Mycobiota of Almond Seeds and the Toxigenicity of Some Involved Genera.

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Abstract: The myco-contaminants associated with almond seeds were surveyed and the fungal isolation frequencies were statistically analyzed. HPLC was used to assess the mycotoxins production of some involved fungi. Nine species belonging to six fungal genera were recovered from a total of 20 random samples of almond collected from different locations in Riyadh, Kingdom of Saudi Arabia. *Aspergillus flavus, A. niger, A. ochraceus, A. ustus, Chaetomium globosum, Penicillium aurantiogriseum, Phoma sp. Rhizopus sp.* and *Ulocladium atrum* were isolated. *A. niger* was the most predominant while, *C. globosum* was the least frequent species. Significant positive and negative correlations were found among isolation frequencies of some isolated fungal species. About 40% of *A. flavus* isolates produced aflatrem (2-3 ppb), while 60% were sterigmatocystin (300-440 ppb) and maltoryzine (7-10 ppb) producers. Meanwhile, 57% of *A. niger* isolates were capable of producing oxalic acids (47-430 mg/ml). On the other hand, 60% of *P. aurantiogriseum* isolates were citrinin producers (5-22 ppb) and 40% were citrovirdin producers (20-45 ppb). Due to the ideal nutrient composition of almond, as well as inappropriate processing and storage conditions, the risk of fungal and mycotoxin contamination could be increased. To avoid almond contamination with toxigenic fungi, and prevent hazards to human and animal health; rigorous quarantine, accurate diagnosing methods and healthy storage conditions should be undertaken.

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1. Introduction

Almond fruit is a drupe, consisting of an outer hard shell with the seed inside. Commoditized almonds are shelled, unshelled and blanched that shelled almonds have been treated with hot water to soften the seed coat to reveal the white embryo. An edible seed of almond is significant source of mineral elements, vitamins, fibers, proteins and monounsaturated fatty acids. Almond seed provides high nutritional value for fresh consuming or a raw material of many industrial applications, and its oils could be medicinally employed (Ali *et al.*, 2009; Akpabio, 2012).

Like other oilseeds, commoditized almond seeds are vulnerable to fungal infection. Particularly, inappropriate marketing and storage conditions; may lead to extra mycofloral contamination (Gürses, 2006, Bruce *et al.,* 2003).

Numerous seed deteriorating fungi could attack almond seeds during storage under improper conditions. *Aspergillus,* Penicillium, Fusarium and Rhizopus are the most dominant fungi could invade commoditized almond seeds (Khosravi *et al*., 2007; Deabes, 2010).

Mycotoxigenic are the most serious fungal genera contaminate the almond seeds. However; Aspergillus, Penicillium and Fusarium fungi are responsible for secretion of different metabolic toxic compounds (Yassin *et al.,* 2010; El-Samawaty *et al.,* 2011). Accumulation of such mycotoxic compounds could affect nut quality (Arrusa *et al.*, 2005; Kumar *et al.*, 2008) and harm both human and animal consumers (Palanee *et al.*, 2001; David *et al.*, 2005).

The present study aimed to investigate the natural occurrence of myco-contaminants in almond seeds imported to Saudi Arabia for food purposes and to evaluate toxin-producing abilities of isolated myco-flora.

2. Materials and Methods

Fungi

Almond samples were collected from 20 different locations in Riyadh city, Kingdom of Saudi Arabia to be used for fungal detection and isolation. Almond seed samples were randomly seeded onto Petri dishes containing PDA, in quadruples. Plates were incubated at 27±2°C and examined daily for 7 days, after which the developing colonies were counted. Developing fungi were purified and identified to the species level by the aid of stereo microscope then maintained in slanted PDA. Identification of fungal isolates was carried out based on morphological and microscopic characteristics in the Mycological Center, Assiut University, Egypt.

Aflatrem

Production medium of aflatrem (Zhang *et al.,* 2004) was inoculated with *A. flavus* isolates and statically incubated at 30°C, under light or dark conditions. *A. flavus* mycelia were separated by filtration after 7 days. One-gram aliquots were lyophilized and homogenized in 10 ml of a 2:1 mixture of chloroform: methanol, vortexed several times and centrifuged at 2900×g for 10 min at room temperature to pellet insoluble material. The supernatants were transferred to new tubes and the solvent allowed evaporating overnight. Five hundred microliters of acetonitrile: water (9:1) was added to each of the dried extracts and vortexed until the samples were totally re-suspended. Samples were centrifuged at 150×g at room temperature for 20 min, and the supernatants were analyzed by HPLC to quantify the aflatrem (Duran *et al*., 2007).

