

MYCOFLORA AND AFLATOXINS ASSOCIATED WITH SAIDY DATE AS AFFECTED BY TECHNOLOGICAL PROCESSES

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ABSTRACT

The mycobiota of Saily date (Semi-dry) was studied in 40 samples taken from the different production line stages of the Date Packing Factory at El-Kharja Oasis, New Valley Governorate, Egypt. There was a remarkable variance in the fungal count and diversity between the studied samples. Eleven species belonging to five genera were isolated on 20% sucrose-Czapek's agar medium at 28°C. Samples of date paste (Agwa) and flesh supplemented with sesame or peanut were highly polluted (650-3030 colonies/g). The genera of highest occurrence and their respective species were *Aspergillus* (*A. niger*, *A. flavus*, *A. flavipes*, *A. ochraceus*, *A. oryzae* and *A. terreus*); *Cladosporium* (*C. cladosporioides* and *C. sphaerospermum*) and *Penicillium* (*P. chrysogenum*). Thin-layer chromatographic analysis of the 40 different date samples revealed that 2 out of 5 samples of pitted date fruits stuffed with peanut were contaminated by aflatoxin B₁ (4.8 and 6.2 µg/kg). Experimental infection of the different date samples by four isolates of *A. flavus* as well as *A. flavus* CMI 89717, the highly aflatoxin-producing strain, indicated that samples supplemented with sesame or peanut were more susceptible for fungal growth and aflatoxin contamination.

Additional Index Words: New-Valley Date - Semi-dry date - Date fungi - Aflatoxins.

INTRODUCTION

Dates are among the most important horticultural crops in Egypt. Seven millions of fruitful date palms representing about 20 varieties are grown over the Nile Valley and Delta region which yield annually about 615000 tons of fresh, semi-dry and dry native dates (FAO, 1992 and 1993). The prevailing climatic conditions of the New Valley Governorate, Egypt is considered ideal for growing and fruiting of date palms especially soft and semi-dry fruit varieties. The good quality productive

date palms including “Saidy” or “Siwy” variety are approximately as 450000 trees (Hussein *et al.*, 1979 and Shubbar, 1984).

Dates contain high sugar content and a moderate percentages of minerals and vitamins. On the other hand, dates are relatively low in protein and fat, therefore, the addition of a protein and fat source such as, peanut, sesame and almond will enhance the protein and fat content of dates and give a highly nutritious food (Yousif *et al.*, 1987).

Most of dates produced are consumed directly with little or no further processing. Recently, the date producing countries gave some attention to the improvement and development of date processing. However, new products are recently searched to establish outlets of the surplus dates and to present more assortments in consumption forms (El-Shaarawy *et al.*, 1986).

Fungi are of ubiquitous distribution and regarded more or less a source of contamination of foods leading to spoilage and/or food-borne mycotoxins. Owing to the role played by fungi, whether from economic or public health point of view, advanced countries considered mold and yeast counts as a standard test for checking general sanitary conditions (Foster *et al.*, 1958). Mold growth on foods that are to be consumed directly can result in direct exposure to mycotoxins. Aflatoxins are the most potent toxic, mutagenic, teratogenic and carcinogenic metabolites produced by some strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Kurtzman *et al.*, 1987). Aflatoxins B₁, B₂, G₁ and G₂ are the most commonly encountered forms, with the former being the most potent (Eaten and Ramsdell, 1992).

The present investigation aimed to determine the frequency and specific taxa of fungi contaminating the semi-dry date fruits and the succession of fungal population under processing conditions. Also, the level of natural occurrence of aflatoxins and the potentialities of the isolated fungi for aflatoxin production on dates under investigation was conducted.

MATERIALS AND METHODS

Date samples

Forty samples of semi-dry dates and date products (Saidy variety) were taken from different production line stages of the Date Packing Factory at El-Kharja Oasis, The New Valley Governorate, Egypt. Five samples were taken at random from each of the following stages:

- 1 - Raw date fruits.
- 2 - Fumigated date fruits.
- 3 - Treated date fruits (fumigated; washed and partially dried).
- 4 - Treated date fruits, selected and packaged for export purpose.
- 5 - Date paste (Agwa), blocked in ½ kg and 1.0 kg cellophane bags.
- 6 - Date paste mixed with sesame, packaged in one kg cellophane bags.
- 7 - Pitted date fruits stuffed with peanut.
- 8 - Pitted date fruits stuffed with almond.

