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Aflatoxin contamination and mineral profile of almond seeds

[*]Ehsan Ali[1], Saleemullah[2], Farida Anjum[2], Barkat Ali Khan[2] and Ahmed Zubair[1]

[1]NWFP, Agricultural University, Peshawar, Pakistan
[2]Agricultural Research Institute, Tarnab, Peshawar, Pakistan
** Corresponding author's e-mail: ehsanali81@yahoo.com*

Abstract

Samples of almond (*Prunus amygdalus* L.) seeds belonging to Pakistan, Afghanistan and Iran were incubated at 30 °C for fungal count. Bleach disinfected samples were inoculated with *Aspergillus flavus,* isolated from local peanut samples, to assess them for aflatoxins B_1, B_2, G_1, and G_2 by HPLC and change in proximate and mineral contents. Aflatoxins content in inoculated samples indicated the presence of B_1 (21.3 to 106 µg 100 g^{-1}), B_2 (2.28 to 4.14 µg 100 g^{-1}), G_1 (43.84 to 122.72 µg 100 g^{-1}) and G_2 (2.94 to 32.0 µg 100 g^{-1}). A reduction in crude protein (7-10.1%) and crude fat (11.5 to 14.7 %) and an increase in NFE (24.8 to 32.8%) were observed during inoculation. Ash and fiber were not affected while moisture was increased (11.3 to 132.1%) due to addition of spores in solution form. Various minerals like Ca, P, Pb, Mg, Fe, Zn, Se, and K were decreased while Na, Mn, Cu, and Cd increase. Interaction of aflatoxins with proximate and mineral showed that B_1 was positively correlated with moisture, crude fat, NFE, Zn, Mg, and Ca while negatively correlated with ash, Fe, Cu, Mn, Cd, Se, Na, and K. Aflatoxin B_2 showed positive relation with protein, Cd, Zn, Na, and P. An inverse proportion of this toxin was observed with fiber, Fe, Cu, Pb, K, Mg, and Ca. The response of G_1 was positive towards moisture, crude fat, NFE, Zn, Mg, and Ca. A negative effect of Iron, Cu, Mn, Cd, Se, Na, and K was noted on G_1. Aflatoxin G_2 showed an inclining behavior towards ash, Fe, Mn, Pb, Se, and K. A decline of this toxin was found with Zn and P. It is concluded that growth of *A. flavus* and aflatoxin production had a significant effect on the chemical composition of the substrate. Any supplementation or reduction of certain constituent genetically or chemically may cause reduction in aflatoxin production, which would be a possible way of risk management of aflatoxins.

Keywords: Aflatoxin, almond seeds, *Aspergillus flavus,* mineral profile.

Introduction

Almond is a member of Rosaceae family. The coat in the almond is hard and juiceless of a dingy green tinged with dull red. Misconceptionally they are known as nuts. The almond tree is a native of the warmer part of Western Asia (Qatar, Jordan, Cyprus, Yemen) and of North Africa (Algeria, Egypt, Morocco, Sudan) but it has been extremely distributed over the warm temperate region of the old world (Grieve, 1981).

Almonds provide a nutrient-dense source of vitamin E, manganese, magnesium, copper, phosphorus, fiber, riboflavin, monounsaturated fatty acids and protein. On expression, almonds yield nearly half their weight in a bland fixed oil, which is employed medicinally for allaying acrid juice, softening and relaxing solids and in bronchial diseases, in tickling coughs, hoarseness, costiveness, nephritic pains and because they contain practically no starch and being rich in protein, they are often made into flour for cakes and biscuits for diabetic patients (Grieve, 1981).

