TUJNAS

Thamar University Journal of Natural & Applied Sciences ISSN: 2073-0764
2009 A(**1**) 11-19

Mycoflora and Total Aflatoxin Isolated from Dry Dates in Sana'a, Yemen

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ABSTRACT

This study is designed to record the mycoflora and total aflatoxin isolated from dry dates in Yemen Republic. Thirty four date samples collected from different shops and markets in Sana'a city were analyzed mycologically for the presence of fungi cultivated on three types of media. Eight species belonging to 7 genera were isolated from analyzed date samples on the three cultural media. *Aspergillus* genera was the most dominant fungi and grew well on the three type of media of which *A. niger* is the most common species, *A. flavus* was isolated in rare, low and rare frequency on 1% and 20% sucrose Czapek's and Sabouraud dextrose agar media. *Rhizopus stolonifer* was isolated in rare, low and low frequency on 1, and 20% sucrose Czapek's and Sabouraud dextrose agar media. The date samples were analyzed for the presence of total aflatoxin using ELISA technique which revealed that 4 out of 5 date samples were contaminated with aflatoxin ranged from 3059.89-7585.96 ppt (ng Kg^{-1}).
Keyword: Dry dates, Yemen, fungi, mycoflora, aflatoxin.

INTRODUCTION

Date fruit (*Phoenix dactylifera*) is an important food commodity consumed in large amounts particularly in Islamic countries (Shenasi *et al.,* 2002). Mycotoxigenic fungi, particularly aflatoxigenic Aspergilli, have been associated with dates and date products (Emam *et al*., 1994; Aidoo *et al.,* 1996; Ahmed *et al.,* 1997; and Ragab *et al.,* 2001). Aflatoxins are highly toxic, mutagenic and carcinogenic secondary metabolites predominantly produced by *Aspergillus flavus* and *A. parasiticus*. Both fungal species infect several agricultural products like cereals, cereals hay, straw, corn, oily seeds, tree nuts, drained fruits and spices. Thus food and feed which is contaminated by aflatoxin producing fungi is a serious problem, not only due to the economic losses resulting from significant yield reduction and low quality of food, but mainly due to the serious worldwide health hazard to both human and livestock (Smith, 1997 and Chu, 2002).

MATERIALS AND METHODS

1. Collection of samples:

Thirty four samples (250 g of each) of dates were collected from different markets and shops at Sana'a city, Yemen Republic during 2005. Kind, number, and source of product of the collected samples are shown in Table (1). Each sample was put in a sterile polyethylene bag, sealed, and put in another polyethylene bag, and transferred to the laboratory (Biology Department, Faculty of Science, Sana'a University) for mycoflora and aflatoxin analysis.

2. Isolation and identification of fungi:

Fungi were isolated by using the dilution plate method as described by Johnson and Curel (1972). Twenty-five g. of each sample were suspended into 250 ml. of sterile physiological solutions 0.85% NaCl in a sterile conical flask of 500 ml. volume. The flasks were shaken, using a mechanical shaker, for 20-30 minutes. Dilutions from 10^{-1} to 10^{-3} were made under aseptic conditions. One ml of appropriate dilution was transferred into Petri dish plat, from each sample, nine Petri dishes were used (three for every medium). Twenty ml. of melted agar media were added after being cooled to 45-50° C. The Petri dishes were incubated for 7-14 days at 28° C.

Table (1): List of dates kind, source and number of samples collected.

Kind of dates	Source of dates	No. of samples tested
White dates	Yemen	10
	Saudi Arabia	16
	Iraq	2
Black dates	Yemen	1
	Saudi Arabia	4
Brown dates	Yemen	1
Total	34	