Samples were transferred to the laboratory and kept in a refrigerator until fungal and mycotoxins analysis.

Chemicals:

Standard aflatoxins B₁, B₂, G₁ and G₂ were obtained from the Southern Regional Research Center, New Orleans, Louisiana, USA. TLC aluminum plates 20x20 cm precoated with 0.25 mm silica gel G-25 HR as well as, silica gel for column chromatography were obtained from Sigma Chemicals Co. Other chemicals were reagent grade.

Aflatoxin-producing culture:

A highly aflatoxin-producing strain (*Aspergillus flavus* CMI 89717) was obtained from the Commonwealth Mycological Institute, Kew Surrey, England. It was maintained on potato dextrose agar slant, at 5°C.

Media used:

- Czapek's-sucrose agar medium (200.0 g sucrose, 3.0 g sodium nitrate, 0.5 g potassium chloride, 0.5 g magnesium sulphate, 1.0 g dibasic potassium phosphate, 0.01 g ferrous sulphate and 20.0 g agar in 1.0 L distilled water) was used for isolation of fungi. (Raper and Fennell, 1977).

- Sabouraud-yeast extract broth medium (20.0 g glucose, 10.0 g peptone and 10.0 g yeast extract in 1.0 L distilled water) was used for aflatoxin production as a control of the studied substrates.
- Sabouraud-yeast extract agar medium (the same components of the previous medium with adding 20 g. agar) was used for the preliminary detection of aflatoxin-producing fungi.

Methods:

Moisture determination:

Moisture content of date samples was determined by oven drying at 65°C according to Auda *et al.* (1976).

Fungal analysis:

The dilution-plate method (Johnson and Curl, 1972) was applied for isolation of fungi. 20% (w/v) sucrose-Czapek's agar medium was employed. Chloramphenicol (20 µg/ml) and rose bengal (30 ppm) were used as bacteriostatic agents. Four plates of each sample were prepared and incubated at 28°C for one week. The developing fungi were counted (per g date fruits) and identified according to the following references: Booth (1971); Ellis (1976); Raper and Fennell (1977); Pitt (1979); Domsch *et al.* (1980); Kozakiewicz (1989); Moubasher (1993) and Samson *et al.* (1995).

Chromatographic analysis of aflatoxins:

Thin-layer chromatography was routinely used for qualitative and quantitative estimations of aflatoxins (if any) in the resulting chloroform extracts.

Extraction and purification:

Aflatoxins were extracted and purified according the method of AOAC (1984). The extraction was performed using chloroform:water (10:1 v/v) mixture. The obtained crude extracts were purified by column chromatography containing anhydrous sodium sulphate (15 g) and silica gel (10 g).

Qualitative estimation of aflatoxins:

Rectangular glass jar (30x15x30 cm) was used for developing chromatoplates. A suitable volume of solvent mixture (chloroform:

methanol, 97:3 v/v) was placed in the bottom of the jar so that the starting spots on the plates would be 1 cm above the upper surface of the solvent mixture. Chromatographic plates (20x20 cm) were activated by heating 1 h at 120°C in a hot air oven, and removed immediately to a desiccator to cool. Parallel starting spots, 2 cm from each side of the plate and 1.5 cm apart, were made with micropipets from chloroform extracts with authentic reference aflatoxins. Spots were left to dry in air. Prepared plates were then transferred to the chromatographic jar, developed to a suitable distance (10 cm), and removed. The solvent front was marked and the plates were dried in air. Spots were viewed under UV light (366 nm) and the outline of each fluorescent spots was marked by sharp pin. R_f values, colors, and intensities of the unknown spots were compared with those of the authentic reference aflatoxins (El-Bazza *et al.*, 1982).

Quantitative Determination of Aflatoxins

The dilution-to-extinction (Coomes *et al.*, 1965) and comparison of standards (AOAC, 1984) techniques were used for estimation of aflatoxins concentrations.

Preliminary detection of aflatoxin-producing fungi:

Isolates of *A. flavus* recovered from the studied date samples were screened for their ability to produce aflatoxin(s) on Sabouraud-yeast extract agar plates, using the fluorescent agar technique of Hara *et al.* (1974). Each of the isolated molds was inoculated as a single short streak at the center of the plate surface. Plates were then incubated at 25°C for 7 days and viewed under UV light (366 nm); the presence of any fluorescence in the medium surrounding the fungal growth was recorded. A plate of non-inoculated medium was similarly incubated and viewed under UV light as a control. This control was used to rule out any fluorescence that may be produced by the constituents of the medium.

