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Susceptibility of Some Varieties of Date Fruits to Support the Production of Aflatoxins: Analysis by High Performance Liquid Chromatography

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Abstract: Six varieties of date palm (*Phoenix dactylifera* L.) namely Nabtet-Aly, Sakei, Succary, Rashody, Rothana and Menepthy of the most widely grown in Al-Qassem region, Saudi Arabia were selected to survey the microflora present in these varieties and studied its susceptibility to support the production of aflatoxins. The total number of spores present in the fruits of these varieties were in descending order: Menepthy > Succary > Rashody > Sakei > Nabtet-Aly = Rothana. The most abundant genus found on dates was *Aspergillus*. Also, similar degrees of microflora growth were found when fruits were inoculated for 5 and 10 days with either *A. flavus* or *A. parasiticus*. The date fruits of all the tested varieties were able to support the production of different forms of aflatoxin such as B₁, B₂, G₁ and G₂. The contents of aflatoxin G₂ produced by either *A. flavus* or *A. parasiticus* were higher than the other forms. Menepthy and Sakei varieties contained less levels of the total aflatoxins when inoculated with either *A. flavus* or *A. parasiticus*, respectively. It is suggested that care must be exercised to avoid the poor conditions during the storage of dates.

Key words: Date fruits, fungi, inoculation, aflatoxin, analysis

INTRODUCTION

Fungi grown on field and stored products can cause heavy damage, resulting in reduction in quality and quantity. In addition, many species are capable of producing mycotoxins, secondary metabolites highly toxic to humans and animals. Mycotoxins often spread in agricultural commodities in the field before harvest or during storage when certain weather conditions prevail. Aflatoxin is a potent hepatocarcinogenic secondary metabolites produce by the fungi, *Aspergillus flavus* Link: Fr. and *A. parasiticus* Speare. Both fungi spread worldwide on a number of agricultural commodities including peanuts, almond, pistachios, cotton seed, wheat, maize and date fruits^[1-3]. Different forms of aflatoxin, including B₁, B₂, G₁ and G₂, which are found in many forms of human food and are known for their toxicity and carcinogenicity^[4].

Date fruits (*Phoenix dactylifera* L.) are vital components of the diet in the Arabian Peninsula especially Saudi Arabia. In addition to the direct

consumption of the fruits, dates are used in many food industries^[5-7]. Molds are considered to be the major causative agents of the spoilage of date fruits at all stages of ripening on trees as well as during storage and processing^[8]. The microflora of date fruits depends on the maturation stage. Microbial counts were high at the first stage of maturation (Kimri) and increased sharply at the second stage (Rutab), then decreased significantly at the final dried stage of maturation (Tamr) which has low water activity and high sugar content^[3]. *A. flavus* and *A. parasiticus* which produce aflatoxins have been found to invade dates at all stages of maturation as well as date products^[9,10]. Another species that also is widely distributed is *A. niger* Tiegh. Although this species does not produce aflatoxin, it is considered as a pathogen to many plants and had antagonistic effect to *A. flavus*^[11].

Al-Qassem region is one of the largest agricultural areas in the Kingdom of Saudi Arabia where much of its arable and fertile land is under date palm orchards. Al-Qassem Community produces the majority of date

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fruits consumed locally and much of those exported outside the country. During the 2002 season, date fruits production was greater than 130,000 tons, of which approximately 1,092 tones were destined for export^[12]. Because fruits of the date palm are vital components of the diet in Saudi Arabia, consumers are increasingly concerned about aflatoxins in date fruits and their carryover to processed products. Unfortunately, no data are available on the forms of aflatoxin on date fruits collected from Al-Qassem markets, therefore the present study was carried out to survey the microflora present in different varieties of date fruits as well as study the susceptibility of these varieties to support the production of aflatoxins when inoculated with either *A. flavus* or *A. parasiticus*

MATERIALS AND METHODS

Chemicals: Aflatoxin B₁, B₂, G₁ and G₂ were purchased from Winab Limited, Company UK. Certified HPLC-grade methanol and acetonitrile and granular AR anhydrous sodium sulfate were purchased from BDH Company, while Florisil (60-100 mesh) was purchased from Riedel-de Haën (Germany). Deionized water of 15 MΩ.cm resistivity was obtained from a water purification system (PURELAB Option-R, ELGA, UK). All solvents and solutions were passed before used through a 0.45 µm sterile disposable nylon filter (AcroCp, Gelman Sciences). All other chemicals used in this study were of the highest grade available.

