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Mycobiota and Mycotoxins Associated With Fresh and Dried Grape Fruits in Egypt

Khashaba, Reham A.[1]; M.K. Farag [1]; M.A. Al-Geddawy[1]; A.A. Zohri[2] and W.S. Ragab [1]

[1]Food Science and Technology Department, Faculty of Agriculture, Assiut University, Egypt.
[2]Botany and Microbiology Department, Faculty of Science, Assiut University, Egypt.

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Abstract

The aimed to evaluate the extent of contamination of fresh grape, dried grape fruits (raisin) with molds and their mycotoxins. Three hundred and forty five fungal isolates were obtained from 40 samples of fresh fruits (243 isolates) and 30 samples of dried fruits (102 isolates). Twelve fungal genera namely, *Alternaria, Aspergillus, Cladosporium, Curvularia, Emericella, Fusarium, Geotricum, Gibberella, Mocur, Pinicillum, Rhizopus* and *Trichoderma* were identified from fresh grape samples. All the previous fungal genera except *Geotricum* and *Gibberella* were also isolated from dried grape samples. *Aspergillus* was the most predominant genus followed by *Penicillum, Alternaria* and *Fusarium.* Most of the isolated *Aspergillus* species were found to be positive producers of one or more toxin of aflatoxins and ochratoxin A. Thin layer chromatographic analysis of 14 tested samples of fresh and dried grape showed that one out of four samples of fresh grape was found to be contaminated with aflatoxins B_1 and G_1. Meanwhile, 7 out of 10 samples of dried grape were naturally contaminated with ochratoxin A and aflatoxins B_1, B_2 and G_1.

Keywords*: Fresh grape fruits, Raisin, Aflatoxin, Ochratoxin, Fungi.*

Introduction

Fresh grape are prone to fungal contamination in the field, during harvesting, transporting, marketing and during storage under domestic conditions. Post-harvest fruit spoilage results in significant economic losses. Additionally, if the spoiling fungi are toxigenic or pathogenic, they could pose a health risk for the consumer. Grape fruit contain high levels of sugars and other nutrients, and they possess an ideal water activity for microbial growth; their low pH makes them particularly susceptible to fungal spoilage. Some fungi are plant pathogens and can start the spoilage from the field; they proliferate and cause substantial spoilage only after harvest. On the other hand, many fungi can grow at low temperatures and cause substantial damage especially if the grape fruits are stored for extended periods of time. Toxigenic fungi have been isolated from decayed grape fruits. Some of these molds could produce mycotoxins while grown on fruits even during refrigeration. Beside, some molds could cause infections or allergies in susceptible individuals (Tournas and Katsoudas, 2005).

Dried grape fruits (raisin) have become an increasingly attractive snack food because of containing essential amino acid, vitamin, minerals and rich dietary fiber which are beneficial for keeping health **(**Asghar *et al.,* 2017). According to data from the European commission RASFF, 2015

(Rapid Alert System for Food and Feed), dried grape fruits and their products have been one of the top safety-patrolled foods as a result of mycotoxin contamination (Miao and Zhou, 2014, Dai *et al*., 2015 and RASFF, 2015). As for dried grape, mycotoxin contamination has also been perpetually reported (Alghalibi and Shater, 2004, Juan, *et al*., 2008 and Asghar *et al.,* 2017).

Mycotoxins are fungal secondary metabolites mainly produced by *Fusarium* spp*., Aspergillus* spp*., Penicillium* spp*., Claviceps* spp*.,* and *Alternaria* spp*.,* and more than 400 types of mycotoxins have been described (Tolosa *et al.,* 2013). Due to the variable molecular structure of these mycotoxins, these metabolites exhibit a wide range of effects on human health, such as immune suppressive disorders, hormonal teratogenic and mutagenic effects as well as carcinogenic effects on liver and kidney tissues (Pitt, 2013; Rychlik, 2017 and Asam *et al.,* 2017).

