JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. **7**(Spl. Edn.), p. 361-367

Myco-Contaminants Associated with Pistachio Nut and the Aspergillii Mycotoxicity

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(Received: 15 September 2013; accepted: 31 October 2013)

The aim of the present work was to survey the myco-contaminants associated with pistachio nut consumed in Riyadh, Kingdom of Saudi Arabia. A total of forty commercially available samples, randomly collected from different locations were investigated and the isolation frequencies of myco-contaminants were statistically analyzed. Mycotoxins productivities of the isolated fungi were analyzed using HPLC. Nine fungal species belonging to five genera were found to be associated with pistachio nut samples. Distributions of isolated fungi indicated that *Aspergillus niger, Rhizopus sp.* and *A. flavus* were predominant with isolation frequencies of 67.7%, 57.5% and 32.5% respectively. Highly significant positive and negative correlations were observed among some fungal species when compared with the frequency of the others. The mycotoxins; Aflatrem, maltoryzine and sterigmatocystin were produced by 60%, 40% and 60% of the *A. flavus* isolates in this study. Meanwhile, 50% of the tested A. niger isolates were oxalic acids producers. Neither citrinin nor citreoviridin could be produced by any of the tested *Penicillium spp.* in this study.

Key words: Storage fungi, HPLC, Deterioration, Mycotoxins.

Mold has been reported to affect about 25% of the world's crops[1]. The agricultural and storage practice conditions conducive to fungal growth and toxin production could magnify yield losses[2,3]. Fungal contamination of various foodstuffs and agricultural products is a major problem and results in human and livestock poisoning[4,5].Nuts that provide high nutritional value for fresh consuming or raw materials of many industrial applications could be attacked by several myco-contaminants[6,7]. Numerous seed deteriorating fungi could attack nuts during storage under improper conditions. *Aspergillus, Penicillium, Fusarium* and *Rhizopus* are the most dominant fungi could invade commoditized nuts[8,9].

The pistachio (*Pistacia vera L.*) is also a perishable nut vulnerable to pre and/or post-harvest fungal infection. *A. niger, A. flavus* and *Penicillium spp.* were the dominant pre harvest external mycoflora of the immature pistachio nuts[10]. Several other genera; *Alternaria, Aspergillus, Chladosporium, Eurotium, Fusarium, Penicilium, Trichoderma, Ulocladium, Epicoccum* and *Rhizopus* found to be associated with pistachio kernels [11].

Mycotoxigenic *Aspergillus* and *Penicillium* are the most serious fungal genera that responsible for secretion of different metabolic

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toxic compounds[12,13]. Accumulation of such mycotoxic compounds affect nut quality and harm both human and animal consumers[14-16]. The myco-contaminants in pistachio nuts imported to Saudi Arabia for food purposes were surveyed and toxin-productivity of isolated fungi was also investigated in this study.

MATERIALS AND METHODS

Sampling and mycoflora

Forty samples of pistachio nuts were collected from forty different locations in Riyadh city, Kingdom of Saudi Arabia. Samples were randomly seeded onto Petri dishes containing PDA, in quadruples. Plates were incubated at 27±2ºC 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.

Mycotoxin analyses

Aflatrem assay

A. flavus isolates were grown on aflatrem production medium[17]. Cultures were incubated statically under light or dark conditions at 30ºC for 7 days after which, mycelium was separated by filtration. 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 at room temperature for 10 min 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 resuspended. Samples were centrifuged at 150×g at room temperature for 20 min, and the supernatants were analyzed by HPLC to quantify the aflatrem[18].

Maltoryzine assay

HPLC was performed to quantify maltoryzine produced by *A. flavus* grown in Czapek-Dox broth medium containing malt sprout extract[19].

Sterigmatocystin assay

According to Delgado and Guzman[20], *A. flavus* isolates were grown on Kafer medium, and incubated at 37ºC under static conditions for 7 days. By the end of the incubation period, mycelium was separated by filtration. 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 500 µL HPLC grade methanol and filtered through C-18 columns. Analysis of ST were performed by HPLC.

Oxalic acid assay

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 CLCC825 CM caption exchange column; mobile phase, 90% H2O and 10% CH3OH; flow rate, 1 ml/min and temperature 35°C[21].

Patulin and citreoviridin assay

Penicillium isolates were aseptically cultured onto malt extract broth and incubated at 27±2ºC for 7-10 days. Then, mycotoxins were extracted using acetonitrile/water solution (5/95, v:v). Solvent was then evaporated under vacuum at 35ºC. Dried residues were dissolved in 1 ml of the same mobile phase, and then filtered through a 0.45-µm micro-filter prior to HPLC analyses[22].

