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The ability of date extracts to support the production of aflatoxins

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Abstract

Aqueous extracts of the fruit of the date palm (*Phoenix dactylifera* L.) are being used widely in the Middle East, and they are often stored at high ambient temperatures and in vessels of dubious hygenic standards. It was shown that, if a toxigenic mould like *Aspergillus parasiticus* grew on the surface of such an extract, then aflatoxins could be synthesised. The levels of aflatoxin generated in experimental extracts depended upon the sugar content (glucose and fructose) of the solution, but extracts from all the varieties tested were able to support mycelial growth and aflatoxin production. It is suggested that care must be exercised during the commercial processing of dates for syrups and similar products.

1. Introduction

The date palm (*Phoenix dactylifera* L.) is the major fruit tree in most Arabian countries, eg. Iraq, Saudi Arabia, Egypt, Iran, Algeria and the Arabian Peninsula. In the Gulf region, during the pre-oil period, the date palm was the main source of food, together with camel's milk and fish. Even today, in spite of the drastic socio-economic changes in the region, dates continue to play an essential role in the diet of the local inhabitants of the Arabian Peninsula, and Elarosi, Mussa, and Jaheen (1983) concluded that dates constitute the most important basic dietary component for people in the Gulf region.

Besides the direct consumption of the fruit, dates are utilized locally in many ways, including the production of date extract (depis), date syrup (El-Shaarawy, Mesallam, Saber & Al-Johar, 1989), salami-like rolls and fruit cakes, and vinegar (Shinwari, 1992). In addition, fruits at Khalal stage are cooked to prepare what is known as 'Khalal Matbuuk', which is consumed in the Gulf region and Iraq or exported to India and Pakistan (Yousif, Hassan, Saed & Elia, 1983).

More recently, feasibility studies on the utilization of dates in modern, large-scale factories have been published, and among the proposals have been: the incorporation of date syrup into jam and butter (Mustafa, Hamad & Al-Kahtani, 1983), the production of sugar and alcohol (Shinwari, 1992), the manufacture of chutney and glaces (Sawaya, Khatchadourian, Khalil & Al-Shalhat, 1989), ice cream making (Hamad, Mustafa & Al-Kahtani, 1983), the production of high quality caramel colour (Mikki, Bukhaev & Zaki, 1983) and the preparation of "Tamrudeen"—dehydrated sheets of fruit—as a substitute for the well-known apricot sheet "Qamarudeen" (El-Nakhal, Mesallam & El-Shaarawy, 1989).

Most of these products incorporate date extract/syrup and, as a consequence, the production and marketing of date syrup has increased steadily in recent years—production of date syrup is estimated to be about 6500 tonnes/year in the United Arab Emirates. Traditionally, the syrup was marketed in tins or drums made of metal, clay pots or animal skins, and was often stored under very poor conditions and at temperatures between 25 and 35°C.

Previous work has confirmed: (a) the natural presence of toxigenic fungi on date fruits (Ahmed & Robinson, 1997); (b) the ability of date fruits of some varieties to support both the growth of toxigenic aspergilli, following penetration of intact fruits or invasion of the exposed pulp; and (c) aflatoxin production (Ahmed, Ahmed & Robinson, 1997). It is clear also that date varieties show, in addition to differences between the stages of ripening, distinct characteristics with respect to morphology and chemical composition, and that these differences can affect the levels of aflatoxin generated in

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the tissues (Ahmed, Ahmed & Robinson, 1995). Whether or not these differences would remain after extraction of the syrup was not apparent, and consequently, it was decided to establish whether the formation of aflatoxins would occur in date syrups of different varieties and stages of ripening.

2. Materials and methods

2.1. Materials

Fruits of the date palm (*Phoenix dactylifera* L.) from 12 varieties and at the four stages of ripening were collected directly from trees on three commercial farms and one Government experimental orchard; these sites represented three of the four major date-producing regions in UAE. The orchards are located in the Emirates of Dubai, Sharjah and Ras Al-Khaimah.

For each variety/stage of ripening, stalks of dates were removed at random from different bunches and trees using clean scissors, and kept in clean paper bags. The bags were then taken immediately to the laboratory for subsequent analysis. The Arabic terms Kimri, Khalal, Rutab and Tamr were used to denote the green immature stage, the stage with the typical fruit colour, the soft mature stage and the dry raisin-like stage of ripening, respectively.