Almond like other nuts are infected by fungi, include *Aspergillus flavus, Aspergillus* species cause production of aflatoxin. Aflatoxins are suspected to be involved in human liver cancer. Aflatoxicoses in human has been reported in many Afro-Asian countries. Aflatoxins have been implicated in hepatocellular carcinoma, acute hepatitis, Reye's syndrome, cirrhosis in malnourished children, and kwashiorkor (Saleemullah *et al.,* 2006). The overall toxicity of aflatoxin in an animal appears to be determined by the rate of formation of the reactive intermediate, its binding to the largest macromolecules (DNA, RNA) and the rate of detoxification and other competing reactions (Sermand *et al.,* 1999). Concern for human and animal health has led to regulatory limitations on the quantity of aflatoxins in foods and feeds throughout most of the world. According to WHO and FAO regulation the

content of aflatoxin B should be less than 0.5 µg Kg^{-1} in milk (Reddy, 2000).

Keeping in view the hazards of aflatoxin contamination in food products especially in tree nuts and the wide spread use of almond, it was imperative to initiated this research to assess the nature of the problem and suggest ways and means to control aflatoxin contamination of food and nuts in North Western Frontier Province (NWFP).

Materials and Methods

Sample collection

Different almond samples were collected from local market of NWFP, Pakistan to make a composite sample of one kg, which was then reduced to small lab. Sized samples for analysis.

Promixate composition

All samples were analyzed for proximate composition i.e. moisture, crude protein, crude fat, crude fiber, ash and nitrogen free extract (NFE) by standard method of AOAC (1990). Moisture determination was carried out by oven drying method, for which 1.0 g sample was oven dried at 105 °C for 6 hrs. Crude fat was determined by ether extract method using Sox let apparatus. For crude fiber analysis fat free sample was digested using 2.5% HCl solution, it was then subjected to alkali digestion using 2.5% NaOH solution. The residue was then ignited in furnace at 600 °C till ashing. For crude protein determination the sample was digested using digestion mixture of K_2SO_4 and $CuSO_4$ (8:1) and conc. H_2SO_4. The digested sample was subjected to distillation process using 40% NaOH and 4% boric acid solutions and was titrated against 0.1 N HCl solution.. Ash was determined in muffle furnace at 660 °C for one hour. Nitrogen free extract represents the digestible carbohydrates. This value was obtained by subtracting the sum of the percentages of moisture, crude protein, crude fat, ash and crude fiber from 100.

Mineral determination

For mineral determination, acid digest was prepared using 10 ml perchloric acid ($HCLO_4$). Afterward spectrophotometric determination of phosphorus, iron, sodium and potassium was carried out using KH_2PO_4, $FeSO_4(NH_4)6H_2O$, NaCl, and KCl respectively.

Atomic absorption spectrophotometeric determination

For the determination of selected trace elements such as, copper (Cu), magnesium (Mg), zinc (Zn), and calcium (Ca), lead (Pb), Maganese (Mn) by AAs, the method adopted by Jorhem, L. (2000), modified for macro-levels (Sattar and Chaudary, 1978) was employed.

Preparation of media for fungal growth

Malt extract medium (MEA) was prepared by dissolving 50 g MEA in 1 liter distilled water. One gram *streptomycin* was added and was autoclaved (Prestige Medical 2075) at 121 °C for 20 minutes.

For the preparation of coconut media, 1 kg powdered coconut were taken and dissolved in 1 liter distilled water. Added 3 g agar and 1 g streptomycin to it and were sterilized at 121^0C for 20 minutes in an autoclave.

Frequency of isolation and total fungal counts in samples

Four seeds were randomly taken from samples of all the selected commodities and directly plated on Petri plates containing MEA and malt salt agar medium. All the inoculations were made in triplicate. The total fungal populations and dominant genera were isolated from the samples using either Malt Extract Agar (MEA) and Coconut Agar media (CAM) or Malt Salt Agar (MS) media (Christensen, 1957).

Isolation and purification of fungus

After a period of one week, the incubated Petri dishes were checked for *Aspergillus flavus*. Heads (conidia) observed under microscope were matched with the standard *A, flavus* colonized in one of the Petri dish. Spores from identified *A. flavus* colonies were transferred on coconut media in already filled Petri dishes. They were incubated for a period of one week at 23-25 °C. The incubated Petri dishes were examined under UV light for toxic strains of *A. flavus* which reflected blue fluorescence. Spores from purified culture of *A. flavus* were taken in 5 ml detergent solution. The detergent with spores was transferred to sterilized vials and stored in refrigerator at 4^o C for using as inoculum of samples.