Aflatoxins (AF) are the most important mycotoxins in grape products and the AFB_1 being the highest carcinogenic natural compound known. The mycotoxins exhibit hepatocarcinogenicity and hepatotoxicity. Acute aflatoxicosis occurs when moderate to high levels are consumed. The disease symptoms may include haemorrhage, acute liver damage, edema, alternation of digestion, absorption and/or metabolism of nutrients and may result in death. The International Agency for Research on Cancer (IARC) has classified AF as groupI carcinogens (Varga *et al.,* 2015). These mycotoxins are produced predominantly by *A. flavus* and *A. parasiticus* (Paterson and Lima, 2010 a and b and Paterson *et al.,* 2014).

Ochratoxin A (OTA) is a widespread mycotoxin in grape products that is produced by several *Aspergillus* and *Penicillium* species (Gil-Serna *et al.,* 2011). The OTA is a cyclic, chlorinated pentaketidedihydroisocoumarin derivate linked to L-β-phenylalanine by an amide bond, which has been detected from many agricultural products. Several nephropathies affecting animals and humans have been attributed to OTA. (Varga *et al*., 2015). It is widely known that OTA presence in raisins is closely related to fungal contamination in the vineyards. Many studies have been performed to unravel the most important ochratoxigenic species occurring in grape. Several reports evidenced the contribution of *Aspergillus* section Nigri species to OTA contamination of grape and their occurrence on the surface of healthy berries (Cabañes *et al.,* 2002). However, their prevalence differs depending on the geographical region where the grapes are cultivated (Varga *et al.,* 2006). In general, *A. carbonarius* is considered as the most important ochratoxigenic species in grape due to its common occurrence and its high production ability (almost all of the strains are able to produce the toxin at different levels).

Thus, The study aimed to evaluate the contamination extent of fresh and dried grape fruits (raisin) with molds and their mycotoxins. The ability of the isolated fungi to produce mycotoxins was also studied.

Materials and Methods

Collection of samples

Forty samples of fresh grape and thirty samples of dried grape (raisin) were collected from different markets of several locationsat Assiut governorate, Egypt. Each sample was put in a sterilized polyethylene bag, sealed and put in another bag which was also sealed to minimize the loss of water content and give sufficient aeration. All samples were transferred immediately to the laboratory and kept in a refrigerator (3-5ºC) till microbiological and mycotoxin analysis.

Isolation and identification of fungi

Fungi were isolated using the dilution plate method as described by Tournas *et al.,* (2006). Using 20% sucrose-Czapek's agar medium containingchloramphenicol (20 µg/ml) as bacteriostatic agent. The plates were incubated at 28ºC for 10 days and the developing fungi were counted, isolated and identified based on their macro- and microscopic characteristics using the following references: (Reper and Fennell, 1965, Ainsworth, 1971, Ellis, 1976, Booth, 1977, Pitt, 1979, Klich and Pitt, 1992, Moubasher, 1993, and Pitt and Hocking, 2009).

Mycotoxins analysis

Testing the toxicity of the isolated molds

A total of 10 different isolates of filamentous fungi from different samples during the first part of this study belonging to *Aspergillus* spp (8 isolates) and *Penicillum* spp (2 isolates) were examined for their ability on mycotoxins production. Each individual fungal isolate was cultivated on liquid medium (5g difco mycological peptone, 20g yeast extract, 40g sucrose in 1liter deionized water " pH 5.5"). Erlenmeyer flasks of 250 ml capacity were used. Each flask contained 50 ml medium. The flasks were sterilized at 1.5 atmosphere for 20 min and inoculated after cooling with two ml of the inoculum suspension of 10 days old culture of the pure organism. The culture were incubated at $28^{\pm\circ}$C as static cultivation for 10 days (Dorner *et al.,* 1984). The extraction and determination of the released mycotoxinswas carried out according to the method of AOAC (1980).