Maltoryzine

Tested isolates of *A. flavus* were grown in Czapek-Dox broth medium containing malt sprout extract (Iizuka and Iida, 1962). Production of maltoryzine in the culture media was examined using HPLC (Bahkali *et al.*, 2013).

Sterigmatocystin

Analysis and quantitation of ST were performed by HPLC. *A. flavus* isolates grown on Kafer medium (Bahkali *et al*., 2013) for 7 days at 37°C under static conditions were investigated (Delgado and Guzman, 2009). Mycelium was separated by filtration and ST was extracted, with 50 ml acetone for 30 min, followed by 50 ml chloroform by further 30 min. The organic phase was separated, filtered through anhydrous sodium sulfate and evaporated in a fume hood in a water boiling bath. The residue was re-suspended in 500µL HPLC grade methanol and filtered through C-18 columns prior to analysis.

Oxalic acid

The concentration of oxalic acids produced by *A. niger* cultivated on Czapek-Dox broth medium was determined by HPLC. Separation of oxalic acids was carried out in a CLC-C825 CM caption exchange column; mobile phase, 90% H2O and 10% CH3OH; flow rate, 1 ml/min and temperature 35°C (Ghorbani *et al.,* 2007).

Citrinin

Tested isolates of *Penicillium* spp. were grown on sterilized malt extract at 27±2°C for 7–10 days. Cultures were blended for 2 min using a high speed homogenizer and filtered using glass filter paper. Citrinin was extracted from homogenized filtrate using dichloromethane with the addition of phosphoric acid, and extract was then cleaned up on polyamide columns. HPLC analysis of citrinin employed the method described by Franco *et al.* (1996). The mobile phase consisted of methanol/acetonitrile/water (3:3:4 v:v:v), the pH value of the mixture was 2.5, and the flow rate was the 0.15 ml/min.

Citreoviridin

A reliable analytical quantitative method described by Stubblefield *et al*. (1988) was used for determination of citreoviridin produced by *Penicillium* spp. The toxin was extracted with dichloromethane, and the extract was partially purified on silica and amino solid phase extraction (SPE) columns. The extract was analyzed for citreoviridin by normal-phase liquid chromatography, using a mobile phase of ethyl acetate: hexane (75:25) at 1.5 ml/min.

Statistical Analysis

MSTAT-C statistical package, Michigan State Univ., USA was used for ANOVA and correlation analysis of the fungal isolation frequency. Duncan's multiple test was used to compare means. Cluster analysis by the unweighted pair-group method based on arithmetic mean (UPGMA) was performed using SPSS6.0 software package.

3. Results

Fungi

Nine species belonging to six fungal genera were isolated from a total of 20 random samples of almond collected from different locations in Riyadh city. *Aspergillus flavus, A. niger, A. ochraceus, A. ustus, C. globosum, P. aurantiogriseum, Phoma sp. Rhizopus sp.* and *U. atrum* were obtained. *A. niger* was the most predominant while, *C. gluposum* was the least frequent species.

ANOVA of the fungal isolation frequency (Table 1) revealed that fungus, sample and (F x S) interactions were all highly significant sources of variation in frequencies of isolated fungi. The fungus was the first in importance as a source of variation in isolation frequency, while F x S interaction was the second important topic (Fig. 1).

Table 1. Analysis of variance of the fungal isolation frequencies from almond seed samples.

