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Toxigenic Fungal Biota Associated with Walnut in Saudi Arabia

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Mycoflora associated with 120 walnut samples was examined using agar plate method. Data of isolation frequency were statistically analyzed. Mycotoxin productivity of obtained fungi was assayed using HPLC. Twelve species belonging to six fungal genera were isolated in this work. *Aspergillus flavus, Aspergillus niger* and *Rhizopus stolonifer* were the most predominant, with highest distribution over samples. Significant to highly significant positive correlation was found among some isolated fungi. Most of *Aspergillus flavus* isolates were capable of producing sterigmatocystin, maltoryzine and aflatrem. Meanwhile, all tested isolates of *Aspergillus niger* were capable of producing oxalic acids ranged from 300-850 mg/ml in the culture media. Both of *Fusarium proliferatum* and *Fusarium subglutinans* were toxigenic and varied in their productivities of ipomeanine, neosolaniol, nivalenol and NT-2 toxin. In respect to citrinin and citreovirdin, *Penicillium aurantiogriseum* was more productive than *Penicillium brevicompactum*.

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

Walnut (*Juglans regia* L) is one of the finest nuts imports to the Arabian market from temperate regions. It has highly nutritional values, very positive effects on human health (Ozcan, 2009, Ali *et al.,* 2010) and appreciated medicinal importance (Papoutsi *et al.,* 2008; Spaccarotella *et al.,* 2008). Walnut is a significant component in a variety of traditional dishes that eaten raw or roasted in some countries (Gecgel *et al.,* 2011). Meanwhile, it has significant economic interest for the food industry (Martinez *et al.,* 2008).

Unfortunately, walnut is sensitive to pre and post-harvest fungal invasion and as a result of inappropriate storage conditions; it may be contaminated by extra mycoflora (Gürses, 2006). Infection can occur during growth, harvesting, transportation or storage of walnuts (Bruce *et al.,* 2003). Numerous fungi could attack walnut during storage under improper conditions (Al-Bachir, 2004). *Aspergillus, Penicillium, Fusarium and Rhizopus* are the most dominant fungi invade commoditized walnut (Deabes, 2010).

Toxin-producing fungal genera are the most serious fungi could invade walnut. Members of *Aspergillus, Penicillium* and *Fusarium* fungi are responsible for secretion of different metabolic toxic compounds (Yassin *et al.,* 2010; El-Samawaty *et al.,* 2011). Unfortunately; accumulation of such mycotoxic compounds could affect nut quality (Arrusa *et al.,* 2005; Kumar *et al.,* 2008) and harm both human and animal consumer (Palanee *et al.,* 2001; David *et al.,* 2005). The present study aimed to investigate the natural occurrence of toxin-

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producing fungi in walnuts imported to Saudi Arabia for food purposes and to evaluate toxin-producing abilities of isolated fungi.

MATERIALS AND METHODS

Mycoflora analysis

Mycoflora associated with walnut kernels was examined using the agar plate method recommended by International Seed Testing Association (ISTA, 1999). Forty walnut samples, collected from forty different locations of Riyadh city were used for detection of deteriorating fungi. Kernels were randomly separated and directly seeded onto Petri dishes containing PDA, in quadruples. Plates were incubated at 27±2ºC and examined daily for five 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.

Mycotoxin analyses

Aflatrem assay

Aspergillus flavus isolates were grown on aflatrem production medium (Zhang *et al.,* 2004). Cultures were incubated statically under light or dark conditions at 30 °C 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 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).

Sterigmatocystin assay

According to Delgado and Guzman (2009) *A. flavus* isolates were grown on Kafer, (1977) medium, and incubated at 37 °C 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 and quantitation of ST were performed by HPLC.

Maltoryzine assay

HPLC was performed to quantify maltoryzine produced by *Aspergillus flavus* grown in Czapek-Dox broth medium containing malt sprout extract (Iizuka and Iida, 1962).