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.

RESULTS

Mycoflora analysis

Nine fungal species belonging to five genera were isolated from a total of 40 random samples of pistachio nut. Distributions of isolated fungi indicated that *A. niger, Rhizopus sp.* and *A. flavus* were predominant with isolation frequencies of 67.7%, 57.5% and 32.5% respectively (Table 1).

Analysis of variance of the fungal isolation frequency (Table 2) revealed that fungus,

sample and fungus × sample interactions were all highly significant sources of variation in frequencies of fungi isolated from pistachio nut. The fungus was the least important as a source of variation in isolation frequency, while fungus × sample interaction was the most important topic (Fig. 1).

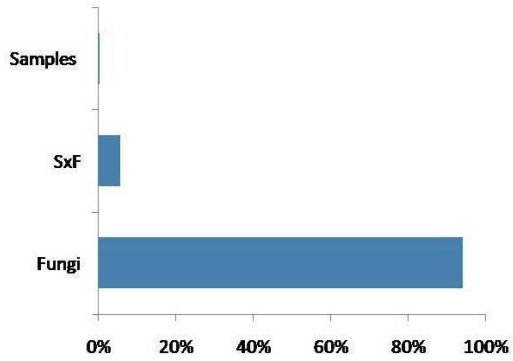

Fig. 1. Relative contribution of samples (S), fungus (F) and their interactions (S x F) to variation in frequencies of fungi isolated from Pistachio nut samples

The significance of fungus × sample interactions indicates that isolation frequencies of fungal species are varies according to the sample sources. For example, isolation frequency of *A. flavus* was significantly higher than *P. chrysogenum* frequency in sample No. 25 and the vice versa in sample No. 38. Although, *Rihizopus* sp. and *P. chrysogenum* showed an equal isolation frequencies in sample No. 8. The isolation frequency of *Rihizopus* sp. was significantly smaller than *P. chrysogenum* (Table 3).

Table 1. Frequencies (%) of the fungi isolated from Pistachio nut samples

Fungi	Frequencies %
1. *A. flavus*	32.5
2. *A. niger*	67.5
3. *P. aurantiogriseum*	5.0
4. *P. brevicompactum*	150
5. *P. chrysogenum*	22.5
6. *P. funiculosum*	2.5
7. *Phoma eupyrena*	5.0
8. *Urrotium amstelodam*	7.5
9. *Rhizopus* sp.	57.5

Table 2. ANOVA of isolation frequencies of fungi isolated from Pistachio nut samples.

Source of variation	Df	MS	F. value	P
Replication	2	0.317	0.425	
Samples	39	0.733	0.983	0.000
Fungi	8	286.443	384.310	0.000
SxF	312	16.747	22.468	0.000
Error	718	0.745		

Highly significant positive and negative correlations were observed among some fungal species when compared with the frequency of the others. Highly significant positive correlation was found among *Rhizopus* sp. and *A. niger*, indicating similar colonizing conditions of these fungal species in the pistachio nut. On the other hand, highly significant negative correlation was among *Rhizopus* sp. and *A. flavus* indicating different colonizing conditions of these fungal species in the pistachio nut (Table 4).

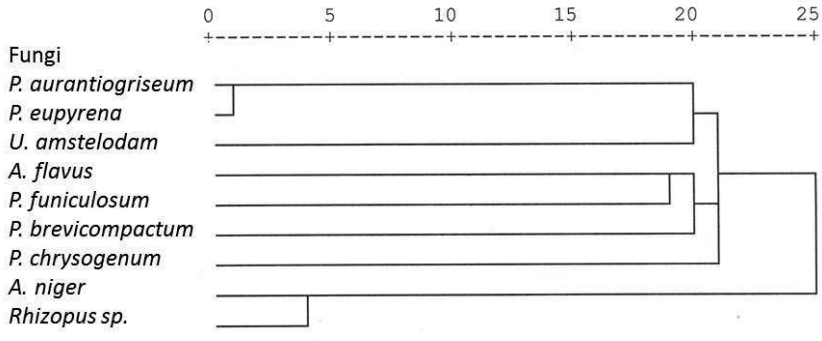

Fig. 2. Phenogram based on average linkage cluster analysis of frequencies of fungi recovered from almond samples

Table 3. Comparable isolation frequencies of fungi isolated from Pistachio nut samples