2.2. Methods

2.2.1. Preparation of the date extracts

All glassware, utensils and equipment used in this experiment were sterilized unless otherwise specified. Two concentrations of date extract, namely 10 and 20% (weight of date/volume of water) were used in this experiment. The selection of these concentrations was based on the studies by Delucca, Mayne, Franz and Ory (1977) of mycelial growth on extracts of different agricultural crops, and the same concentrations were used by Sallal and Ashkenani (1989) in studying the effect of date extracts on the growth of *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi* and *Pseudomonas aeruginosa*.

The initial extract was prepared by soaking 200 g of stone-free, surface-sterilized date fruits in 500 ml sterile distilled water for 30 min. The soaked dates were then aseptically blended in a Waring blender for 1 min at low speed, and for a further 1 min at high speed. The homogenized extract was filtered through a double layer of cheese-cloth. The residue was then washed with hot water and the solution made up to the volume required for a concentration of 20%; subsequent dilutions to 10% were made with sterile distilled water. The date extract solutions were distributed in 50 ml portions into 250 ml Erlenmeyer conical flasks with screw caps.

2.2.2. Preparation of the inoculum

An ampoule of freeze-dried *Aspergillus parasiticus* IMI 9109b, which produces the four major aflatoxins, was obtained from the International Mycological Institute, Surrey. In the laboratory, the ampoule was first marked, with a file, near the middle of the cotton wool plug and then cracked with a hot glass rod. After allowing air to filter through the plug into the ampoule, the plug was removed aseptically with sterile forceps. A few drops of sterile distilled water were added to the culture, the cotton plug was replaced, and ampoule left for 30 min for the fungus to rehydrate. Using a sterile pasteur pipette, the rehydrated spores were transferred to pre-poured plates of potato dextrose agar (PDA) and incubated at 28°C for 5–7 days.

Ten McCartney bottles containing slants of PDA were then inoculated with spores from the PDA plates, and incubated at 28°C for 5–7 days. At the end of the incubation period, the spores were harvested by adding 10 ml of sterile 'wetting solution' (0.05% Tween 80, Sigma Chemicals, USA) to each bottle; a sterile, cotton wool swab was used to dislodge the spores. The spore suspensions from all ten bottles were collected into a sterile 250 ml screw-capped bottle, and the spore count determined by plating duplicate aliquots (1 ml) of serial dilutions onto pre-poured plates of PDA. The stock suspension was held at 4°C for 72 h while the colony count was obtained, and the spore count was then adjusted to a final concentration of about 1×10^6 spores per ml. The spore suspension was then distributed into 25 ml screw-capped bottles in 15–20 ml portions and stored at −18°C for later use.

2.2.3. Inoculation of the extracts

Six flasks of each concentration (10 or 20%) were inoculated with 1 ml of the previously prepared inoculum; the flasks were then shaken so as to mix the spores with the solution. A control flask at each concentration was inoculated with sterile distilled water (1 ml). All flasks were incubated at 28°C for 10 days; the flasks were shaken twice daily for the first 2 days, and then left stationary. At the end of the incubation period, all the flasks were examined for aflatoxins.

2.2.4. Extraction of aflatoxins

At the end of the incubation period, 50 ml of chloroform (Ajax Chemicals, Australia) were added to each flask and shaken vigorously with a wrist-action shaker (Gallenkamp, England) for 30 min. The mixture was then filtered through Whatman No.4 filter paper into a separating funnel. After separation, the lower chloroform layer was drained into a 250 ml stainless steel beaker through an anhydrous sodium sulphate column. Each original flask was rinsed with additional 2×25 ml amounts of chloroform which were then transferred through the same filter paper to the aqueous layer

remaining in the separating funnel. The separator was stoppered and shaken vigorously for 30–60 s, and the lower chloroform layer was added to the previous chloroform in the stainless steel beaker through the same anhydrous sodium sulphate column (Beebe, 1978).

After adding two to three boiling chips, the beaker was placed on a water bath and the chloroform was evaporated to a few millilitres under a gentle stream of nitrogen. The extract was then transferred to a 4-dram vial. The beaker was rinsed with 2 to 3 ml of chloroform which were then added to the vial. The chloroform in each vial was evaporated under a gentle stream of nitrogen, and the vials were stored at −18°C for determination of aflatoxins by high pressure liquid chromatography (HPLC).