Sampling: Six varieties namely Nabtet-Aly, Sakei, Succary, Rashody, Rothana and Menephy at the final stages of maturation (Tamr) of the most widely grown date varieties in Al-Qassem region were collected from the local markets of Buraidah city during March–April, 2004, immediately transported to the laboratory and kept at 4°C.

Isolation and identification of fungi: All glass and equipments used during this study were sterilized. Twenty grams from each variety were cut into small pieces, placed into flask containing 100 mL of sterile distilled water and shaken for 10 min. Three replicates were used for each variety with one sterile distilled water blank was run concurrently. One mL of the prepared inoculum from each variety was put into petridish containing Potato Dextrose Agar (PDA), then incubated at 28±1°C for 7 days. At the end of incubation period, the total number of microflora and the number of *Aspergillus* spores were determined.

Inoculation techniques for date varieties: The date fruits were immersed in 1% silver nitrate solution for 1 min followed by immersing in 1% sodium chloride solution,

rinsed with sterile distilled water, cut into small pieces with a sterilized disposable blade and then subsampled into 20 g. *A. flavus* and *A. parasiticus* were grown on PDA at 28±1°C for 10 days, then the dense spore suspension was prepared by adding 10 mL of sterile distilled water to the culture and agitating the surface of the colony with a sterile glass rod. The spore count mL⁻¹ (1×10⁶ spores) was made using a haemocytometer. One mL of each suspension was introduced into 250 mL sterile, Erlenmeyer conical flask containing 20 g of subsamples. Three replicates were used for either *A. flavus* or *A. parasiticus* for each variety with one sterile distilled water blank. Ten days after incubation at 28±1°C, the growth density was determined according to the following scale: (-), (+), (++) and (+++) mean no growth, slight growth, moderately growth and highly growth, respectively.

Sample extraction and cleanup: High Performance Liquid Chromatography (HPLC) analysis was performed according to the official method of AOAC^[13] with some modifications. At the end of inoculation period, 20 g of inoculated fruits were extracted by blending for 2 min with 100 mL of acetonitrile: H₂O (90:10). The extracts were vacuum filtered through a Whatman No. 2 filter paper. After filtration, the pellets were washed with another 100 mL of the extraction solvent and then filtered. The combined extracts were passed through anhydrous sodium sulfate and then evaporated to just dryness at 35-40°C using a rotary evaporator. The residue was dissolved in 5 mL methanol and purified through a glass column chromatography (10 cm x 10 mm i.d.) packed with activated Florisil and topped with 1 cm layer of anhydrous sodium sulfate. Column was washed with 5 mL of acetonitrile: H₂O (90:10) before loading the extract and eluted with 10 mL of acetonitrile: H₂O (90:10). The elute was evaporated to just dryness and then dissolved in 5 mL of acetonitrile.

Derivatization of the extract: The purified extracts (200 µL) were derivatized by adding 700 µL of the derivatizing agent (10 mL of trifluoroacetic acid, with 9 mL of glacial acetic acid and 35 mL of deionized water of 15 MΩ cm, resistivity), heated in water bath at 65°C and then 20 µL was injected into HPLC system containing precolumn.

The analysis was performed using a Perkin Elmer HPLC system model 200 equipped with a degasser, quaternary LC pump model 2000Q/410, 20 µL loop, Spheri-5 RP-18 column (25 cm x 4.6 mm i.d., 5 µm, Perkin Elmer), oven column, a fluorescence detector set at excitation 360 nm and emission 440 nm. The Turbochrom Workstation Software package was used for instrument control, data acquisition and data analysis. The column

temperature was kept at 25°C. The mobile phase was acetonitrile: methanol: H₂O at ratio of 1:1:4 (v/v) and pumped at a flow rate of 0.8 mL min. The obtained corresponding retention times (R_t) for B₁, B₂, G₁ and G₂ were 6.2, 8, 9.1 and 10.5 min, respectively.

Preparation of standard solutions: Ten-calibration solution of each forms of aflatoxin with concentration ranging from 0.0 to 10 ppm were prepared in methanol and 20 µL of each was derivatized as previously described and then injected in HPLC. Areas under the peak (uV.sec) versus concentrations were plotted and fit by simple linear regression to obtain an equation for the standard curve. The amount of aflatoxins in each sample was thus calculated based on the slope of the standard curve.