Sample	Isolation frequencies %								
No.	1	2	3	4	5	6	7	8	9
1	0.00^{u}	61.11^{b-g}	0.00^{u}	0.00^{u}	20.83^{n-s}	0.00^{u}	0.00^{u}	18.05^{m-s}	0.00^{u}
2	0.00^{u}	83.33^{eb}	0.00^{u}	0.00^{u}	16.67^{m-s}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
3	0.00^{u}	68.89^{a-f}	0.00^{u}	0.00^{u}	19.76^{m-s}	0.0^{u}	0.00^{u}	11.43^{r-t}	0.00^{u}
4	0.00^{u}	71.11^{a-f}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	28.89^{k-o}	0.00^{u}
5	0.00^{u}	50.5^{d-j}	0.00^{u}	25.0^{m-s}	0.00^{u}	0.00^{u}	0.00^{u}	12.5^{r-t}	0.00^{u}
6	0.00^{u}	68.25^{b-g}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	31.74^{i-o}	0.00^{u}
7	0.00^{u}	81.94^{a-c}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	18.05^{m-s}	0.00^{u}
8	0.00^{u}	60.47^{b-g}	0.00^{u}	0.00^{u}	19.76^{m-s}	0.00^{u}	0.00^{u}	19.76^{m-s}	0.00^{u}
9	0.00^{u}	75^{a-e}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	25.00^{m-s}	0.00^{u}
10	0.00^{u}	0.00^{u}	48.33^{f-k}	0.00^{u}	0.00^{u}	51.66^{d-j}	0.00^{u}	0.00^{u}	0.00^{u}
11	0.00^{u}	72.22^{a-f}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	27.78^{k-p}	0.00^{u}
12	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	100.0^{a}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
13	86.67^{ab}	0.00^{u}	0.00^{u}	0.00^{u}	13.33^{q-t}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
14	0.00^{u}	83.33^{a-c}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	16.67^{st}	0.00^{u}
15	0.00^{u}	0.00^{u}	85.00^{ab}	0.00^{u}	0.00^{u}	15.00^{p-s}	0.00^{u}	0.00^{u}	0.00^{u}
16	65.18^{b-g}	0.00^{u}	0.00^{u}	24.44^{l-q}	0.00^{u}	0.00^{u}	0.00^{u}	10.37^{st}	0.00^{u}
17	0.00^{u}	71.31^{a-f}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	28.69^{j-o}	0.00^{u}
18	0.00^{u}	59.52^{b-g}	0.00^{u}	22.62^{m-r}	0.00^{u}	0.00^{u}	0.00^{u}	17.85^{m-s}	0.00^{u}
19	0.00^{u}	80.00^{a-c}	0.00^{u}	16.67^{st}	83.33^{a-c}	0.00^{u}	0.00^{u}	20.00^{m-s}	0.00^{u}
20	0.00^{u}	83.33^{a-c}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	16.6^{st}	0.00^{u}
21	0.00^{u}	0.00^{u}	0.00^{u}	100.00^{a}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
22	0.00^{u}	88.89^{ab}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	11.11^{st}	0.00^{u}
23	0.00^{u}	51.85^{d-i}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	48.15^{e-j}	0.00^{u}
24	85.00^{ab}	0.00^{u}	0.00^{u}	15.00^{p-s}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
25	75.47^{a-d}	0.00^{u}	0.00^{u}	0.00^{u}	19.67^{m-s}	0.00^{u}	0.00^{u}	4.76^{tu}	0.00^{u}
26	0.00^{u}	76.66^{a-e}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	23.33^{m-s}	0.00^{u}
27	67.58^{b-g}	32.41^{i-n}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
28	35.98^{h-m}	44.31^{g-l}	0.00^{u}	0.00^{u}	19.70^{m-s}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
29	37.73^{h-m}	62.27^{b-g}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
30	0.00^{u}	55.55^{d-i}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	44.44^{i-n}	0.00^{u}
31	0.00^{u}	64.89^{b-g}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	35.18^{k-o}	0.00^{u}
32	43.33^{i-o}	34.44^{h-m}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	22.22^{r-t}	0.00^{u}
33	100.00^{a}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
34	0.00^{u}	54.54^{c-h}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	45.45^{f-k}	0.00^{u}
35	50.00^{d-i}	50.00^{d-i}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
36	19.44^{o-s}	80.55^{a-c}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
37	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	100.00^{a}
38	15.00^{p-s}	0.00^{u}	0.00^{u}	0.00^{u}	85.00^{ab}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}
39	100.00^{a}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	100.00^{a}
40	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	0.00^{u}	100.00^{a}	0.00^{u}	100.00^{a}