Preparation of inoculums

Seven days old cultures of *A. flavus* used as a source of spores (conidia). The suspension was filtered and centrifuged twice for 5 min and re-suspended in sterile distilled water to remove germination inhibition factors that appear to be present in the conidial matrix (Railey et al 1997).

About 50 g whole nuts were uniformly inoculated with diluted spores solution. The inoculated nuts were transferred to sterilized

brown bottles. The bottles were capped and were incubated at 30 °C for 15 days. The nuts were dried at room temperature and were tested for aflatoxin.

Standardization of HPLC

50 µl of each of aflatoxin standards (Sigma Aldrich, USA) containing 400 ng g^{-1} of AFB_1 & AFG_1 and 100 ng g^{-1} of AFB_2 & AFG_2 were run through HPLC system and a calibration curve was prepared.

Sample assay

Aflatoxins were determined by the method of Devries and Chang. (1982). For this purpose 10 gm grinded almond sample was taken in 250 ml conical flask. 100 ml methylene chloride and 5gm cupric carbonate were added. The sample was shaken and filtered; 30 ml of the filtrate was evaporated. The residue was redissolved in 4 ml acetonitrile and transferred to 250 ml separator. 25 ml petroleum ether was added to the separator which given rise to two layers. The lower layer was collected and transferred to another separator. Again 25 ml petroleum ether was added to it and the lower layer was collected and dried. The residue was redissolved immediately in 1 ml methylene chloride and evaporated to complete dryness. 50 microliter TFA (trifloroacetic acid) and 4 ml HPLC solvent was added to the residue. HPLC solvent is the solution of acetonitrile, water, acetic acid in the ration of (10:90:1). The extracted solution was then stored in black colour ependorff tubes. Then HPLC Perkin elmer with C_{18} column, and the flourosence 450 detector were used for sample extract analysis.

Flow rate was set at 1 ml/minute and the column was equilibrated for 30 minutes. A pressure of about 824 pa was developed. Aflatoxin standard (mixed) was injected through a loop of 20 µl. Chromatograms of aflatoxin G1, B1, G2, B2 were obtained. The peak height and area was automatically calculated by the CSW32 software. The samples were injected one by one and the resulting chromatograms were identified for toxin peaks.

Results and Discussion

The fungal profile of almond seeds from various locations showed the presence of *Fusarium, Alternaria, Eurotium, A. niger,* pink yeast, and *Penicillium* (Table-1). Some of the fungal colonies were remained un-identified due to lake of facilities. *A. niger* population was highest on all the three samples followed by *Alternaria*. No contamination of *A. flavus* was found on any of the almond seeds. Total fungal count showed that there was no significant difference between the seeds belonging to Pakistan and Afghan while Pak and Iran seeds were significantly different from each other with respect to fungal count.

Proximate composition of control and inoculated samples indicated that the crude protein and crude fat were reduced during inoculation period by the values of 7.00-10.10% and 11.50 to14.70% respectively. The affect of inoculation period was negligible on ash, and fiber content ($P \leq 0.05$). Moisture content was increased (113.3-132.1%) due to inoculation with moist spores of *A. flavus*. The content of nitrogen free (NFE) extract was also increased by a range of 24.8 to 32.8% in all the three samples (Table 2). The present data was in line with (Saleem *et al.,* 2006). The mineral content of control and inoculated samples (Table 2 and 3) were found to have similar trend of increase and decrease. Among the mineral content Ca, P, Pb, Mg, Fe, Zn, Se and K, showed % decrease while, Na, Mn, Cu and Cd were increased. The present data agreed with (Javed *et al.,* 2007). The toxin content (Table: 4) of the inoculated samples showed that aflatoxin were present in all of the three samples. Aflatoxin G_1 was found in range of 43.84 to 122.72 µg 100 g^{-1}. Aflatoxin B_1 was ranged from 21.3 to 106.0 µg 100 g^{-1}. Aflatoxin G_2 content was 2.94-32.0 µg 100 g^{-1}. While aflatoxin B_2 was 2.28-4.14 µg 100 g^{-1} in the inoculated samples. The data agreed with (Magbanua *et al.,* 2007).