Statistical analysis: The data were calculated as mean±S.D. and analyzed using analysis of variance technique (ANOVA). Probability of 0.05 or less was considered significant. All statistical analysis was done with Costat Program [14] on a personal computer.

RESULTS AND DISCUSSION

Four genus of fungi namely, *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp. and *Fusarium* sp. were identified in the tested date fruits (Table 1). The higher recorder numbers of spores were recorded in Menepthy and Succary varieties (160 and 103 spores/gram of date fruits, respectively), while Rashody variety contained 74 spores/gram of date fruits. On the contrary, the remaining varieties had lower number of spores (18-31 spores/gram of date fruits). *Aspergillus* species were identified in all the tested varieties. *Aspergillus* was found to be the most abundant genus on dates at all stages of ripening [9,15] as well as associated with date

products [10]. In addition, many genera were identified as fungal contaminants of date palm tissue culture [16,17], where the most frequently genus was *Penicillium* sp., followed by *Currularia* sp., *Cladosporium* sp., *Aspergillus* sp., *Acremonium* sp., *Fusarium* sp. and *Alternaria* sp. Three species of *Aspergillus* namely, *A. niger*, *A. flavus* and *A. parasiticus* were identified in all the varieties under investigation (Table 1). Menepthy and Succary varieties were found to contain more number of spores of either *A. flavus* or *A. parasiticus*, while Rothana, Nabtet-Aly and Sakei varieties contained less number of these spores. Rashody variety was found to contain 46 and 18 spores/gram of date fruit of *A. flavus* and *A. parasiticus*, respectively. The frequency of *A. flavus*, *A. niger* and *A. tamari* in date palm tissue culture were 1.7, 5 and 6.7%, respectively [17,18]. Similar degrees of growth were found when fruits were inoculated for 5 and 10 days with either *A. flavus* or *A. parasiticus* (Table 2). Rashody and Menepthy varieties showed slightly and moderately degrees of growth after inoculation for 5 and 10 days, respectively, while the remaining varieties showed moderately and highly degrees of growth after inoculation for 5 and 10 days, respectively.

Production of aflatoxins: The production of aflatoxins (B₁, B₂, G₁ and G₂) were determined in the selected varieties of date fruits after inoculated with either *A. flavus* or *A. parasiticus* (Table 3). It was found that aflatoxins naturally occurring in control samples of all tested varieties. The total means values of aflatoxins naturally occurring in Succary, Menepthy, Rashody, Rothana, Nabtet-Aly and Sakei varieties were 0.90, 1.10, 1.26, 1.26, 1.31 and 1.60 ppm, respectively. In addition, *A. flavus* isolate was found to support the production of aflatoxins with degree greater than that the isolate of *A. parasiticus* in all the varieties except the variety of Menepthy. The

Table 1: Number and types of microflora and *Aspergillus* spores present in different varieties of date fruits

Varieties	Total number of microflora spores	Types of fungus present	No. of spores after inoculation with	
			<i>A. niger</i>	<i>A. flavus</i> + <i>A. parasiticus</i>
Nabtet-Aly	18±7ab	<i>Aspergillus</i> sp. <i>Penicillium</i> sp.	13±11a	2±4a
Sakei	31±5c	<i>Aspergillus</i> sp. <i>Penicillium</i> sp.	4±5a	20±13ab
Succary	103±30bc	<i>Fusarium</i> sp. <i>Aspergillus</i> sp.	72±34b	20±15ab
Rashody	74±40ab	<i>Fusarium</i> sp. <i>Aspergillus</i> sp.	46±18b	18±24ab
Rothana	18±7a	<i>Alternaria</i> sp. <i>Aspergillus</i> sp.	11±1a	2±4a
Menepthy	160±31d	<i>Penicillium</i> sp. <i>Aspergillus</i> sp.	116±29c	37±15b
		<i>Penicillium</i> sp. <i>Alternaria</i> sp.		
LSD _{0.05}	56		31	21

Data are expressed as mean±S.D. (n=3) Means within the same column and having the same letter(s) are not significantly different from each other

Table 2: Growth density of microflora on different varieties of date fruits inoculated with either *A. flavus* or *A. parasiticus*