Oxalic acid assay

The concentration of oxalic acids produced by *Aspergillus niger* cultivated on Czapek-Dox broth medium was determined by high performance Liquid chromatography (HPLC). Separation of oxalic acids was carried out in a CLC-C825 CM caption exchange column; mobile phase, 90% H_2O and 10% CH_3OH; flow rate, 1 ml/min and temperature 35°C (Ghorbani *et al.,* 2007).

Fusaria toxins assay

Fusaria were grown on rice sterile culture medium (87.5 g rice and 37.5 g distilled water) and incubated for 14 days at 28°C (Geraldo *et al.,* 2006). Toxins were extracted in 40 ml acetonitrile/water (84:16) samples were incubated and shaken vigorously overnight in a wrist-action shaker, centrifuged at 4500 rpm for 10 min and decanted. One milliliter of the supernatant was pipetted into 1.5 ml Eppendorf tubes, evaporated to dryness at 45 °C in a Speed vacuum chamber and stored in the freezer at -20 °C. Samples were prepared for HPLC analysis by dissolving in 375 µL methanol, 375 µL buffer (10 mM ammonium acetate, pH 4.5 (adjusted with formic acid and 1% acetonitrile) and 500 µL of hexane. The solution was shaken and centrifuged for phase separation (20 °C, 1400 rpm for 5 min) and the lower phase was filtered using the OPTIFLOW 13 PTFE 0.2 µm into HPLC vial, and mycotoxins (neosolaniol, nivalenol and T-2) were quantified using the HPLC/MS (Klötzel *et al.,* 2006).

Patulin assay

Penicillium isolates were grown on sterilized malt extract for 7–10 days at 27±2°C. Cultures were blended for 2 min using a high speed homogenizer and filtered using glass filter paper. Patulin was extracted from homogenized filtrate using acetonitrile: water (5:95 v:v) solution. The solvent was then evaporated at 35°C under vacuum. The dried residues were dissolved in 1 ml

of acetonitrile: water (5:95 v:v) solution. HPLC was used to quantify patulin (Christian, 1990).

Citreoviridin assay

A reliable analytical quantitative method described by Stubblefield *et al*. (1988) was used for citreoviridin determination. The toxin was extracted with dichloromethane, and the extract was partially purified on silica and amino solidphase 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

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

RESULTS

Mycoflora analysis

Twelve species belonging to six fungal genera were isolated from a total of 120 walnut random samples collected from different locations in Riyadh city. Of these, *Aspergillus flavus* was the most predominant and significantly differed

Table 1. Isolation frequency means of fungi recovered from walnut

S. No.	Fungus	Isolation frequency means
1.	*Aspergillus flavus*	43.17a
2.	*Aspergillus fumigatus*	5.71d
3.	*Aspergillus niger*	11.99 c
4.	*Aspergillus ochraceus*	2.89 efg
5.	*Fusarium nygamai*	1.95fg
6.	*Fusarium proliferatum*	1.95fg
7.	*Fusarium subglutinans.*	3.34ef
8.	*Penicillium aurantiogriseum*	5.83d
9.	*P.brevicompactum*	1.28fg
10.	*Neurospora sitophila*	1.64fg
11.	*Rhizopus stolonifer*	18.33b
12.	*Syncephalastrum racemosum*	0.8g

Values followed by the same letter are not significantly different.

Table 2. Correlation among frequencies of fungi isolated from walnut fruits collected from different location in Riyadh city

S. No	Isolation *frequencies of*	Isolation frequencies of 1	2	3	4	5	6	7	8	9	10	11	12
1.	*A. flavus*		-0.376*	-0.312*	-0.4166	-0.203	-0.064	-0.342*	-0.359*	-0.203	-0.242	-0.160	-0.203
2.	*A. fumigatus*			-0.223	-0.055	-0.187	-0.090	0.445**	-0.112	-0.008	0.059	-0.134	0.191
3.	*A. niger*				-0.185	-0.099	-0.141	-0.166	-0.175	0.261	0.297	0.010	-0.099
4.	*A. ochraceus*					-0.048	0.100	0.078	-0.085	-0.048	-0.057	0.160	0.205
5.	*F. nygamai*						-0.037	-0.043	-0.045	-0.026	-0.031	-0.177	-0.026
6.	*F. proliferatum*							-0.062	0.316*	-0.037	-0.044	-0.169	-0.037
7.	*F. subglutinans.*								-0.077	-0.043	0.017	-0.168	0.601**
8.	*P. aurantiogriseum*									-0.045	-0.054	-0.037	-0.045
9.	*P. brevicompactum*										-0.031	-0.177	-0.026
10.	*N. sitophila*											-0.211	-0.031
11.	*R. stolonifer*												-0.177
12.	*S. racemosum*												