The three culture media used for growing fungi were 1% sucrose Czapek's agar, 20% sucrose Czapek's agar, and Sabouraud dextrose agar. 1% sucrose Czapek's agar medium (Sucrose 10.00 g., sodium nitrate 3.00 g., potassium dihydrogen phosphate 1.00 g., magnesium sulphate 0.50 g, potassium chloride 0.50 g, ferrous sulphate 0.01g and agar 20.00 g $l^{-1)}$, 20% sucrose Czapek's agar medium(Sucrose 200.00 g, sodium nitrate 3.00 g., potassium dihydrogen phosphate 1.00 g, magnesium sulphate 0.50 g., potassium chloride 0.50 g., ferrous sulphate 0.01g. and agar 20.00 g $l^{-1)}$ and Sabouraud dextrose agar medium (Dextrose 40 g., Peptone 10 g. and agar 20 g l^{-1}). The pH of all media was adjusted to 5.5. These media were sterilized by autoclaving at 121° C and 1.5 bar for 30 minutes. Cloramphenichol (500 mg l^{-1}) was added to the medium after sterilizing as bacteriostatic agent. The growing fungi were counted, identified, and isolated.

The isolated fungi were identified up to genus and species level based on macro- and microscopic characteristics. The identification of fungal genera and species was made depending on the following references:

Booth (1971), for the genus *Fusarium*, Ellis (1971), for dematiaceous hyphomycetes, Raper and Fennell (1977), for the genus *Aspergillus,* Pitt (1979), for the genus *Penicillium,* Moubasher (1993) and Samson *et al.* (1995), for other isolates.

Potato dextrose agar medium contain: Potato 200 g., dextrose 20 g. and agar 20 g l^{-1} was used for purification of fungi. The purified fungi were transferred to slants of the same medium for the good sporulation, and kept in refrigerator at 4° C.

3. Determination of total aflatoxins in dried fruit samples:

Five date samples were analyzed to determine their content of Total aflatoxins by enzyme-linked immunosorbent assay (ELISA) technique using R-biopharm-Germany kits (RIDASCREEN FAST). The samples were analyzed in Laboratory of Molecular Neurology and Functional Neuroproteomics, Swiss Federal Institute of Technology Lausanne (EPFL), Switzerland.

RESULTS

1. Fungi isolated from dates on 1 % sucrose Czapek's agar:

Data in Table (2) shows that 8 fungi species belonging to 6 genera were isolated from date samples on 1 % sucrose Czapek's agar medium at 28º C. The total count of fungi was 40745 g^{-1} in all samples. *Aspergillus* was the most frequently isolated genus. It was occurred in 97% of the samples comprising 99.74 % of total fungi in dried dates. *A. niger* was the prevalent species. The remaining species were isolated in rare frequency. *Rhizopus* sp*., Penicillium* sp*., Cochliobolus sativus, Phoma* sp. sterile mycelium and *Scopulariopsis candida* were found in rare frequency.

2. Fungi isolated from dates on 20 % sucrose Czapek's agar:

Data in Table (2) shows that 2 genera and 6 species were isolated from date samples on 20 % sucrose Czapek's agar medium. The total count of fungi was 343210 g^{-1} in all samples. *Aspergillus* was the highest frequent genera representing 99.645 % (341990 g^{-1}) of total count. It occurred in 97% of the samples. *A. niger* was the most prevalent species, which isolated in high frequency representing 61.361 % of total isolates. *A. flavus* species was found in low frequency. Other species of *Aspergillus*, which were recovered from date samples, were *A. terreus, A. fumigatus* and *A. parasiticus. Rhizopus stolonifer* was isolated in low frequency.

3. Fungi isolated from date samples on Sabouraud dextrose agar:

Data in Table (2) shows that 6 fungal species belonging to 4 genera were isolated from date samples on Sabouraud dextrose agar. The total count of fungi was 326720 per g in all samples. *Aspergillus* was the most common genus isolated from date samples representing 99.25 % of total count g^{-1} of date in all samples. It occurred in 97 % of the date samples and the total count of *Aspergillus* was 324290. *A. niger* was the most common species recovered from date samples. It was found in high frequency representing 63.6538 % of total count. *A. flavus*, *A. terreus* and *A. parasiticus* were occurred in rare frequency.

Rhizopus stolonifer was found in low frequency. It's occurred in 17.64 % of date samples. *Chrysosporium sp.* and *Mucor fuscus* were recovered in rare frequency.