2.2.5. Analysis by HPLC

The dry extracts of the samples were re-dissolved in 1 ml acetonitrile-190 (UniChrom, Ajax Chemicals, Australia) and water (Hi PerSolv-BDH Chemicals) in the ratio of 40:60 by volume (injection solvent). After shaking vigorously with a vortex mixer, the dissolved extract was filtered through a 0.45 μm disposable PTFE membrane microfilter unit (3 mm diameter)—Supelco Inc., USA. The filtered samples were collected into 3.7 ml vials with Teflon-lined screw caps (Supelco Inc., USA).

The LC instrument used in this experiment was a Hewlett–Packard (HP) 1090 Liquid Chromatograph equipped with a PV5 Ternary SDS three channel pump. A manual injector, with Rheodyne(tm) model 7010 valve was built into the frame together with the thermocontrolled column compartment. The LC was connected to an HP-1046A programmable fluorescence detector (PFD). The LC and PFD were controlled via an HP 79994A Analytical Work Station consisting of an HP 9000/300 computer, HP9153 integrator and HP 3574A colour monitor. A stainless steel RP column (250×4.6 mm) packed with 5 μm Spherisorb ODS1 (Phase Sep Ltd, UK) was used. The mobile phase was water: acetonitrile: methanol (60/30/10 v/v/v, all HPLC grade) at a flow rate of 0.75 ml min^{-1}. In order to enhance the fluorescence characteristics of aflatoxins B1 and G1, a post-column derivatization (PCD) reaction with saturated solution of iodine, as described by Shepherd and Gilbert (1984), was employed. The instrument used for PCD was the PC X 3100 Reactor (Pickering Laboratory, USA). The reactor, which was connected between the LC and PFD, consisted of an adjustable reagent metering pump, thermostated reaction tee-fitting and coil (ambient to 100 ± 4°C), and backflow prevention devices. Externally, a pressurized reagent reservoir coated with tough plastic film (for safety reasons), was connected to the gas supply (helium) and to the reactor through a tan-coloured inlet tube and reagent supply tube.

The method of Beaver and Wilson (1990) was followed for the preparation of the saturated solution of iodine: 2 g of Analar Grade iodine (P-Park Scientific Limited, UK) were first dissolved in 10 ml HPLC grade methanol. About 1 l of HPLC grade water was added and the mixture stirred overnight with a magnetic stirrer. The supernatant iodine solution was filtered through a 0.45 μm microfilter using a 50 ml disposable plastic syringe.

All the data were analysed using the general linear model procedure of SAS (1987), and differences between treatments were tested using Duncan's multiple range test.

3. Results and discussion

Extracts prepared from fruits at Kimri stage were poor substrates for aflatoxin production and, while the 10% extracts showed traces of toxins in three varieties only, little production occurred in the 20% extracts either. In fact, date extracts at Kimri stage supported much lower aflatoxin production compared with the corresponding whole fruit segments (Ahmed et al., 1997); the dilution of the already low levels of nutrients contained in the fruits at Kimri stage may well explain the results observed. For example, the typical sugar levels (glucose + fructose) in fruits at Kimri are 3–7 g 100 g^{-1} depending on the variety, so that the reduction during extraction might not have left sufficient carbohydrate to support both growth of the fungus and aflatoxin production (Benson, Kurtzman, Halbrook & McCready, 1975; Lacey, 1986).

At Khalal stage, the sugar contents of the 20% extracts were estimated to be 4–6 g 100 ml^{-1}, and the results shown in Table 1 indicate an overall increase in the amount of aflatoxins produced compared with the Kimri stage. However, the pattern was not uniform so that, while a ~25% increase in available sugars produced a significantly higher level of aflatoxins in the extract from Naghal, similar changes in the sugar contents of extracts from Khulas and Lulu had no significant effect. A number of other inter-varietal differences are evident from Table 1, and the low levels of aflatoxins produced by Fard and Gush Rabie are, given the sugar levels predicted in Table 2, of particular note. What factor(s) contribute the apparent inhibition of aflatoxin production in extracts from these two varieties is not clear, but it is notable that Gush Rabie does not support appreciable aflatoxin synthesis until the Tamr stage; tissue metabolism in the date may well change dramatically at the fully mature stage.