Tables 6 and 7, showed interaction of aflatoxin with proximate composition and minerals. The data explained that Aflatoxin B_1 was positively correlated to moisture, fat, NFE, Zn, Mg and Ca. A negative correlation of B_1 was observed with % ash content, where Fe, Cu, Mn, Cd, Se, Na and K were remarkable. Similarly, B_2 had positively correlated with protein, Cd, Zn, Na, P and inversely related to fiber, Fe, Cu, Pb, K, Mg and Ca. The relation of G_1 with moisture, crude fat, and NFE, Zn, Mg, and Ca was positive. A decreasing trend of G_1 was observed with Fe, Cu, Mn, Cd, Se, Na and K. The aflatoxin G_2 showed an inclining behavior towards Ash, Fe, Mn, Pb, Se, and K constituents. While this toxin was declined with moisture, crude protein, crude fat, and Nitrgen free extract (NFE). The minerals Zn, and P negatively affected the G_2 content during inoculation. All other relation of aflatoxins with composition was not significant. The data was in lined with (Malini *et al.,* 1983).

The present data showed a dependence of the mold on the composition of the substrate.

Although in these results there was decreasing or increasing affect of the component on the toxin production, but scientist had proved and some might have to prove that a particular component is responsible for attraction or repelling the *A. flavus*. This would be a step toward the hypothesis that *A. flavus* is depended on substrate composition and also the production of aflatoxin and paved a way for further confirmatory research in this regard. This will be a better step toward the preventive strategies of the fungus and aflatoxin and with addition or deletion of that particular component the danger of toxin would be eliminate.

Table 1: Molds Isolated from selected almond varieties.

Fungus	Occurrence of fungi (%)			
	Pakistan	Afghanistan	Iran	Total
Aspergillus flavus	_	_	_	-
Fusarium sp.	21	19.3	17.0	57.3
Alternaria sp.	28	17.1	12.7	57.7
Eurotium sp.	10.5	-	14.8	25.3
Aspergillus niger	24.5	25.8	22.3	72.6
Pink yeast	15.7	21.4	10.6	38.7
Penicillium sp.	-	16.1	22.3	38.4

Table 2: Proximate composition (%) on dry weight basis of artificially inoculated almond seeds.

Proximate composition		Pak	Afghan	Iran	Mean
Moist (%)	Control	2.8	3.0	2.8	2.87±0.12a
	Inoculated	6.1	6.4	6.5	6.33±0.21b
Protein(%)	Control	21.0	20.2	20.6	20.60±0.40a
	Inoculated	19.0	18.6	18.5	18.70±0.26b
Fats (%)	Control	57.1	57.8	56.9	57.27±0.47a
	Inoculated	50.5	50.5	48.5	49.83±1.15b
Ash (%)	Control	2.7	2.8	2.6	2.70±0.10a
	Inoculated	2.8	2.7	2.6	2.70±0.10a
Fiber (%)	Control	2.5	2.6	2.6	2.57±0.06a
	Inoculated	2.6	2.6	2.5	2.57±0.06a
NFE (%)	Control	13.9	13.6	14.5	14.0±0.46a
	Inoculated	19.0	19.2	21.4	19.87±1.33b

Controlled: Before *A. flavus* inoculation. Inoculated: Inoculated with *A. flavus*
The mean values of control and inoculated followed by similar later have no significant differences at 5% probability. Std± : Standard Deviation
± indicates the standard error of means.