Linear Correlation coefficient (r) is significant at Pd" 0.05 (*) or Pd" 0.01(**)

from other species. In addition, *Syncephalastrum racemosum* was the least frequent species. *Aspergillus fumigatus* and *P. aurantiogriseum* were exhibited the same frequency (Table 1).

Relative distribution of isolated fungi over samples revealed that *Rhizopus stolonifer* was the highest distributed followed by *A. flavus* and *A. niger* species. Meanwhile; *F. subglutinans*, *P. aurantiogriseum* and *Fusarium proliferatum* were almost equal distributed species (Fig. 1).

Significant positive and negative correlations were observed among some fungal species when compared with the frequency of others. Highly significant positive correlation was found among *F. subglutinans* and *Aspergillus fumigatus* but significant positive correlation was occurred among *F. proliferatum* and *P. aurantiogriseum*. On the other hand *A. flavus* exhibited significant negative correlation with *Aspergillus fumigatus*, *Aspergillus niger, F. subglutinans* and *P. aurantiogriseum* (Table 2).

Based on average linkage cluster analysis of fungal isolation frequencies (%); isolated fungi appear to form 3 distinct groups (divided into subgroups)**.** Of these, *A. flavus* was formed a separate group (Figure 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 3. Aflatrem, maltoryzine and sterigmatocystin productivity of *Aspergillus flavus*

S. No	Fungus	Mycotoxin (ppb)		
		Sterigmatocystin	maltoryzine	Aflatren
1.	*Aspergillus flavus*	500	5	2.5
2.	*Aspergillus flavus*	-	-	-
3.	*Aspergillus flavus*	-	-	-
4.	*Aspergillus flavus*	250	7	1
5.	*Aspergillus flavus*	200	12	1

Table 4. Oxalic acid productivity of *Aspergillus niger*

S. No.	Fungus	Oxalic acid (mg/ml)
1.	*Aspergillus niger*	650
2.	*Aspergillus niger*	700
3.	*Aspergillus niger*	850
4.	*Aspergillus niger*	450
5.	*Aspergillus niger*	300

Table 5. Ipomeanine, Neosolaniol , Nivalenol and NT-2 toxin productivity of *Fusarium* species

S. No	Fungus	Mycotoxin (ppb)			
		Neosolaniol	NT-2 toxin	Nivalenol	Ipomeanine
1.	*Fusarium proliferatum*	1	3	200	6
2.	*Fusarium subglutinans.*	4.5	5	150	2.5