Extraction of mycotoxins

Fifty grams of each fruit sample were transferred into blender jar and 100 ml chloroform were added. The contents were homogenized for 5 minutes at low speed and 3 minutes at high speed. The extract was filtered through filter paper. The extraction procedure was repeated twice with the same volume of chloroform. The combined chloroform extracts were washed with equal volume of distilled water, dried over sodium sulphateanhydrous and then evaporated to near dryness on steam bath. The residue was transferred quantitatively to a small vial with 1ml chloroform.

Mycotoxins detection

Mycotoxins extracted from grape fruits tissue and fungal isolates cultures were determined by thin layer chromatographic technique on pre-coated silica gel plate 60 F254 (Merck) as described by El-Kady and Moubasher (1982). Mycotoxins were identified by comparison with appropriate reference standards of mycotoxins using solvent system of chloroform: acetone (90:10, v/v) for

Aflatoxins and toluene: ethylacetate: 90% formic acid (6:30:10, v/v/v) for ochratoxins.

Chemical confirmatory tests for mycotoxins

Additional confirmatory tests were needed to differentiate unambiguously between the mycotoxins and other fluoresces compounds which may be present in an extract. Chemical confirmatory tests for positive samples was carried out using various treatments on TLC plates in accordance with the method of Golinski and Grabarkiewica-szczesna(1984).

Results and Discussion

Occurrence and percentage of fungal frequency isolated from fresh and dried grape fruits.

A survey study was conducted on 40 samples of fresh grape and 30 samples of dried grape (raisin) which collected from shops and markets of different locations at Assiut governorate, Egypt.

Isolation of fungi contaminated fresh and dried grape resulted in collecting of 345 fungal isolates (243 isolates from fresh and 102 fungal isolates from dried grape). Data in Table (1) show that, twelve fungal genera namely; *Alternaria, Aspergillus, Cladosporium, Curvularia, Emericella, Fusarium, Geotricum, Gibberella, Mocur, Pinicillum, Rhizopus* and *Trichoderma* were identified from fresh grape samples. All the previous fungal genera except *Geotricum* and *Gibberella* were also isolated from dried grape samples. Data in the same Table showed that, *Aspergillus* was the most frequently occurring genus which record 40 isolates with frequency occurrence 100% in fresh fruits and 26 isolates with frequency occurrence 86.7% in dried fruits. *Penicillum* was the second predominant genus which recorded frequency of 92.5% in fresh fruits and 56.7% in dried fruits. *Alternaria* and *Fusarium* were moderate fungal frequency occurred in fresh and dried samples of grape. *Cladosporium, Emericella, Mocur, Trichoderm* and *Ulocladium* were less fungal frequency occurred in all grape samples.

Similar results were obtained by several studies (Magnoli *et al.,* 2004, Tournas and Katsoudas, 2005, Alisa *et al.,* 2007, Fredj *et al.,* 2007 and Alghalibi *et al.,* 2008). The data of Alisa *et al.*(2007) obtained from the grape samples revealed a high diversity (812 isolates) of fungal genera including *Aspergillus* spp*., B. cinerea, Alternaria* spp*., Penicillium* spp*., Cladosporium* spp*., Sphaeropsis* spp*., Trichoderma* spp*., Rhizopus* spp*., Epicoccum* spp. and *Fusarium* spp. Al-Ghalibi *et al.* (2008) reported that prevalence of *Aspergillus* and *Penicillum* on dry raisins, *Fusarium, Cladosporium, Emericella* and *Mocur* were less frequently isolated.

During maturation, the spoilage agents, *Aspergillus, Penicilliumand Rhizopus,* increase their incidence. When the temperature is higher than 37°C, species in *Aspergillus* section Nigri, usually called black Aspergilli become predominant (Valero *et al.,* 2005). At harvest time, the conditions are optimal for fungal invasion, especially if physical damage had occurred on grape. After harvest, grape is subjected to different processes, depending on the intended use.