At the Rutab stage (see Table 1), more aflatoxins were produced than at the previous ripening stages, probably due to the fact the mean (all varieties) concentration of sugars had reached 45.2 ± 1.64 g 100 g^{-1}

Table 1
Mean total levels of aflatoxin formed by *A. parasiticus* grown on extracts (20% total solids) made from dates of the varieties indicated and at the four stages of ripening[a]

Variety	Stage of ripening			
	Kimri[d]	Khalal	Rutab	Tamr
Naghal[e]	$0.43 \pm 0.41a_1$	$12.38 \pm 0.98e_2$	Not available[c]	$29.86 \pm 1.42b_3$
Buchibal	$0.51 \pm 0.33a_1$	$5.34 \pm 0.68bc_{12}$	$24.33 \pm 2.53abcd_3$	$10.42 \pm 1.79a_2$
Khunaizy	$0.14 \pm 0.01a_1$	$19.8 \pm 0.86g_2$	$21.15 \pm 3.16abcd_2$	$28.16 \pm 3.39b_2$
Khulas	$2.84 \pm 0.49b_1$	$6.94 \pm 1.81cd_1$	$24.87 \pm 3.43bcd_2$	$23.97 \pm 1.37b_2$
Gush Rabei	ND[b]	$0.20 \pm 0.31a_1$	$16.09 \pm 0.76a_2$	$42.24 \pm 1.42c_3$
Hilali Ahmr	ND	$6.81 \pm 1.79bcd_1$	$25.75 \pm 1.08cd_2$	$32.29 \pm 3.91bc_2$
Barhi	$1.97 \pm 0.48b_1$	$16.00 \pm 0.90f_2$	$27.29 \pm 3.59d_3$	Not available
Lulu	$2.70 \pm 0.30b_1$	$3.44 \pm 1.29ab_1$	$16.85 \pm 2.93ab_2$	$63.07 \pm 5.46d_3$
Fard	ND	$0.20 \pm 0.44a_1$	$24.98 \pm 1.06bcd_2$	$41.98 \pm 4.45c_3$
Naghal Hilali	ND	$9.88 \pm 0.84de_1$	$18.33 \pm 3.15abc_{12}$	$26.90 \pm 3.56b_2$
Khasab	ND	$0.79 \pm 0.61a_1$	$28.23 \pm 1.17d_2$	Not available
Hilali Pakistan	$0.23 \pm 0.14a_1$	$12.40 \pm 0.99e_2$	$28.90 \pm 1.14d_3$	$31.47 \pm 3.85bc_3$
Overall means	1.26 ± 1.05	7.84 ± 2.47	23.34 ± 2.08	33.04 ± 3.63

[a] All figures as μg ml^{-1} ± standard error.
[b] ND = none detected.
[c] Not available = overgrowth by yeasts in all trials or no Tamr stage (Khasab).
[d] Means within a column with different letters are significantly different ($p < 0.05$).
[e] Means within a row with different subscript numerals are significantly different ($p < 0.05$).

Table 2
Mean levels of total sugars (g 100 g^{-1} fresh weight) at the different stages of ripening[a,b]

Stage/variety	Kimri	Khalal	Rutab	Tamr
Naghal	5.1	30.6	44.2	44.3
Buchibal	5.1	18.8	49.0	55.1
Khunaizy	6.4	23.4	46.2	53.9
Khulas	7.0	31.9	46.1	57.0
Gush Rabei	5.3	24.9	48.1	49.9
Hilali Ahmr	3.4	23.0	43.6	64.1
Barhi	7.7	31.1	40.8	57.2
Lulu	7.6	29.7	43.9	57.7
Fard	5.6	27.1	50.1	59.5
Naghal Hilali	7.0	31.8	44.8	52.7
Khasab	7.6	22.9	41.7	Not formed
Hilali Pakistan	6.6	23.8	44.1	51.4
Overall means	6.2 ± 1.12	26.6 ± 2.04	45.2 ± 1.64	53.7 ± 2.45

[a] All the figures are estimates based on the summation of individual sugars (glucose, fructose, sucrose) determined in three replicates of fruits from two consecutive seasons.
[b] After Ahmed et al. (1995).

of fresh fruit (see Table 2). Khunaizy, which produced the highest concentration of aflatoxins at the Khalal stage, was not so prolific at Rhutab, even though the anticipated carbohydrate concentration in the original fruit/extract should have been above average. Hilali Pakistan continued to display a high level of toxin formation and, even at the lower concentration (10%), the extract from Hilali Pakistan supported the secretion of 21.8 μg ml^{-1} of aflatoxins (data not shown). Some of the extracts made at the Rutab stage supported the production of more aflatoxin at the 10 and 20% concentrations than on the whole fruit substrates (Ahmed et al., 1997), a contrast that could be due to the high sugar contents of some of the fruits at Rutab stage (see Table 2). These high levels can adversely affect toxin formation, for similar results were obtained by Delucca et al. (1977), who reported that more aflatoxin was produced in groundnut broth than on the whole nuts.

