtained from United States Department of Agriculture, Peoria, Illinois, USA. Cultures were maintained on potato dextrose agar slants for 8 days at 28.0 ± 1°C in a BOD incubator.

2.3. Inoculation of Millets and Sorghum with A. flavus

To maintain an optimum moisture content of the samples for aflatoxin elaboration, (10 g) dried grains were washed 2-3 times with sterilized glass distilled water and with sodium hypochlorite solution (1% v/v) and washed thoroughly with distilled water 2-3 times to remove any residual sodium hypochlorite solution. The seeds (10 g) in triplicate were presoaked in distilled water and transferred into a clean Erlenmeyer flask (250 ml) presterilized in a laboratory autoclave. Fungal spore inoculums in a volume of 500 µl containing 1×10^6 spores prepared in 0.01% Tween-20 was added to 10.0 g of grain samples, in the laminar flow hood under aseptic conditions. These samples were incubated at 28 ± 1°C in a cooling incubator and the fungus was allowed to grow for a period of 12 days. At four different time points of fungal infection, i.e., 3, 6, 9, and 12 days, samples were drawn and processed for further analysis. The samples were maintained in duplicate flasks, and two subsamples were drawn from each of the duplicate flasks for analysis. The samples were dried at 40-45°C for 48 h. After drying, samples were powdered in a mechanical grinder to a particle size of 0.4 mm.

Figure 1: Digital photograph of various small millet grains and sorghum.

2.4. Extraction and Estimation of Aflatoxins

Extraction and estimation of aflatoxins were done as described by Tanuja *et al*., and Ratnavathi *et al*. [17,18]. The infected seeds were ground in a high-speed mechanical blender to a fine powder. The powdered samples were defatted using a Soxhlet apparatus using n-hexane as a solvent. The defatted powder (1.0 g) was extracted with 5.0 ml of methanol:water (55:45) using a mechanical shaker for 1 h. Later, the samples were centrifuged, the aqueous methanolic phase was transferred into a separating funnel and equal volume of chloroform was added and mixed thoroughly. The chloroform layer containing the toxin was separated and dried using flash evaporator. The dried samples were stored at −20°C until further analysis by TLC.

AFB_1 was estimated by TLC/Fluoro-densitometric method as per the procedure reported by Egan [18]. The samples were redissolved in benzene:acetonitrile (98:2) and were spotted (10 µl) on the activated TLC plates. The TLC plates were developed in toluene:ethyl acetate:formic acid (6:3:1) and visualized under long wave ultraviolet (UV) light (365 nm). Annotated digital density images of the spots were recorded by the CCD camera (UVItec, Cambridge, UK) and saved in PC-compatible file format (TIF file). Later,

Table 1: Aflatoxin B_1 production in small millets after infestation with *A. flavus* in different conditions.

Millet	Condition	AFB_1 (ng g^{-1})			
		Period of incubation (in days)			
		3	6	9	12
Finger millet	Pre-harvesting	ND	ND	ND	ND
	Harvesting	ND	ND	7±1	5±1
	Post-harvesting (market)	10±2	30±2	33±3	18±2
Foxtail millet	Pre-harvesting	ND	ND	ND	ND
	Harvesting	ND	18±2	20±3	ND
	Post-harvesting (market)	34±2	47±2	45±3	33±3
Little millet	Pre-harvesting	ND	ND	ND	ND
	Harvesting	ND	ND	ND	ND
	Post-harvesting (market)	ND	ND	ND	ND
Kodo millet	Pre-harvesting	ND	ND	ND	ND
	Harvesting	ND	ND	ND	ND
	Post-harvesting (market)	ND	ND	ND	ND

Values are mean±SD of triplicate analysis. ND=Not detected, SD=Standard deviation. *A. flavus*=*Aspergillus flavus*

Table 2: Aflatoxin B_1 production in pearl millet and sorghum after infestation with *A. flavus* in different conditions.