Grapes can be eaten fresh, dried by sunlight for raisin production.

Each of these treatments is characterized by contamination with different fungal species. Both grape form for table consumption, are mainly contaminated in the field by *Aspergillus, Botrytis,* and *Penicillium* species, which often can be isolated from symptomless berries (Battilani and Pietri, 2002), and successively by black Aspergilli and *Botrytis cinerea* in post-harvest cold storage (Guzev *et al.,* 2008).

On dried fruits as well, *Aspergillus* and *Penicillium* species are often present (Valero *et al.,* 2005); in particular the predominance of *Aspergillus* species on dried fruits is reported worldwide, including Italy, Spain (Abarca *et al.,* 2003), Brazil (Iamanaka *et al.,* 2005), Argentina (Da Rocha Rosa *et al.,* 2002), and California (Palumbo *et al.,* 2011). Determination of the mycobiota occurring on grape fruits at the different stages of growing and processing is important to establish an adequate program of treatments for the prevention of fungal contamination in the vineyard and storage. Some of the fungal species occurring on grape and their products can produce mycotoxins, so species identification is critical to predict the potential mycotoxin contamination of grape and raisin. Certainly the *Aspergillus* species are present worldwide, in all the grape products and under all environmental conditions.

Table 1. Number of case of isolations (NCI), frequency percent (F %), occurrence remarks (OR*) and species number (SN) of each of the isolated fungal genera

Genera	Grape fruit 40 samples				Raisin 30 samples			
	SN	NCI	F%	OR*	SN	NCI	F%	OR*
Alternaria	1	20	50	H	1	7	23	M
Aspergillus	20	40	100	H	20	26	86.7	H
Cladosporium	1	16	40	M	1	8	26.7	M
Curvularia	1	14	35	M	1	6	20	L
Emericella	1	18	45	M	1	14	46.7	M
Fusarium	1	22	55	H	1	6	20	L
Geotrichum	1	9	22.5	L	-	0.0	0.0	0.0
Gibberella	1	15	37.5	M	-	0.0	0.0	0.0
Mucor	1	14	35	M	1	4	13	L
Pinicillum	10	37	92.5	H	5	17	56.7	H
Rhizopus	1	24	60	H	1	10	33	M
Trichoderma	1	14	35	M	1	4	13	L
Total	40	**243**	**-**	-	33	**102**	-	-

***OR= Occurrence remark:**
-For grape fruit samples: H= high, between 20-40 cases (out of 40); M= moderate, between 10-19 cases; L= low, between 5-9 cases; R= rare, 4-1 cases.
-For raisin samples: H= high, between 15-30 cases (out of 30); M= moderate, between 7-14 cases; L= low, between 3-6 cases; R= rare, 1 or 2 cases.

The isolated fungi ability to produce mycotoxins.

A total of 10 different isolates of filamentous fungi isolated from different samples during the first part of this study belonging to *Aspergillus* (8 isolates) and *Penicillum* (2 isolates) were examined to assess their ability on mycotoxins production. Data recorded in Table (2) showed that, all the examined isolates of *Aspergillus* except *A. niger* proved to be

positive producers of mycotoxins. Data also indicated that, two *Aspergillusflavus* isolated from dried and fresh grape samples produced aflatoxin B_1 and aflatoxins B_1 plus B_2, respectively. While *A. parasiticus* isolated from fresh grape samples produced aflatoxinsB_1, B_2, G_1 andG_2. Also, anisolate of *A. awamori* isolated from raisin samples showed positive reaction for aflatoxin B_1 production. Toxigenic strains of *A. flavus, A. parasiticus* and *A. awamorii* forming aflatoxins were found in fresh grape and products from grape (raisin) by many investigators (Antonio *et al.,* 2003, Heperkan, 2006, Alghalibi *et al.,* 2008, Barkai and Paster, 2008 and Karbaneoglu and Heperkan, 2009).