1. *A. flavus*, 2. *A. niger*, 3. *P. aurantiogriseum*, 4. *P. brevicompactum*, 5. *P. chrysogenum*, 6. *P. eupyrena* 7. *P. funiculosum*, 8. *Rihizopus sp.*, 9. *U. amstelodam.*

Cluster analysis of fungal isolation frequencies (%) showed that isolated fungi appear to form 2 distinct groups. The first group including *A. flavus* and *A. niger* while the second was divided into 3 subgroups (Fig 2). Within each group, fungi were associated strongly and positively in their distribution patterns over samples, whereas between groups, they were associated weakly or negatively. This result implies the potential existence of sample (environment) related fungi.

Table 4. Correlation among frequencies of fungi isolated from Pistachio nut samples

Fungi	1	2	3	4	5	6	7	8	9
1. *A. niger*		0.540**	-0.285	-0.257	-0.295	-0.214	-0.283	-0.535**	a
2. *Rhizopus sp.*			-0.236	-0.161	-0.206	-0.149	-0.183	-0.457**	a
3. *P. chrysogenum*				-0.107	-0.084	-0.061	-0.075	-0.057	a
4. *P. brevicompactum*					-0.062	-0.045	-0.055	-0.037	a
5. *P. aurantiogriseum*						-0.035	0.684**	-0.134	a
6. *Urrotium amstelodam*							-0.031	-0.097	a
7. *Phoma eupyrena*								-0.119	a
8. *A. flavus*									a
9.*P. funiculosum*									

a= cannot be computed because at least one of the variables is constant.
Linear Correlation coefficient (r) is significant at P≤ 0.05 (*) or P≤0.01(**)

Table 5. Production of Aflatrem, maltoryzine and sterigmatocystin by *A. flavus*

A. flavus	Mycotoxin (ppb)		
isolates	Aflatrem	Maltoryzine	Sterigmatocystin
A.f.1	2.00	11.00	325.00
A.f.2	1.00	0.00	650.00
A.f.3	1.00	4.00	225.00
A.f.4	0.00	0.00	0.00
A.f.5	0.00	0.00	0.00

Table 6. Production of oxalic acid by *A. niger* isolates

A. niger isolates	Oxalic acid (ppm)
*A.n.*1	450.00
A.n. 2	0.00
*A.n.*3	0.00
*A.n.*4	150.00
*A.n.*5	80.00
A.n. 6	350.00
*A.n.*7	0.00
*A.n.*8	0.00
*A.n.*9	0.00
*A.n.*10	630.00

Mycotoxins assay

Aspergillus toxins

HPLC analyses of *Aspergillii* mycotoxigenicity revealed that Aflatrem (1-2 ppb), maltoryzine (4- 11ppb) and sterigmatocystin (225-650 ppb), were produced in the culture media by 60%, 40% and 60% of the *A. flavus* isolates in this study (Table 5). Meanwhile, 50% of the tested *A. niger* isolates were capable of producing oxalic acids ranged from 80-630 mg/ml in their culture media (Table 6).

Penicillium toxins

Neither citrinin nor citreoviridin could be produced by any of the tested *Penicillium* spp. in this study.

DISCUSSION

Isolation of seed deteriorating fungi from pistachio nut was agreed with the documented results of several investigators[8,9,23,24]. The infection by such fungal genera might be occurred during growth, harvesting, transportation or storage periods due to the ideal nutritional characteristics of pistachio nut[7,25,26]. Inappropriate conditions of post-harvest, marketing and/or storage periods could result in increasing fungal population[3,19].

Most of *A. flavus* isolates in this survey were capable of producing aflatrem, maltoryzine and sterigmatocystin mycotoxins in their culture media[18,27,28]. This finding was agreed with those of many investigators[17,29,30,31]. Most of the tested *A. niger* isolates also were capable of producing oxalic acids in the culture media[27,32].

Tested isolates of *Penicillia* failed to produce any citrinin and/or citreoviridin mycotoxins in this study. This result was disagreed with those of many investigators[12,13,19].

CONCLUSION

Myco-contamination of pistachio nut could be increased due to the ideal nutrient composition, as well as inappropriate processing

and storage conditions. To minimize the risk of contamination with mycotoxigenic fungi, and prevent hazards to human health; rigorous quarantine, accurate diagnosing methods and healthy storage conditions should be undertaken.

ACKNOWLEDGMNTS

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP-VPP-298.

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