Experimental production of aflatoxins on date samples:

50 g samples of each date product were placed in 250 ml Erlenmeyer flasks and inoculated by 1 ml spores suspension (approximately 10^6 conidia) of each of the four *A. flavus* isolates, which proved to be aflatoxigenic using the fluorescent agar technique as well as, *A. flavus* CMI 89717 strain. Another flasks containing 50 ml of Sabouraud-yeast extract broth medium were similarly inoculated as control. The infected flasks were incubated at $25\pm 1^\circ\text{C}$ for two weeks. Extraction and estimation of aflatoxins were made as previously mentioned.

RESULTS AND DISCUSSION

Fungal flora of date samples:

The mycological analysis of semi-dry dates “Saidy variety” during different stages of processing and packing revealed that the total count of fungi in all samples ranged from 310-3030 colonies/g dates product (Table 1). Samples of date paste (Agwa) and paste mixed with sesame were highly contaminated with fungi in comparison with the intact and pitted date fruits. There was a remarkable low incidence of diverse fungal contamination of the analyzed samples. These results were in contrast with those observed by Abu-Zinada and Ali (1977); Nassar (1986) and Abdel-Sater and Saber (1999). They reported that the dry dates were highly polluted with various fungal genera and species.

Eleven species appertaining to five genera were isolated from the studied samples. Members of *Aspergillus* and *Cladosporium* were the most prevalent (Table 1).

Aspergillus was the first predominant genus, encountered in 70.9-100.0% of total fungi. Six species of *Aspergillus* were identified of which *A. niger* was the most prevalent species in all the studied date products (58.1-100% of total fungi) followed by *A. flavus* (1.5-7.0%). Whereas, *A. flavipes* (0.75-7.4%) and *A. oryzae* (4.0-5.8%) were only isolated from two treated samples. The remaining *Aspergillus* species (*A. ochraceus* and *A. terreus*) were encountered only from the selected date fruits (Table 1). These results are in agreement with those obtained by Nassar (1986) and Abdel-Sater and Saber (1999). They found that *Aspergillus* was the predominant genus on dry dates and the most prevalent species were *A. niger*, *A. flavus* and *A. fumigatus*. Similarly, the genus incidence and its respective numbers represented the highest contaminants of other dried fruits and kernels such as peanuts, hazelnut, walnut and figs as indicated by Moubasher *et al.* (1979); El-Maghraby and El-Maraghy (1987); Jimenez *et al.* (1991); Abdel-Hafez and Saber (1993) and Abd-Alla *et al.* (1999).

The second higher incidence rate was represented by the genus *Cladosporium*. It recovered from raw, fumigated and washed dates in addition to the pitted date fruits stuffed with peanuts at levels of 2.6, 10.0, 23.2 and 1.5% of the total fungi, respectively. Among the two isolated *Cladosporium* species, *C. cladosporioides* was the most common whereas, *C. sphaerospermum* rarely occurred (Table 1). This genus was also isolated at various levels of occurrence from different foodstuffs including dry and semi-dry dates as reported by Abu-Zinada (1977);

Pruski and Ben-Arie (1985); Nassar (1986); Samson *et al.* (1988, 1995); Benkhemmar *et al.* (1992); Reiss (1993); Abdel-Sater *et al.* (1996) and Abdel-Sater and Saber (1999).

Data in table (1) also showed that the following species were detected as a single representatives of genera and infrequently encountered: *Penicillium chrysogenum*, *Rhizopus stolonifer* and *Ulocladium botrytis*. These species were also isolated, but with various frequencies and occurrences, from similar foods in Egypt (Nassar, 1986; El-Maghraby and El-Maraghy, 1987; Abdel-Gawad and Zohri, 1993; Abdel-Hafez and Saber, 1993 and Abd-Alla *et al.*, 1999), and in other parts of the world (Abu-Zinada and Ali, 1977; Pitt, 1985; Pruski and Ben-Arie, 1985; Benkhemmar *et al.*, 1992, 1993; Samson *et al.*, 1988, 1995 and Reiss, 1993).