Data in Table (2) revealed that, two *A.carbonarius* and one isolate of *A.ochraceus* had the ability to produce ochratoxin A. On the other hand, no mycotoxin was formed by the examined two *Penicillum* species (*P.citrinum* and *P.glabrum*) isolated from samples of fresh and dried grape. Toxigenic strains of *A. carbonarius* and ochratoxin A were often found associated with black rot of grape (Antonio *et al.,* 2003). A survey carried out in Lebanon also reported that *A. carbonarius* was the most important OTA producer in dried grape reaching 100% of producing isolates (El Khoury *et al.,* 2008). Similar results were obtained by Fredj *et al.* (2009) who reported, *A. carbonarius* and *A.ochraceus* represented an important risk for OTA contamination with more than 80% of producing strains isolated from raisin. Garmendia and Vero (2016) reported that, all *A. carbonarius* isolates from grape fruits and their products had the ability to produce OTA.

However, mycotoxigenic fungi are spread on grape fruits during fruit growth, ripening and at especially during the ripening and over ripening phase. The formation of mycotoxinsin dried grape is mainly due to contamination by *Aspergillus* species and particularly *A. flavus* and *A. parasiticus*. Thus, toxigenic fungi may grow and form mycotoxinson the outer surface or inside the cavity even if no damage occurs on the skin. The critical periods for aflatoxin formation in dried grape fruits starts with grape ripening on the tree, continues during the over-ripe period when they lose water, shrivel and fall down onto the ground and until they are fully dried on drying trays. Some insect pests that are active at fruit ripening stage may act as vectors in transferring the mycotoxigenic fungi to the fruit cavity (CAC/RCP65, 2008). However, Current concerns mostly apply to mycotoxins (Aflatoxins and Ochratoxin A) contamination in grapes and their products (Li *et al.,* 2017).

Table 2. Mycotoxins formation by some fungi isolated from fresh and dried grape fruit samples.

Type of Mycotoxins	Source of isolation		Fungal species	Fungal Genera
	Dry grape	Fresh grape		
Aflatoxin B_1	+	-	*A.flavus*	***Aspergillus***
Aflatoxins B_1, B_2,G_1& G_2	-	+	*A.parasiticus*	
Aflatoxins B_1& B_2	-	+	*A.flavus*	
Aflatoxin B_1	+	-	*A.awamorii*	
Ochratoxin A	-	+	*A.carbonarius*	
Ochratoxin A	+	-	*A.carbonarius*	
Ochratoxin A	+	-	*A.ochraceus*	
-	+	-	*A.niger*	
-	-	+	*p.citrinum*	***Penicillum***
-	+	-	*p.glabrum*	

Natural occurrence of mycotoxins in fresh grape fruits and raisin samples.

As shown in Table (3), thin layer chromatographic analysis of 14 tested samples (10 raisins samples) and 4 fresh grape fruits samples, showed that 8/14 positive samples were found to be contaminated with mycotoxins; 7 of raisin and one sample of fresh grape fruits were naturally contaminated with mycotoxins.

Results in Table (3) indicated that aflatoxins were detected in four samples (40%) of raisin and one sample (25%) of fresh grape fruits. Also, ochratoxin A was found in three out of the ten samples of raisin (30%). Similar results were recorded by Luttfullah and Hussain (2011) who detected aflatoxins in raisin samples and found contamination in 25% of the tested samples. Another study also revealed that 48% of 110 raisin samples analyzed for ochratoxin A were contaminated (Heperkan, 2008). According to Varga and Kozakiewicz (2006), ochratoxin A contamination of dried grape is usually much higher than that of grape fruits. Formation of OTA in raisin is mostly considered to be a post-harvest problem and around 50% of the *A. carbonarius* contamination occurred during the first to the second week of sun-drying stage (Magan and Aldred, 2005).