Table 3: Micro-minerals composition on dry weight basis (mg 100 g^{-1}) of artificially inoculated almond Seeds.

mineral		Pak	Afghan	Iran	Mean	
Fe	Control	189.3	162.3	165.0	172.20±14.87a	
	Inoculated	55.0	97.2	126.7	92.97±36.04a	
Cu	Control	19.2	14.4	17.1	16.90±2.41a	
	Inoculated	153.3	75.8	77.5	102.20±44.26a	
Mn	Control	13.5	13.3	15.1	13.97±0.99a	
	Inoculated	12.2	14.2	15.3	13.90±1.57a	
Cd	Control	9.6	16.5	6.9	11.00±4.95a	
						0.90
	Inoculated	17.7	25.7	13.0	18.80±6.42b	
Zn	Control	47.6	47.0	50.3	48.30±18.50a	
						9.65
	Inoculated	46.1	42.6	45.4	44.70±1.85a	
Pb	Control	19.3	16.7	17.4	17.80±1.35a	
						2.80

	Inoculated	13.2	16.0	21.0	16.73±3.95a	
Se	Control	215.5	192.5	177.9	195.30±18.96a	
						54.98
	Inoculated	343.2	135.5	172.9	217.20±110.71a	

Controlled: Before *A. flavus* inoculation. Inoculated: Inoculated with *A. flavus*
The mean values of (control and inoculated) followed by similar later
have no significant differences at 5% probability. ± : Standard Deviation

Table 4: Macro-Minerals comp on dry weight basis (mg/100g)of artificially inoculated almond seeds.

Minerals		Pak	Afghan	Iran	Mean Std±
Na	Control	68	71	69	69.30±1.5a
	Inoculated	74	52	72	66.00±12.2a
K	Control	782	763	771	772.00±9.5a
	Inoculated	752	762	772	762.00±10.0a
P	Control	305.3	376.3	371.4	351.00±39.65a
	Inoculated	295.7	317.9	355.6	323.07±30.28a
Mg	Control	1165.6	1151.6	1188.6	1168.60±18.68a
	Inoculated	153.5	151.5	151.7	152.23±1.10b
Ca	Control	2129.3	2429.3	2218.2	2258.93±154.09a
	Inoculated	592.2	1073.4	640.8	768.80±264.90b

Controlled: Before *A. flavus* inoculation. Inoculated: Inoculated with *A. flavus*
The mean values of (control and inoculated) followed by similar later
have no significant differences at 5% probability. Std± : Standard Deviation

Table 5: Aflatoxin contents (µg/100g) of Selected almond Varieties.

Varieties	G_1	B_1	G_2	B_2	Total
Afghan	43.84	21.30	32.00	2.28	99.42
Gilgit	46.56	34.23	5.28	4.14	90.212
Iran	122.33	106.34	2.94	2.88	234.49
Control	-	-	-	-	-

Table 6: Correlation of aflatoxins with proximate composition differences.

Toxins	Moisture	Protein	fats	Ash	Fiber	NFE
G_1	0.98	-0.25	0.93	-1.00	-1.00	0.97
B_1	0.99	-0.14	0.97	-0.99	0.37	0.99
G_2	-0.74	-0.64	-0.84	0.56	0.44	-0.76
B_2	0.04	0.99	0.19	0.19	-0.95	0.07

Table 7: Correlation of aflatoxins with micro-minerals.

Toxins	Fe	Cu	Mn	Cd	Zn	Pb	Se
G_1	-0.74	-0.86	-0.94	-0.93	0.64	0.01	-0.84
B_1	-0.81	-0.63	-0.97	-0.88	0.72	-0.09	-0.89
G_2	0.98	1.00	0.82	0.24	-0.99	0.80	0.94
B_2	-0.54	-0.74	-0.17	0.53	0.65	-0.99	-0.40

Table 8: Correlation of aflatoxins with macro-minerals.

Toxins	Na	K	P	Mg	Ca
G_1	-0.62	-0.52	-0.37	0.94	0.62
B_1	-0.53	-0.62	-0.26	0.89	0.53
G_2	-0.27	0.99	-0.54	-0.27	0.28
B_2	0.88	-0.75	0.97	-0.50	-0.88

correlation coefficients are significant.

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