Source of variation	Df	MS	F. value	P
Replication	2	0.68	0.574	
Samples	19	4.719	3.988	0.000
Fungi	8	263.112	222.394	0.000
SxF	152	8.830	7.464	0.000
Error	358	1.183		

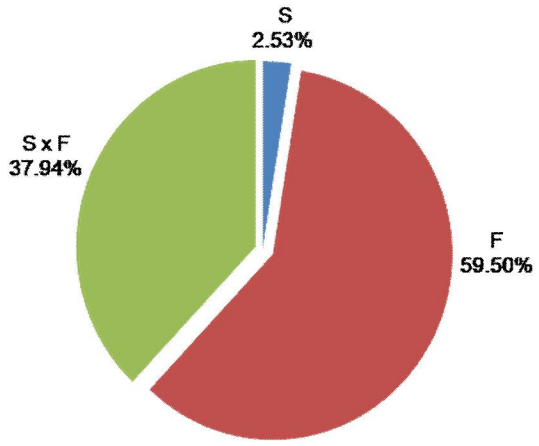

Figure 1. Relative contribution of samples (S), fungus (F) and their interactions (S x F) to variation in frequencies of fungi isolated from almond samples.

The significant effect of fungus × sample interaction indicates that the isolation frequencies are different according to the source of almond samples. For example, the isolation frequencies of *A. flavus* and *A. niger* were insignificantly different from each other in the sample No. 7. Meanwhile, isolation frequency of *A. niger* was higher (with highly significant difference) in sample No. 7 than in sample No. 6, and the vice versa for *A. flavus* (Table 2).

Significant positive and negative correlations were found among some fungal species when compared with the frequency of the others. Highly significant positive correlation was found among *P. aurantiogriseum* and both of *A. ochraceus* and *U. atrum*. Similar results were observed among *A. ustus* and those fungi. The significant positive correlation among some species indicates that those fungi have similar growth and colonizing conditions. On the other hand *A. niger* exhibited significant negative correlation with *Rhizopus* spp. indicating different growth conditions (Table 3).

Cluster analysis based on average linkage, of isolation frequencies (%); appear to form main distinct group (divided into subgroups)**.** *A. niger* was formed a separate group (Figure 2). Within particular group, fungi were associated strongly and positively in their distribution patterns over samples. This result implies the potential existence of sample (environment) related fungi.

Table 2. Comparable isolation frequencies of fungi isolated from almond seed samples.

Sample	Isolated fungi								
No.	1	2	3	4	5	6	7	8	9
1	2.08^{r}	81.30^{a-c}	5.13^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	18.70^{j-o}	0.00^{r}
2	3.03^{r}	67.72^{a-e}	2.78^{r}	0.00^{r}	0.00^{r}	12.22^{o-q}	0.00^{r}	20.55^{i-o}	0.00^{r}
3	0.00^{r}	61.13^{b-f}	0.00^{r}	4.76^{r}	0.00^{r}	4.17^{r}	0.00^{r}	38.87^{e-k}	5.13^{r}
4	0.00^{r}	73.90^{a-d}	0.00^{r}	4.17^{r}	0.00^{r}	3.03^{r}	0.00^{r}	26.10^{h-o}	0.00^{r}
5	2.78^{r}	66.97^{a-e}	0.00^{r}	5.13^{r}	0.00^{r}	0.00^{r}	0.00^{r}	33.03^{g-n}	2.78^{r}
6	4.71^{r}	80.24^{a-c}	2.08^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	19.76^{i-o}	0.00^{r}
7	32.42^{g-m}	27.73^{g-o}	2.78^{r}	12.73^{o-q}	0.00^{r}	14.39^{o-q}	0.00^{r}	0.00^{r}	12.73^{o-q}
8	0.00^{r}	22.22^{l-q}	77.78^{a-c}	0.00^{r}	0.00^{r}	2.08^{r}	0.00^{r}	0.00^{r}	0.00^{r}
9	41.85^{e-j}	44.1^{j-i}	0.00^{r}	0.00^{r}	7.41^{p-r}	4.76^{r}	6.67^{p-r}	0.00^{r}	0.00^{r}
10	0.00^{r}	75.00^{a-d}	3.03^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	25.00^{j-o}	0.00^{r}
11	5.13^{r}	76.67^{a-c}	2.78^{r}	4.17^{r}	0.00^{r}	23.33^{n-k}	0.00^{r}	0.00^{r}	0.00^{r}
12	2.78^{r}	85.00^{a-c}	0.00^{r}	0.00^{r}	0.00^{r}	2.08^{r}	0.00^{r}	15.00^{n-q}	0.00^{r}
13	0.00^{r}	30.95^{i-o}	5.13^{r}	0.00^{r}	0.00^{r}	5.13^{r}	69.05^{a-e}	0.00^{r}	0.00^{r}
14	40.00^{h-o}	47.78^{f-l}	4.17^{r}	12.22^{o-q}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}
15	0.00^{r}	23.33^{l-p}	0.00^{r}	3.03^{r}	0.00^{r}	0.00^{r}	0.00^{r}	76.67^{a-c}	0.00^{r}
16	66.67^{a-e}	33.33^{i-o}	0.00^{r}	0.00^{r}	0.00^{r}	2.08^{r}	0.00^{r}	0.00^{r}	4.76^{r}
17	0.00^{r}	93.33^{a}	0.00^{r}	6.67^{p-r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}
18	0.00^{r}	50.00^{c-h}	2.08^{r}	50.00^{c-h}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}
19	58.89^{c-g}	23.33^{m-q}	17.78^{k-p}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}	0.00^{r}
20	25.00^{j-o}	0.00^{r}	25.00^{j-o}	0.00^{r}	0.00^{r}	50.00^{c-h}	0.00^{r}	0.00^{r}	4.76^{r}
	1. *A. flavus*		2. *A. niger*		3. *A. ochraceus*		4. *A. ustus*		
	5. *C. gloposum*		6. *P. aurantiogriseum*		7. *Phoma sp.*		8. *Rihizopus sp.*		
	9. *U. atrum.*								