At both the Rutab and Tamr stages, significant inter-varietal differences were evident with respect to the levels of aflatoxins formed and, in these cases, concentrations of carbohydrate did not appear to be important. For example, the fresh fruits of Khulas and Lulu both contain around 57 g of total sugars 100 g^{-1} but, while the extract from Lulu supported ~63 μg ml^{-1} of aflatoxins, the extract from Khulas only contained ~23 μg ml^{-1}.

Two anomalous results were the low levels of toxin supported by the extract (20%) from the variety Naghal at Rutab and from Bahri at Tamr (same concentration). These contrasts were due to a yeast fermentation which took place during the incubation of the broths, as it was found difficult to achieve adequate surface sterilization of these varieties, particularly following infiltration of microbial cells into the internal part of the fruit; no Tamr stage is produced by the variety Khasab.

At the Tamr stage, the date fruit extracts supported the highest total aflatoxin production (see Table 1) compared to the other stages, and it is likely that the elevated sugar contents—typically 10–12 g 100 g^{-1} in the 20% date extracts—explain this increase. These latter results confirm that commercial extracts made from

Table 3
Overall mean levels (all varieties) of the different types of aflatoxin formed in extracts (20% total solids) made from dates at the stages of ripening indicated[a]

Stage	Aflatoxin B_1[c,d]	Aflatoxin B_2[d]	Aflatoxin G_1[d]	Aflatoxin G_1[d]
Kimri	0.50 ± 0.85a	0.06 ± 1.34	0.15 ± 0.53a	ND
Khalal	5.32 ± 1.85ab	0.48 ± 0.62	2.63 ± 1.46a	0.06 ± 0.26
Rutab	10.72 ± 1.16b	0.74 ± 0.44	11.60 ± 1.96b	0.28 ± 0.91
Tamr	12.80 ± 2.22b	0.82 ± 0.57	18.95 ± 2.91b	0.41 ± 0.50

[a] All figures as μg ml^{-1} ± standard error.
[b] ND = none detected.
[c] Means within a column with different letters are significantly different ($p < 0.05$).
[d] Note: whilst the levels of aflatoxin B_1 and G_1 were significantly higher ($p < 0.05$) than the levels of aflatoxin B_2 and G_2 at Rutab and Tamr, the differences between aflatoxins B_1 and G_1 were not significant.

Tamr fruits would probably support aflatoxin production if not handled and stored correctly.

The proportion of aflatoxins B_1 to G_1 did not show any significant pattern but, generally, aflatoxin B_1 tended to be more in evidence than aflatoxin G_1 in poor substrates, such as Kimri and Khalal (see Table 2); at the later stages of the fruit development, eg. Rutab and Tamr which were characterized by substantial aflatoxin production, aflatoxin B_1 was often equal to, or lower in concentration than, G_1. This trend is in line with the findings of Bennett, Lee, Shoss & Boudreaus (1980); Davis, Diener, and Eldridge (1966), Lacey (1986); Reddy, Viswanathan, and Venkitasubramanian (1971).

Overall, it would appear that the pattern of aflatoxin production within each stage of ripening is broadly in line with predictions that could be made on the basis of sugar content/chemical composition. Nevertheless, the experiment has confirmed the ability of date extracts to support aflatoxin production, a point that could, along with intervarietal differences, be of some practical importance. Thus, in date producing regions, date extracts are produced in the form of syrup, drinks, carbonated beverages and juices in small or commercial-scale operations (Mohamed & Ahmed, 1981; El-Shaarawy et al., 1989), and date syrup is also incorporated into some bakery products (Al-Obaidi, Aziz, Al-Hakkak, & Al-Hilli, 1987). If such extracts are stored prior to utilization, a surface film of mould could provide a source of aflatoxins.

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