Food Chemistry **17** (1985) 33–39

Production of Aflatoxins on Amaranth Seeds by *Aspergillus* Spp.*

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(Received: 1 October, 1984)

ABSTRACT

Five varieties of amaranth seeds, cooked and uncooked, were inoculated with toxigenic strains of Aspergillus *spp. and the levels of aflatoxins were quantitated. Cooking seeds prior to inoculation increased the level of aflatoxins; aflatoxin levels were the same in all the amaranth varieties inoculated. Aflatoxin level was much lower on amaranth than on rice, corn or winged bean.*

INTRODUCTION

A recent publication by the US Academy of Sciences stated that the amaranth plant, *Amaranthus* spp., could be important as a source of protein in the developing countries where protein-rich animal foods are not available. The amaranth seed has a relatively high protein level, i.e. 13·1 % (Osuntogan & Oke, 1983), and contains exceptionally high levels of lysine, a critical amino acid usually deficient in plant protein (Anon., 1975). We have recently completed analyses of the sterols and fatty acids of the leaves, seeds and stems of *Amaranthus tricolor* (Fernando & Bean, 1984) plus seeds of weedy and vegetable varieties of *Amaranthus* spp. (results unpublished). The major sterol was spinasterol with lesser

* Scientific article No. A-3927; Contribution No. 6910 of the Maryland Agricultural Experiment Station.

amounts of Δ^7 ergosterol, stigmasterol and Δ^7 stigmasterol whereas 24-methylene cycloartanol was present in seeds only. The major unsaturated fatty acid in seeds and stems was linoleic acid whilst, in leaves, linolenic acid was the most prevalent. The major saturated fatty acid was palmitic acid in seeds, stems and leaves. In addition, the ratio of saturated to unsaturated fatty acids was at a level considered to be safe (i.e. 0·41). All of the previous studies indicate that the *Amaranthus* could safely be used as a source of plant protein.

In developing countries, the amaranth grain is first parched and milled, the flour then being used to prepare a dough which is made into pancakes or else cooked for gruel and made into confections. The grain is also powdered and used as a drink (Anon., 1975). Thus, seeds are an important edible part of the amaranth and this has led to the current study on the occurrence of aflatoxins in amaranth seeds.

Aflatoxins are hepatotoxic secondary metabolites of some *Aspergillus* spp. which occur whenever agricultural commodities such as nuts and grains are stored improperly, i.e. at high temperatures and/or moisture levels (Hunter, 1969). Aflatoxins are frequently a problem in the developing countries where inadequate storage facilities exist (Kraybill & Shapiro, 1969). The present study compares the level of aflatoxins produced in amaranth seeds when inoculated with toxigenic strains of *Aspergillus* spp.

MATERIALS AND METHODS

Seeds of five varieties, *A. cruentus* RRC 1011, *A. hypochondriacus* RRC 112, *A. hypochondriacus* RRC 1023, *A. hybridus* RRC 1044 and *A. hypochondriacus* RRC 674, were obtained from Rodale Research Center, Kutztown, PA. Four toxigenic isolates of *Aspergillus* spp. were used in this study—two strains of *A. flavus* Link Ex Fries, ATCC 15546 and 6432, and two strains of *A. parasiticus*, ATCC 26871 and NRRL 2999. Stock cultures of the *Aspergillus* spp. isolates were maintained on potato dextrose agar slants at 25 °C.

Seed samples (7 g) were washed three times in sterilized distilled water; excess water was drained off and they were then ground in a sterilized Waring blendor for 5 min according to the method of Sherertz *et al.* (1976). The ground sample was transferred to a sterilized 250-ml Erlenmeyer flask and 20 ml of sterilized distilled water were added. Cooked amaranth seeds were prepared by autoclaving flasks, containing

ground seed, for 15 min at 15 psi and 120 °C. After cooling, the flasks were inoculated with 1 ml of a spore suspension containing 3×10^6 conidia per milliliter. Aflatoxin production was carried out in a semi-synthetic medium containing 200 g of sucrose, 0·5 g of $MgSO_4 . 7H_2O$, 3 g of KNO_3 and 7 g of yeast extract in 1 liter of distilled water (Gupta & Venkitasubramanian, 1975). Aflatoxin production on amaranth seeds was compared with that on polished brown rice, corn, peanuts, soybeans and winged bean.

After 10 days' incubation, 50 ml of chloroform were added to each flask, the chloroform was passed through a Whatman No. 1 filter paper and the residue was washed twice with two 25 ml of chloroform. The combined chloroform fractions were evaporated to dryness by flash evaporation, redissolved in methanol and stored frozen under nitrogen until they were quantitated by high performance liquid chromatography (HPLC).

The HPLC system used in this study was a Varian model 5000 liquid chromatograph equipped with a variable wavelength ultraviolet absorbance detector. An IBM column (4·5 × 25 cm) containing octadecyl (C18) and a water–methanol–acetonitrile (3:1:1 v/v) solvent system was used throughout the study. The qualitative and quantitative examination of aflatoxins in the chloroform extracts was carried out by comparing retention times and peak areas relative to aflatoxin standards purchased from Sigma Chemicals.

RESULTS AND DISCUSSION

The levels of aflatoxins produced on liquid media and media composed of ground raw and cooked amaranth seeds are summarized in Table 1. The types of aflatoxins produced, i.e. B_1, B_2, G_1 or G_2, and the level of each aflatoxin produced, varied, depending upon the isolate of *Aspergillus* spp. used and the variety inoculated. For example, *A. parasiticus* isolate NRRL 2999 produced all four aflatoxins in the semi-synthetic medium and on the cooked amaranth variety RRC 1044, whereas, on cooked variety RRC 674, only B_1 and G_1 were produced. The levels of individual aflatoxins were also highly variable, depending upon the isolate and the amaranth variety. The level of aflatoxin B_1 on cooked seeds varied from 4–140 mg on varieties inoculated with *A. parasiticus* isolate ATCC 26871.