Varieties	Degree of growth after inoculation with				
	Control	<i>A. flavus</i>		<i>A. parasiticus</i>	
		5 days	10 days	5 days	10 days
Nabtet-Aly	-	++	+++	++	+++
Sakei	-	++	+++	++	+++
Succary	-	++	+++	++	+++
Rashody	-	+	++	+	++
Rothana	-	++	+++	++	+++
Menephy	-	+	++	+	++

- No growth+, Slight growth, ++ Moderate growth, +++ Highly growth

Table 3: Mean levels (ppm) of aflatoxins produced in different varieties of date fruits inoculated with either *A. flavus* or *A. parasiticus*

Varieties	Control					<i>Aspergillus flavus</i>					<i>Aspergillus parasiticus</i>				
	B ₁	B ₂	G ₁	G ₂	Total	B ₁	B ₂	G ₁	G ₂	Total	B ₁	B ₂	G ₁	G ₂	Total
Nabtet-Aly	0.05± 0.001a	0.05± 0.002a	0.40± 0.06a	0.81± 0.09a	1.31	1.52± 0.42b	2.20± 0.52b	0.99± 0.09ab	2.67± 0.00c	7.38	0.47± 0.009a	0.61± 0.13a	0.74± 0.08a	1.26± 0.11a	3.08
Sakei	0.11± 0.07a	0.13± 0.05a	0.46± 0.90a	0.09± 0.10a	1.60	1.54± 0.18b	2.45± 0.58b	1.09± 0.03b	3.03± 0.38c	8.11	0.43± 0.009a	0.56± 0.00a	0.56± 0.34a	1.27± 0.18a	2.82
Succary	0.07± 0.01a	0.08± 0.01a	0.27± 0.09a	0.48± 0.05a	0.90	1.22± 0.32b	1.76± 0.59ab	0.92± 0.25b	2.32± 0.23b	6.22	0.86± 0.10c	1.14± 0.20c	0.81± 0.06a	1.16± 0.00a	3.97
Rashody	0.04± 0.01a	0.04± 0.005a	0.32± 0.01a	0.86± 0.10a	1.26	1.97± 0.001b	2.86± 0.00b	1.74± 0.38c	2.97± 0.15c	9.54	0.70± 0.09bc	0.92± 0.11bc	0.94± 0.12a	1.25± 0.06a	3.81
Rothana	0.08± 0.02a	0.12± 0.003a	0.25± 0.007a	0.81± 0.07a	1.26	1.92± 0.08b	2.41± 1.01b	1.89± 0.12c	2.83± 0.23c	9.05	0.55± 0.09ab	0.71± 0.11ab	0.85± 0.15a	1.92± 0.05b	4.03
Menephy	0.04± 0.01a	0.05± 0.002a	0.32± 0.05a	0.69± 0.004a	1.10	0.25± 0.11a	0.32± 0.16a	0.56± 0.11a	1.09± 0.21a	2.22	0.47± 0.06a	0.61± 0.08a	0.89± 0.14a	2.06± 0.35b	4.03
LSD _{0.05}	0.08	0.10	0.19	0.44		0.75	1.50	0.50	0.56		0.20	0.30	0.42	0.42	

Data are expressed as mean ± S.D. (n=3), Means within the same column and having the same letter(s) are not significantly different from each other

contents of all forms of aflatoxin when *A. flavus* inoculated with date fruits were found to be high in Rashody, followed by Rothana, Sakei, Nabtet-Aly, Succary and then Menephy varieties, where the corresponding values were 9.54, 9.05, 8.11, 7.38, 6.22 and 2.22 ppm, respectively. On the contrary, when *A. parasiticus* inoculated with fruits, the levels of total aflatoxin were 4.03, 4.03, 3.97, 3.81, 3.08 and 2.82 ppm in Menephy, Rothana, Succary, Rashody, Nabtet-Aly and Sakei, respectively. In general, the contents of aflatoxin G₂ produced by either *A. flavus* or *A. parasiticus* were higher than the other forms of aflatoxin in all the tested varieties of date fruits. Aflatoxin G₁ was produced at almost twice the level of B₁ at Khalal stage, while the amounts of the aflatoxin G₁ and G₂ were produced by six times than the level of aflatoxin B₁^[18].

In conclusion, all the tested varieties support the growth of microflora and aflatoxins production with different levels. These differences could be attributed to that these samples in our study collected from the local market after 6 months of harvest and therefore might be stored under different conditions. In addition, the

widespread occurrence of the *Aspergillus* mould and high carbohydrate content of fruits, means date fruits are vulnerable to contamination with this type of moulds. It is suggested that care must be exercised to avoid the poor conditions during the storage of dates.