Table 3. Correlation among frequencies of fungi isolated from almond seed samples.

Isolated fungi	Isolation frequencies								
	1	2	3	4	5	6	7	8	9
1. A. niger		-0.690**	-0.406	0.017	-0.243	-0.256	-0.256	-0.108	-0.108
2. Rhizopus sp.			-0.197	-0.330	-0.368	-0.254	-0.254	-0.254	-0.254
3. A. flavus				0.060	0.513*	0.305	0.305	0.427	0.427
4. P. aurantiogriseum					0.352	0.568**	0.568**	-0.096	-0.096
5. A. ustus						0.568**	0.703**	-0.076	-0.076
6. U. atrum							1.00**	-0.053	-0.053
7. A. ochraceus								-0.053	-0.053
8. C. gloposum									1.00**
9. Phoma sp.									

Linear Correlation coefficient (r) is significant at P≤ 0.05 (*) or P≤ 0.01(**)

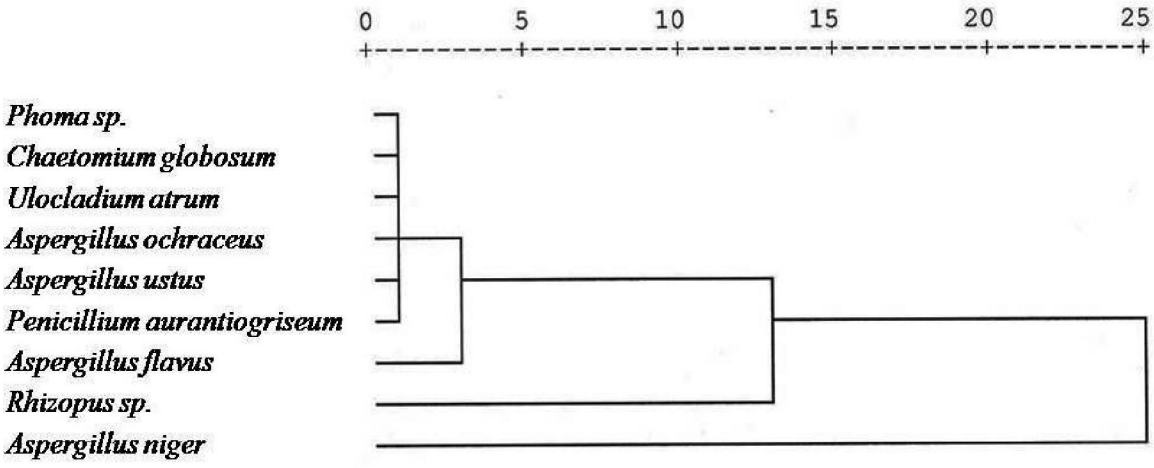

Figure 2. Phenogram based on average linkage cluster analysis of frequencies of fungi recovered from almond seeds.