4. Contamination of date samples by Aflatoxin:

Data in Table (3) shows that 4 out of 5 date samples were contaminated by aflatoxin with total aflatoxin ranged from 3059.89-7585.96 ppt (ng Kg^{-1}).

Table (2): Total counts (TC calculated X 10 g^{-1} of date sample), number of cases of isolation (NCI) and occurrence remarks (OR) of fungal genera and species recovered from 34 date samples on 1 and 20 % sucrose Czapek's and Sabouraud dextrose agar medium at 28ºC.

Fungi	1% sucrose Czapek's agar			20% sucrose Czapek's agar			Sabouraud dextrose agar		
	TC	NCI	OR	TC	NCI	OR	TC	NCI	OR
Aspergillus	40639	33	H	34199	33	H	32429	32	H
A. flavus	11	2	R	133	5	L	30	2	R
A. fumigatus	1	1	R	1	1	R	-	-	-
A. niger	25726	33	H	21060	32	H	20797	32	H
A. parasiticus	1	1	R	1	1	R	1	1	R
A. terreus	14900	1	R	13004	2	R	11601	2	R
Chrysospoium sp	-	-	-	-	-	-	1	1	R
Cochliobolus. sativus	1	1	R	-	-	-	-	-	-
Mucor fuscus	-	-	-	-	-	-	1	1	R
Penicillium sp.	3	1	R	-	-	-	-	-	-
Phoma sp.	2	1	R	-	-	-	-	-	-
Rhizopus	97	2	R	122	5	L	241	6	L
R. stolonifer	11	2	R	122	5	L	241	6	L
Rhizopus sp.	86	1	R	-	-	-	-	-	-
Scopulariopsis candida	1	1	R	-	-	-	-	-	-
Sterile mycelium	2	1	R	-	-	-	-	-	-
Total Count	40745	-	-	68520	-	-	32672	-	-
Number of Genera	6	-	-	2	-	-	4	-	-
Number of Species	8	-	-	6	-	-	6	-	-

Table (3): Total aflatoxin content of date samples.

Sample No.	Sample source	Total Aflatoxins ppt. (ng. Kg^{-1})
1	Saudia Arabia	0
2	Yemen	3059.89
3	Saudia Arabia	5665.11
4	Saudia Arabia	7585.96
5	Yemen	4018.37

DISCUSSION

Data in Table (2) showed that *Aspergillus* was the most predominant genus isolated from dates on 1 and 20% sucrose Czapek's and sabouraud dextrose agar media from date samples representing 99.74, 99.64 and 99.25% of total count of fungi. Abdel-Sater and Saber (1999) found that *Aspergillus* was isolated in high frequency, whereas *Eurotium* and *Penicillium* were isolated in moderate frequency from date samples. Alghalibi and Shater (2004) found that *Aspergillus*, *Eurotium* and *Penicillium* were the most common genera isolated from date samples. The deterioration of dates is associated with the growth of lactic acid bacteria and yeasts (Bolin *et al.,* 1972, Salik *et al.,* 1979 and Nussinovitch *et al.,*1989).Yeasts and moulds were detected in pre-packed dates in Greater Glasgow, United Kingdom. Potential aflatoxin producer's *A. flavus* and *A. parasiticus* were found in only four samples (Aidoo, *et al.,* 1996). Lozada (1995) reported that a fruit contaminated by different moulds occurs during preharvesting, harvesting and grape processing. During these periods, temperature and humidity which they are important factors in mycelial growth and conidia germination. The composition of the fruit influences the likely type of spoilage. Because most fruits are somewhat acid, dry at the surface, and deficient in B vitamins and molds are the most common causes of spoilage. The composition, too, must determine the particular kinds of molds most likely to grow; thus, some kinds of fruits support a large variety of spoilage organisms and other kinds comparatively few (Frazier and Westhoff, 2000). Wareing *et al.* (2001) reported that mould growth is worst when drying times are extended during the rainy season. This may be a result of increased relative humidity during the rainy season, or that products take longer to dry if rewetted. In this study, 4 out of 5 date samples were contaminated with total aflatoxins at levels of 3059.89-7585.96 ppt. (ng kg^{-1}). Abdel-Sater and Saber (1999) analyzed date samples for the presence of aflatoxins by chromatographic analysis, aflatoxin B_1 was detected in dates (two samples, 300-390 µg. kg^{-1}). Alghalibi and Shater (2004) analyzed the presence of mycotoxins in date samples. They found that 2 of date samples were contaminated with aflatoxin B_1 and the concentrations of aflatoxin ranged between 110-180 µg kg^{-1}. Ioannou-Kakouri *et al.* (2004) found that dates analyzed from 1997-2000 were almost negative to aflatoxins.