The obtained results are similar with Özay and Alperden (2005) examined 103 samples collected from various orchards and at various stages of grape processing, including samples of dried grape. Overall, aflatoxins B_1, B_2, G_1, and G_2 were present in 29% of the samples. Ochratoxin A was detected in only 3% of the samples. In samples collected during the sun drying of grape, only aflatoxin B_1 and G_1 were detected.

The high concentration of sugar and low water activity during the drying process creates a very suitable and selective environment for these fungi to grow in dried fruits due to the fact that the molds belonging to the *Aspergillus* and *Penicillum* are xerotolerant (Abarca *et al.,* 2003 and Samson *et al.,* 2006).

Table 3. Natural contamination of fresh and dried grape fruits with mycotoxins.

Kind of mycotoxins	No. + vesamples	No. tested samples	Samples grape
Aflatoxin B_1 and G_2	1	4	**Fresh fruits**
Aflatoxin B_1, B_2 and G_1	4	10	**Dried fruits (raisin)**
Ochratoxin A	3		
-	8	14	**Total samples**

Conclusion

The present work indicated that the examined fresh and dried grape fruits were contaminated with several fungi especially members of *Aspergillus* and *Penicillium*. Many of these fungi are capable of producing mycotoxins such as aflatoxins and ochratoxin A. These findings indicate that there may be a risk of human exposure to mycotoxins through the consumption of grape fruits. So, strict hygiene microbiological must be applied during different stages of harvest, transport, storage, drying and handling to avoid the harmful effects on human health.

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الفطريات والسموم الفطرية المرتبطة بثمار العنب الطازجة والمجففة في مصر

ريهام أحمد خشبة[١]، محمد قدرى أحمد فرج[١]، محمد الأنور حسن الجداوى[١]، عبد الناصر أحمد زهرى[٢]، وفيق سند موسى رجب[١]

[١]قسم علوم وتكنولوجيا الأغذية ، كلية الزراعة ، جامعة أسيوط، مصر.
[٢]قسم النبات والميكروبيولوجيا ، كلية العلوم ، جامعة أسيوط، مصر.

الملخص

يهدف هذا البحث إلي تقييم مدى تلوث ثمار العنب الطازجة والمجففة (الزبيب) بالفطريات وسمومها الفطرية وكذلك دراسة مدى قدرة الفطريات المعزولة علي إنتاج السموم الفطرية وقد تم الحصول على ثلاثمائة وخمس وأربعون عزلة فطرية من أربعون عينة من ثمار العنب الطازجة (٢٤٣ عزلة) وثلاثون عينة من ثمار العنب المجفف(١٠٢ عزلة). وقد تم عزل وتعريف إثني عشر نوعا من الفطريات وهما ألترناريا ، أسبرجلس، كلادوسبوريم، كيرفيولاريا، اميريسيلا، فيوزاريوم، جيبريلا، جبوتريكم، ميوكر، بنيسيليوم ، ريزوبس و تريكوديرما من عينات ثمار العنب الطازج. وأيضا تم عزل وتعريف كل هذه الفطريات من عينات العنب المجفف بإستثناء جنسي الجيوتريكم والجيبريلا. وقد تبين أن الأسبرجيلس هو أكثر الأجناس إنتشارًا يليه، بنيسيليوم ،ألترناريا وفيوزاريوم. كما أظهرت نتائج أن الأسبرجيلس كانت قادرة علي إنتاج واحد أو أكثر من سموم الأفلاتوكسينوالأوكراتوكسين A. كما أظهرت نتائج التحليل الكروماتوجرافى لعدد أربعة عشر عينة من ثمار العنب الطازجة والمجففة تواجد سموم الأفلاتوكسين في عينة واحدة من أصل أربع عينات من ثمار العنب الطازجة بينما كان التلوث عالي جدا في الثمار المجففة(الزبيب) حيث إحتوت سبع عينات من أصل عشرة علي سموم الأفلاتوكسينو الأوكراتوكسين.