From data in table (1), it could be concluded that the primary steps of date processing such as, fumigation, washing and selection (sorting), although it led to an increase in fungal frequency, caused a marked reduction of the total fungal count. On the other hand, additional contamination might occur during pitting, mincing and pressing processes of date fruits to obtain the Agwa products. Therefore, precautionary procedures must be adopted during those steps to avoid contamination with mycoflora.

Natural occurrence of aflatoxins on date samples:

Five samples of each of raw date fruits, treated date fruits (fumigated, washed, selected and packaged) and processed date (Agwa, Agwa mixed with sesame and pitted fruits stuffed with peanut and almond) were analyzed for aflatoxin contamination. Data in table (2) clearly show that all the studied samples were found to be aflatoxin free, except that of pitted fruits stuffed with peanut which contained aflatoxin B₁ in 2 out of 5 analyzed samples. The detected concentrations of aflatoxin B₁ in the two contaminated samples were 4.8 and 6.2 µg/kg. Since all of the studied date samples proved to be aflatoxin free, the presence of aflatoxin B₁ in those two samples may be attributed to a previous contamination of the peanut kernels stuffed in these fruits. Abd-Alla *et al.* (1999) reported that, the occurrence of aflatoxin is more common in oil seeds and cereals than in horticultural crops. However, Abdel-Sater and Saber (1999) reported that aflatoxin B₁ was found in 2 samples out of 20 tested samples of dry date. Except this report, no previous studies confirmed the presence of aflatoxins on date fruits.

Table (2): Natural occurrence of aflatoxins in date samples.

Type of samples	Moisture content (%)	No. of analyzed samples	No. of positive samples	Aflatoxins detected	
				Type	Concentration $\mu\text{g}/\text{kg}$
Raw date fruits	24.3-26.5	5	0/5	Non	ND
Fumigated date fruits	17.8-19.2	5	0/5	Non	ND
Washed and partially dried date fruits	18.6-20.1	5	0/5	Non	ND
Selected and packaged date fruits	16.5-18.7	5	0/5	Non	ND
Date paste (Agwa)	24.8-27.1	5	0/5	Non	ND
Agwa mixed with sesame	23.5-26.4	5	0/5	Non	ND
Pitted fruits stuffed with peanut	20.1-23.3	5	2/5	B ₁	4.8 and 6.2
Pitted fruits stuffed with almond	22.9-25.7	5	0/5	Non	ND

ND : Not detected.

Experimental production of aflatoxins on dates:

In order to illustrate the ability of toxigenic fungi on growth and formation of aflatoxins on date fruits under its compositional state, the studied date samples were inoculated with heavy spore suspension of the highly aflatoxigenic strain *A. flavus* CMI 89717 as well as, four isolates of *A. flavus* which proved to be aflatoxin-producers using the fluorescent agar technique. The infected samples were incubated for two weeks at 25°C which reported as optimal conditions for aflatoxin production by *A. flavus* (Diener and Davis, 1966; Lieu and Bullerman, 1977). Sabouraud-yeast extract broth medium, recommended by El-Bazza *et al.* (1982) as the most suitable medium for aflatoxin production was similarly inoculated and incubated as a control. Data in Table (3) revealed that all fungi failed to grow on all date samples except of Agwa mixed with sesame and flesh stuffed with peanut. In case of the two later substrates, there was very little growth observed only on the particles of sesame and peanut with formation of low levels of aflatoxin B₁. Aflatoxin B₁ concentrations ranged from 0.0 to 8.0 and 6.8 to 14.0 $\mu\text{g}/\text{kg}$ of Agwa mixed with sesame and flesh stuffed with peanut, respectively. On the other hand, the same fungi were extremely grew on the control liquid medium and produced aflatoxins B and G at concentrations higher than that produced on the positive date samples by more than hundred folds. The reason(s) which prevent fungal growth and aflatoxin production on dates are not definite, it may be the low level of moisture content which ranged from 16.5 to 27.1% (Table, 2). In this respect, Pitt and Miscamble

(1995) stated that the minimum available water (a_w) level for germination and growth of *A. flavus* spores was very close to 0.82 at 25°C. On the other hand, similar commodities with high osmotic potential such as dried figs were found to be highly susceptible for fungal growth and aflatoxin contamination (Abd-Alla *et al.*, 1999). Further studies are needed to investigate this contrariety.

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