Mycotoxins

About 60% of the tested *A. flavus* isolates were toxigenic and capable of producing sterigmatocystin (300-440ppb) and maltoryzine (7-10ppb) in the culture media. Meanwhile, 40% of the tested isolates of *A. flavus* were produced aflatrem (2-3ppb) in the culture media (Table 4).

Table 4. Production of Aflatrem, maltoryzine and sterigmatocystin by *A. flavus*.

Fungus	Mycotoxin (ppb)		
	Sterigmatocystin	maltoryzine	Aflatrem
1. *A. flavus*	440.00	10.00	2.00
2. *A. flavus*	0.00	0.00	0.00
3. *A. flavus*	0.00	0.00	0.00
4. *A. flavus*	425.00	8.00	3.00
5. *A. flavus*	300.00	7.00	0.00

Regarding the oxalic acid production; 57% of the tested *A. niger* isolates were oxalic acids producers (47-430 mg/ml) in their culture media (Table 5). *Penicillium* mycotoxin assay (Table 6) showed that 60% of the tested *P. aurantiogriseum* isolates were citrinin producers (5-22 ppb) and 40% were citrovirdin producers (20-45 ppb).

Table 5. Production of oxalic acid by *A. niger*.

Fungus	Oxalic acid (mg/ml)
1. *A. niger*	430.00
2. *A. niger*	0.00
3. *A. niger*	85.00
4. *A. niger*	47.00
5. *A. niger*	110.00
6. *A. niger*	0.00
7. *A. niger*	0.00

Table 6. Production of citrinin and citreoviridin by *Penicillium* species.

Fungus	Mycotoxin (ppb)	
	citrinin	citreoviridin
1. *P. aurantiogriseum*	5.00	20.00
2. *P. aurantiogriseum*	8.00	0.00
3. *P. aurantiogriseum*	0.00	0.00
4. *P. aurantiogriseum*	0.00	0.00
5. *P. aurantiogriseum*	22.00	45.00

4. Discussion

The results indicated that nine species belonging to six fungal genera found to be associated with almond seed samples in this study (Deabes and El-Habib, 2011). The predominant fungi associated with the investigated samples were agreed with those in the documented literatures (Khosravi *et al.,* 2007; Deabes, 2010, Alwakeel and Nasser, 2011). This finding confirms the fact that almond seed is an important substrate for the infection and growth of deteriorating fungi; which may occur in the pre and/or post harvest periods (Bruce *et al.,* 2003). However, due to the ideal nutrient composition of almond, as well as inappropriate post-harvest and storage conditions, increasing fungal population could be occurred (Gecgel *et al.,* 2011).

Production of *A. flavus* mycotoxic metabolites were agreed with those of many investigators (Kato *et al*., 2003; Rank *et al*., 2011). The *A. flavus,* famous toxic secondary metabolites; aflatrem (Zhang *et al.,* 2004; Duran *et al*., 2007; Nicholson *et al*., 2009), maltoryzine (Blumenthal, 2004) and sterigmatocystin (Versilovskis and De Saeger, 2010) had frequently been documented (Bahkali *et al*., 2013). The production of oxalic acids by *A. niger* in the culture media and its possible roles in fungal pathogenicity and ecology had also been reported (Blumenthal, 2004, Hattori *et al*., 2007, Bahkali *et al*., 2013).

The potent mycotoxins; citrinin and citreoviridin; were also produced by the tested Penicillium isolates (Kurtzman and Blackburn, 2005; Bragulat *et al*., 2008). Actually, Plant pathogenic Penicillia may produce these toxins in culture media, and/or in agricultural commodities (Rundberget and Wilkins, 2002; Rundberget *et al*., 2004). The food myco-contaminant; *P. aurantiogriseum* was frequently reported as citrinin and citreoviridin producers (Yassin *et al*., 2010; El-Samawaty *et al*., 2011).

5. Conclusion

Due to the ideal nutrient composition of almond seeds, as well as inappropriate processing and storage conditions, the risk of fungal and mycotoxin contamination could be increased. To avoid contamination with toxigenic fungi, and prevent hazards to human and animal health; rigorous quarantine, accurate diagnosing methods and healthy storage conditions should be undertaken.

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