There was also as much variation within the two isolates of *A. parasiticus* and *A. flavus* as there was amongst all four isolates. *Aspergillus*

TABLE 1
Aflatoxin Production by *Aspergillus* spp. on Raw and Cooked Amaranth Seeds

		Variety RRC 674		RRC 1044		RRC 112		RRC 1011		RRC 1023		*Liquid medium*
		Raw	*Cooked*	*Raw*	*Cooked*	*Raw*	*Cooked*	*Raw*	*Cooked*	*Raw*	*Cooked*	
A. flavus												
(ATCC 15546)	B_1	60[a]	100	90	120	84	113	89	120	3	3	150[b]
	B_2	—	—	—	40	2	—	21	—	1	—	—
	G_1	30	125	70	85	49	75	100	250	3	1	850
	G_2	—	107	23	14	—	28	43	43	—	—	250
	Total	90	332	183	259	135	316	253	413	7	4	1 250
A. flavus												
(ATCC 6432)	B_1	13	20	75	150	23	25	8	13	31	40	175
	B_2	—	—	—	—	—	—	2	—	2	—	—
	G_1	11	15	48	162	—	8	6	—	40	126	103
	G_2	—	3	—	21	—	—	—	—	20	80	—
	Total	24	38	123	333	23	33	16	13	93	246	278
A. parasiticus												
(NRRL 2999)	B_1	69	140	120	150	10	18	50	114	101	118	225
	B_2	2	—	25	56	—	—	2	—	—	—	100
	G_1	50	105	65	163	18	20	70	131	160	200	850
	G_2	—	—	—	32	7	1	20	44	50	67	400
	Total	121	245	210	401	35	39	142	289	311	385	1 575
A. parasiticus												
(ATCC 26871)	B_1	68	100	85	120	6	4	89	123	121	140	200
	B_2	5	—	29	55	—	—	2	—	15	35	—
	G_1	21	—	75	86	—	2	21	248	45	125	106
	G_2	—	—	4	12	2	—	14	41	28	50	0
	Total	94	100	193	273	8	6	126	412	209	350	306

[a] Micrograms of aflatoxin per gram of substrate; values are means of three replicate samples.
[b] Micrograms of aflatoxins per 100 ml of culture filtrate.

TABLE 2
Production of Aflatoxin by *A. parasiticus* (NRRL 2999) on Various Substrates

Aflatoxin	*Substrate*										
	Amaranth[a]		*Rice*		*Winged bean*		*Peanut*		*Corn*		*Liquid medium*
	Raw	*Cooked*	*Raw*	*Cooked*	*Raw*	*Cooked*	*Raw*	*Cooked*	*Raw*	*Cooked*	
B_1	70[a]	108	60[b]	190	41	186	35	105	98	224	225[c]
B_2	6	11	22	81	18	74	12	41	45	87	100
G_1	73	123	300	736	251	690	170	236	389	760	850
G_2	15	29	121	230	92	159	15	60	168	321	400
Total	164	271	503	1 237	402	1 109	232	442	700	1 392	1 575

[a] Micrograms of aflatoxin per gram of seed; values are average of five amaranth varieties.
[b] Micrograms of aflatoxin per gram of seed; values are means of three replications.
[c] Micrograms of aflatoxin per 100 ml of culture filtrate.

flavus isolate ATCC 6432 produced 20 μg on cooked amaranth variety RRC 674 seed, whereas *A. flavus* isolate ATCC 15546 produced 100 μg on the same variety. Similarly, the levels of aflatoxins in the five varieties of amaranth varied according to the isolate of *Aspergillus* they were inoculated with, indicating no 'inherent' resistance in amaranth seeds to either growth or aflatoxin production by *Aspergillus* spp. In all treatments, however, cooking seeds prior to inoculation with *Aspergillus* spp. increased the levels of aflatoxins produced compared with non-cooked seeds. Sherertz *et al.* (1976) found similar results with soybeans.

Aflatoxin production by *A. parasiticus* isolate NRRL 2999 on various substrates is summarized in Table 2. In all cases, cooking increased the levels of aflatoxins produced. This was so even in soybeans which are normally considered resistant to aflatoxin contamination (Shotwell *et al.*, 1969) although, occasionally, aflatoxins can be found as a result of field contamination (Bean *et al.*, 1972). Winged beans were included in this study because they are also considered to be important sources of plant protein (Anon., 1975). From these studies it appears that amaranth is not as susceptible to aflatoxin contamination as are commodities such as rice and corn which are frequently used as substrates to produce aflatoxins under laboratory conditions. Peanuts, however, contained considerably less aflatoxins than corn, rice or winged bean. The lowest levels of aflatoxins occurred in amaranth seeds.

Previous studies indicate that none of the five varieties of amaranth tested was considered resistant to aflatoxin contamination. Aflatoxins can be produced when amaranth seeds are inoculated with toxigenic strains of *Aspergillus* spp. although the levels that occur are much less than on other substrates such as corn and rice, indicating some degree of resistance or tolerance to aflatoxins. Even so, in the event that, in the future, amaranth seeds were stored for long periods of time, particularly in the developing countries where storage facilities are frequently inadequate, care should be taken to prevent the development of mold fungi.

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