Table 6. Citrinin and citreoviridin productivity of *Penicillium* species

S. No.	Fungus	Mycotoxin (ppb)	
		Citrinin	Citreoviridin
1.	*P.brevicompactum*	1	6
2.	*P. aurantiogriseum*	22	45

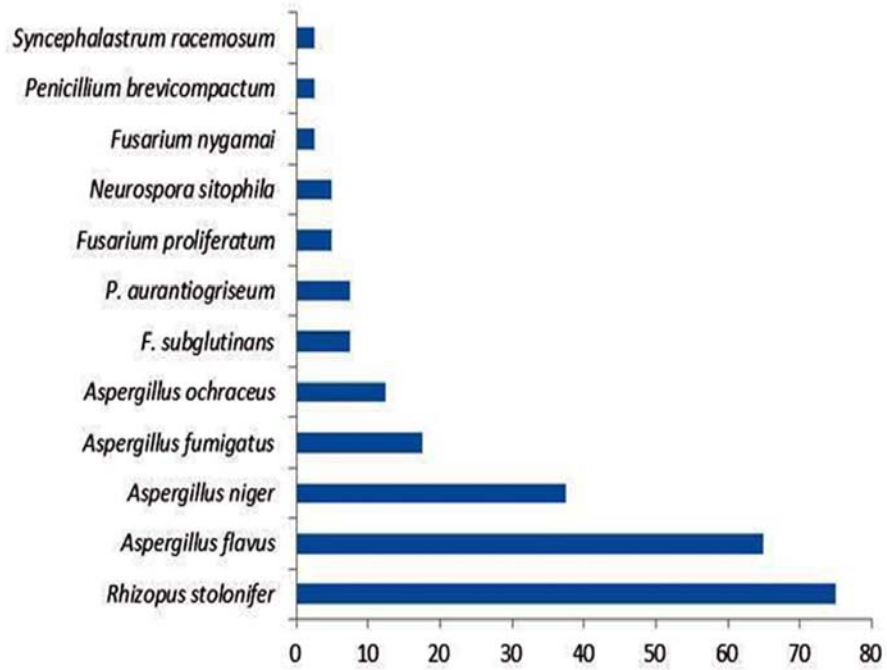

Fig. 1. Relative distribution of fungi over walnut samples

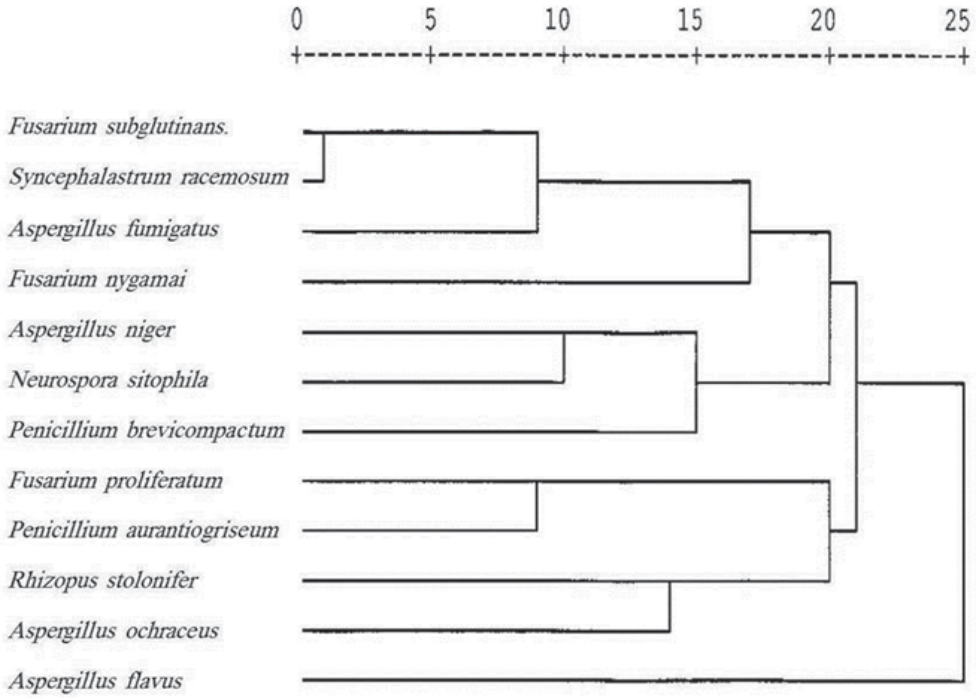

Fig. 2. Phenogram based on average linkage cluster analysis of isolation frequencies of isolated fungi

Mycotoxins assay

Aspergillus toxins

Most of the *Aspergillus flavus* isolates were toxigenic and capable of producing sterigmatocystin (200-500ppb), maltoryzine (5-12ppb) and aflatrem (1-2.5ppb) in their culture media (Table 3). Meanwhile, all tested isolates of *Aspergillus niger* were capable of producing oxalic acids ranged from 300-850 mg/ml. in their culture media (Table 4).