REFERENCES

- Rodriguez-del-Bosque, L.A., 1996. Impact of agronomic factors on aflatoxin contamination in preharvest field corn in Northeastern Mexico. Plant Dis., 80: 988-993.
- Soliman, S.A., N.S. Ahmed, K.A. Osman and M.S. Ibraheem, 2001. Monitoring of zeralenon mycotoxin in some cereals, nuts and dried fruits collected from Alexandria markets using HPLC. J. Egypt. Soc. Toxicol., 24: 103-106.
- Shenasi, M., K.E. Aidoo and A.A.G. Candlish, 2002. Microflora of date fruits and production of aflatoxin at various stages of maturation. Intl. J. Food Microbiol., 79: 113-119.

4. Campbell, T.C. and L. Stoloff, 1974. Implication of mycotoxins for human health. *J. Agric. Food Chem.*, 22: 1006-1014.
5. Mustafa, A.I., A.M. Hamad and M.S. Al-Kahtani, 1983. Date varieties for jam production. In : Proceeding of the Second Symposium on Date Palm in Saudi Arabia, King Faisal University, Al-Hassa; Mars Publishing House: Riyadh, Saudi Arabia, pp: 496-501.
6. Sawaya, W.N., H.H. Khatchadourian, J.K. Khalil and A.F. Al-Shalhat, 1989. Processing of date into date chunty. In: Proceeding of the Second Symposium on Date Palm in Saudi Arabia, King Faisal University, Al-Hassa; Mars Publishing House: Riyadh, Saudi Arabia, pp: 105-111.
7. Shinwari, M.A., 1993. Date Palm. In: Encyclopedia of Food Science, Food Technology and Nutrition. Macrae, R., R.K. Robinson and M. Sadler (Eds.), Academic Press, New York, pp: 1300-1305.
8. Ahmed, I.A. and R. K. Robinson, 1998. Selection of suitable methods for analysis of aflatoxins in dates. *J. Agric. Food Chem.*, 46: 580-584.
9. Al-Shaickly, M.A.S., I.A. Rubaie and A.A. Dulaimi, 1986. Types and extent of microbial contamination on fresh Iraq date during maturation. *Date Palm J.*, 4: 205-220.
10. Ahmed, I.A., A.K. Ahmed and R.K. Robinson, 1997. Susceptibility of date fruits (*Phoenix dactylifera*) to aflatoxin production. *J. Sci. Food. Agric.*, 74: 64-68.
11. Breneman, T.B., D.M. Wison and R.W. Beaver, 1993. Effects of diniconazole on *Aspergillus* populations and aflatoxin formation in peanut under irrigated and nonirrigated conditions. *Plant Dis.*, 77: 608-612.
12. Saudi Ministry of Agriculture and Water, 2003. Statistical Predictions about Agriculture in Al-Qassem Region, Volume 5, Department of Statistical and Economic Researches: Agriculture Administration, Al-Qassem, Saudi Arabia.
13. AOAC, 2002. Official Methods of Analysis of AOAC International, (W. Horwitz, Ed.), 17th Ed., Volume 1: Agriculture Chemical; Contaminants; Drugs. Gaithersburg, Maryland, USA.
14. Costat Program, 1986. Version 2, Cohort Software.
15. Ahmed, I.A. and R. K. Robinson, 1997. Incidence of *Aspergillus flavus* and *A. parasiticus* on date fruits and their ability to produce aflatoxin. *Agric. Equip. Intl.*, 49: 136-139.
16. Blake, J., 1988. Mites and trips as bacterial and fungal vectors between plant tissue culture. *Act. Horticul.*, 225: 163-166.
17. Omamor, I.B., E.O. Asemoto and C.R. Eke, 2003. Fungal contaminants of date palm tissue culture materials in Nifor. In: The International Conference on Date Palm, 16-19 September, 2003, King Saud University, College of Agric. Vet. Med., Qaseem Branch, pp: 301-306.
18. Ahmed, A.A., 2003. Susceptibility of date fruits to aflatoxin production. In: The international conference on date palm, 16-19 September, 2003, King Saud University, College of Agric. Vet. Med., Qaseem Branch, pp: 395-407.