Period of infection (days)	AFB_1 (ng g^{-1})					
	Pearl millet			Sorghum		
	Preharvesting	Harvesting	Postharvesting (market)	Preharvesting	Harvesting	Postharvesting (market)
3	ND	24±2	25±1	277±1	300±5	326±1
6	ND	28±2	30±3	338±8	349±3	356±2
9	ND	37±3	40±4	405±5	416±2	424±4
12	ND	18±1	20±2	303±3	312±2	338±4

Values are mean±SD of triplicate analysis. ND=Not detected, SD=Standard deviation. *A. flavus=Aspergillus flavus*

the digital density images of AFB_1 were analyzed by the software supplied by the instrument manufacturer for determining the net density, which was measured as peak volume. Reference standard AFB_1 was used for calculating the AFB_1 content in the samples. The aflatoxin content in the samples was expressed as ng g^{-1} of defatted powder.

3. RESULTS AND DISCUSSION

AFB_1 was not detected in all the millet samples collected during pre-harvesting, harvesting, and post-harvesting conditions indicating no natural occurrence of AFB_1. However, susceptibility of millet samples to aflatoxin production after *A. flavus* infestation gave varying response under controlled conditions. In pre-harvesting conditions except in sorghum, AFB_1 was not detected in finger millet, foxtail millet, little millet, kodo millet, and pearl millet after infestation with *A. flavus*. In harvesting conditions, little and kodo millet and 3 and 6 days after infection in finger millet and 3 and 12 days after inoculation in foxtail millet, AFB_1 was not detected, whereas in 9 and 12 days after infection in finger millet and 6 and 9 days of infestation in foxtail millet and all the days after infection tested in pearl millet and sorghum showed AFB_1 production. In post-harvest (market) samples, AFB_1 production was seen in finger millet, foxtail millet, pearl millet, and sorghum. However, in market samples of little millet and kodo millet the AFB_1 was not detected, even after infestation (Tables 1 and 2). These results suggest that small millets does not support aflatoxin production when compared to pearl millet and sorghum which may be due to low moisture, high polyphenols, phytate content and they may be regarded as low-risk agriculture commodity. In view of this, small millets can be regarded as safe food grains which are poor substrates for AFB_1 production.

Various factors influencing aflatoxin contamination are soil type, fungal species in the soil, climate, improper agricultural practices, phytoalexin production, maturity, weather condition during harvesting, timely drying, and maintaining safe moisture level during post-harvest conditions. Aflatoxin control and prevention strategies mainly include stopping the infection process of *A. flavus* by host-plant resistance/tolerance, biological control, managing environmental factors, pre-harvest, and post-harvest crop management practices. Aflatoxin contamination occurs more during post-harvest than during pre-harvest conditions [11,13,19]. Improper agricultural practices and adverse climatic conditions at harvest and after harvest are predisposing factors for post-harvest aflatoxin contamination. Significant grain deterioration caused by molds also occurs during storage. Some of the factors such as excessive heat, high humidity, lack of aeration in stores, and insect and rodent damage are the conditions that aggravate aflatoxin production [20, 21]. In general aflatoxin levels in food, commodities increase with storage time. Apart from these factors influencing aflatoxin contamination, strategies to limit aflatoxin biosynthesis by the fungi may also be looked into for better management.

4. CONCLUSION

No natural occurrence of AFB_1 was found in small millets and pearl millet in pre-harvesting, harvesting, and post-harvesting conditions. Millets show varying response after *A. flavus* infestation. AFB_1 production was seen in finger millet, foxtail millet, pearl millet, and sorghum while little and kodo millet showing no response with respect to aflatoxin contamination. Irrespective of the measures taken to control aflatoxin contamination, it continues to be a serious challenge and health hazard to man and in these circumstances millets with no natural occurrence and good resistance offer one of the solutions to combat aflatoxin contamination.

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Bibliographical Sketch

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