The highest concentrations of aflatoxins are produced as a result of post-harvest spoilage of commodities stored under warm moist conditions; significant concentrations may also be produced in the field before harvest. This arises from endophytic association between these moulds and plants, such as maize and groundnut (Hill, *et al.,* 1985). The toxigenic (aflatoxin-producing) strains of *Aspergillus flavus* are distributed worldwide in soil and air which have been reported to contaminate a variety of foods and feeds (Bilgrami, 1984 and Mahmoud, 1993). Firm and ripe fruits show little contamination when

they are dried immediately. Microbiological investigations revealed the presence of aflatoxin-producing strains of *A. flavus* and *A. parasiticus* (Steiner, *et al.,* 1988). Sales *et al.* (2005) found that the presence of *A. flavus* on food-contact surfaces and in the air surrounding the production area for dried Cavendish bananas is indicative of high probability for Philippine dried Cavendish banana chips to be contaminated with aflatoxigenic fungi and aflatoxins.

The Yemeni limit standard for total aflatoxin is 20 µg. g^{-1} for various foodstuffs (Yemeni standard limits, 2001). World Health Organization (WHO) standards for aflatoxin B_1 in various foodstuffs is 5 ng. $g^{-1}$and the total aflatoxin level cannot exceed 10 ng. g^{-1}. Germany, Switzerland, USA, and Hungary limit 4, 5, 20 and 5 ng. g^{-1} for various foodstuffs (Papp, *et al.,* 2002). Current legislation limits 4µg kg^{-1} for total aflatoxins in dried fruits for direct human consumption and 10µg. kg^{-1} to be subjected to sorting or other physical treatment before consumption or use as an ingredient in foodstuff (Commission Regulation, 2003).

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المحتوى الفطري و كمية سموم الافلاتوكسين الكلي للتمر المجفف فى اليمن

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ملخص

تم في هذه الدراسة تشخيص المحتوى الفطري و كمية سموم الافلاتوكسين الكلي للتمر المجفف في الجمهورية اليمنية حيث تم جمع اربعة وثلاثون عينة تمر مجفف من مختلف المحلات و الأسواق في مدينة صنعاء من مختلف الأنواع و المصادر، وتم عزل الفطريات المتواجدة عليها باستخدام ثلاثة أوساط غذائية. تم عزل ثمانية أنواع فطرية تنتمي إلى 7 أجناس من التمر المجفف في الأوساط الغذائية الثلاثة، و كان جنس الاسبرجلس *Aspergillus* أكثر الأجناس شيوعا"بينما كان النوع اسبرجلس نيجر *Aspergillus niger* أكثر الأنواع توجداً من هذا الجنس. تم عزل اسبرجلس فلافس *Aspergillus flavus* بشكل نادر في وسط 1% سكروز شابك آجار وبمعدل منخفض في وسط 20% سكروز شابك آجار بينما عزل بشكل نادر في الوسط الغذائي سابرود دكستروز آجار. تم تحليل عينات التمر المجفف لتواجد سموم الافلاتوكسين بواسطة تحديد الكم الكلي للافلاتوكسين باستخدام طريقة الاليزا والتي تم بواسطتها التأكد من تلوث4 عينات من اصل5 عينات تمر مجففة بسموم الافلاتوكسين بمعدل يتراوح بين 3059.89 و 7585.96 جزء من التريليون (نانو جرام لكل كيلو جرام) من التمر المجفف.