Fusarium toxins

Toxin productivities of *F. proliferatum* and *F. subglutinans* (Table 5) indicated that the former was the highest producer of ipomeanine (6ppb) and nivalenol (200ppb), while the later was the highest producer of neosolaniol (4.5ppb) and NT-2 toxin (5ppb).

Penicillium toxins

Penicillium mycotoxin assay showed that *P. aurantiogriseum* was produced about 22 and 8 fold of citrinin and citrovirdin respectively more than *P. brevicompactum* (Table 6).

DISCUSSION

Results of this work indicated that walnut is important substrate for the infection and growth of fruit molding fungi; which can occur during growth, harvesting, transportation or storage (Bruce *et al.,* 2003). Twelve species belonging to six fungal genera were isolated (Arya, 2003; Singh and Shukla, 2008; Deabes and El-Habib, 2011). Significant to highly significant positive correlation was found among some isolated fungi. Recovering of variant fungal genera from this survey could be attributed to the ideal nutrient composition of nuts, as well as inappropriate post-harvest and storage conditions, which may lead to increasing fungal population (Savage, 2001; Caglarirmak, 2003). Predominant fungi associated with walnut samples were agreed with those in documented literatures (Khosravi *et al.,* 2007; Deabes, 2010 Alwakeel and Nasser, 2011).

Most of our *Aspergillus flavus* isolates were capable of producing sterigmatocystin, maltoryzine and aflatrem. Sterigmatocystin (ST) is a carcinogenic polyketide and a precursor to the even more potent aflatoxins (Versilovskis and De Saeger, 2010) produced by the common food contaminants of *Aspergillus* species (Kato *et al.,* 2003; Rank *et al.,* 2011). Aflatrem the potent tremorgenic toxin produced by *Aspergillus flavus*, and a member of a structurally diverse group of fungal secondary metabolites known as indole-diterpenes had also documented (Zhang *et al.,* 2004; Duran *et al.,* 2007; Nicholson *et al.,* 2009). Maltoryzine had also been recorded as a toxic metabolite produced by *Aspergillii* (Blumenthal, 2004). On the other hand, *Aspergillus niger* tested isolates were capable of producing oxalic acids in the culture media (Hattori *et al.,* 2007). Unlike secondary metabolites, oxalic acid seems to be somehow beneficial to the producing organisms. The production of oxalic acid in fungi and its possible roles in fungal pathogenicity and ecology had been documented (Blumenthal, 2004).

It was also proved that both *F. proliferatum* and *F. subglutinans* were ipomeanine, neosolaniol (Geraldo *et al.,* 2006), nivalenol and NT-2 toxin producers. Fungi of the *Fusarium* genus commonly contaminate agricultural commodities in the temperate climatic zones of the world and contribute to undervalue entering the food and feed chain. Nivalenol (Nakajima, 2007; Pinto *et al.,* 2008), T-2 toxin (Konigs *et al.,* 2009) belong to a group of mycotoxins produced by different *Fusarium* species which can infect agricultural commodities directly on the field or during storage.

Regarding citrinin and citreoviridin; *P. aurantiogriseum* was more productive than *P. brevicompactum* (Yassin *et al.,* 2010; El-Samawaty *et al.,* 2011). Both of these are toxic secondary metabolites produce by *Penicillium* spp. (Kurtzman and Blackburn, 2005; Bragulat, 2008). Plant pathogenic Penicillia may produce these toxins in culture media, and/or in agricultural commodities (Rundberget *et al.,* 2004). Production of such toxins by *Penicillium* species has been frequently reported (Rundberget and Wilkins, 2002).

In conclusion, consumed walnut in KSA was vulnerable to mold colonization. Inappropriate processing and storage condition of walnut could increase the risk of mycotoxin contamination. Rigorous quarantine, more accurate diagnosing methods and healthy storage conditions should be adopted with importing nuts to avoid contamination with toxigenic fungi, and prevent hazards to human and animal health.

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