PAPER

COFFEE BEAN MYCO-CONTAMINANTS AND OXALIC ACID PRODUCING *ASPERGILLUS NIGER*

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ABSTRACT

Coffee bean-contaminating fungi were determined in random samples collected in Riyadh, Kingdom of Saudi Arabia, using the direct plating technique. Forty-five samples were examined and 12 fungal species belonging to 5 genera were isolated. *Aspergillus niger* was the most widely distributed and most frequently isolated fungus (86.67%). The ability of the predominant fungus, *A. niger*, to produce oxalic acid was evaluated using high-performance liquid chromatography. About 50% of the tested *A. niger* isolates produced oxalic acid; the amount produced was in the range of 90–550 ppm of oxalic acid. Because *A. niger* was the predominant and most widely distributed toxigenic fungus in the examined samples, more efforts should be directed to minimize the risk of oxalic acid contamination of commoditized coffee beans in the Kingdom of Saudi Arabia.

- Keywords: mycotoxins, seed-borne, *Coffea arabica* L., high-performance liquid chromatography, metabolites -

INTRODUCTION

Coffea arabica L. is considered to have the best flavour and quality, and coffee is one of the most popular beverages consumed in countries of the Arabian Peninsula. However, coffee beans may become poisonous because of contamination with mycotoxigenic fungi that may occur throughout all pre and/or post-harvest stages (BATISTA *et al.*, 2003; NOONIM *et al.*, 2008). Several fungal genera were found to contaminate coffee beans at each stage, from the farmer to the consumer (VEGA *et al.*, 2008; BATISTA *et al.*, 2009; VILELA *et al.*, 2010).

The main mycotoxigenic fungal genera found to be associated with commoditized coffee beans belong to the genera *Aspergillus, Penicillium,* and *Fusarium* (PARDO *et al.*, 2004; BOKHARI, 2007; LEONG *et al.*, 2007). These fungi not only affect the quality of coffee beans but also produce toxic secondary metabolites that are harmful to the consumers (BERNNETT and KLICH, 2003, VILELA *et al.*, 2010).

One of the most important mycotoxigenic and organotoxic metabolite-producing contaminant is *Aspergillus niger*, which is considered a class 1 containment agent (U.S. NPA, 1977; SCHUSTER *et al.*, 2002; ILIC *et al.,* 2007). This fungus is responsible for the *in vitro* and/or *in vivo* secretion of the nephrotoxic compound oxalic acid (MANDAL *et al.*, 2005; MAGNOLI *et al.,* 2008). The toxicity of oxalic acid is due to the deposition of calcium-oxalate complexes in renal tubules causing renal failure; in humans, the minimum lethal dose of orally ingested oxalic acid is 600 mg/kg (SAFETY OFFICER IN PHYSICAL CHEMISTRY, 2005; BOTHA *et al.*, 2009).

The purpose of this study was to examine coffee beans commoditized in Saudi Arabian markets for the presence of toxigenic fungi. Furthermore, we evaluated the production of oxalic acid by the isolated *A. niger* strains.

MATERIALS AND METHODS

Mycological analysis

Mycotoxin-producing fungi were determined in 45 random samples of coffee beans collected in Riyadh City, Kingdom of Saudi Arabia. For the isolation of fungi associated with coffee beans, the direct plating technique was applied using potato dextrose agar (PDA) medium. Beans were plated directly onto PDA medium after surface disinfection by 5% sodium hypochlorite solution. The plates were incubated for 5–7 days at 25°C and the growing fungal colonies were purified. The obtained fungal isolates were then identified to the species levels at the Mycological Centre, Assiut University, Egypt.

Table 1 - Distribution of isolated fungi in coffee bean samples.

Fungi	Distribution %
1. *A. alternata*	8.89
2. *A. f.columnaris*	17.78
3. *A. niger*	86.67
4. *A. ochraceus*	4.44
5. *A. terreus*	2.22
6. *Nigrospora* sp.	2.22
7. *P. brevicompactum*	8.89
8. *P. corylophilum*	6.67
9. *P. variabile*	2.22
10. *P. suchlasporia*	2.22
11. *R. stolonifer*	44.44
12.*T. flavus*	2.22

Mycotoxigenicity

The oxalic acid production of the tested *A. niger* isolates was determined using high-performance liquid chromatography. Briefly, 50 mL of Czapek-Dox broth medium was placed in 250-mL Erlenmeyer flasks and inoculated with *A. niger*. Inoculated media were incubated in triplicates at 30°C on an orbital shaker maintained at 215 rpm for 7 days. The culture supernatants were then analysed for their oxalic acid content. Separation of oxalic acid was carried out using a CLC-C825 CM cation exchange column. The mobile phase was 90% H_2O and 10% CH_3OH. The flow rate was 1 mL/min and the temperature was 35°C (GHORBANI *et al.,* 2007).

Statistical analysis

The SPSS-16 statistical package was used for the analysis of variance and correlation and cluster analyses. Cluster analysis was performed by the unweighted pair-group method with arithmetic averages. Data were processed by root square transformation of % frequencies + 0.5 to normalize and stabilize the variance before subsequent analyses were carried out. Means were statistically compared using the least significant difference test.

Table 2 - ANOVA of the isolation frequencies of mycotoxigenic fungi in coffee beans.

Source of variance	Df	M S	F	Sig.	R.C.*
Samples	44	2.070	1.558	0.012	0.35
Fungi	11	575.637	433.265	0.000	98.60
Samples x Fungi	484	6.086	4.580	0.000	1.04
Error	1620	1.329			

*Relative contribution.

Table 3 - Comparison of the isolation frequencies of fungi isolated from coffee beans.

S.	F1		F2		F3		F4		F5		F6		F7		F8		F9		F10		F11		F12	
No	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.	Freq.	Trans.
1	0.00	0.71	25.00	3.04	50.00	5.37	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
2	0.00	0.71	25.00	3.04	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
3	7.30	2.29	0.00	0.71	36.46	4.63	0.00	0.71	0.00	0.71	0.00	0.71	0.00	1.79	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
4	0.00	0.71	0.00	0.71	62.50	7.03	0.00	0.71	0.00	0.71	0.00	0.71	6.25	1.79	0.00	0.71	0.00	0.71	0.00	0.71	6.25	1.79	0.00	0.71
5	23.75	4.35	23.75	4.35	46.25	6.76	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	6.25	1.79	0.00	0.71
6	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
7	50.00	5.37	25.00	3.04	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
8	0.00	0.71	5.00	1.66	95.00	9.76	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
9	0.00	0.71	3.57	1.49	42.86	4.98	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	3.57	1.49	0.00	0.71
10	0.00	0.71	0.00	0.71	20.09	3.54	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	6.70	2.22	23.21	3.78
11	0.00	0.71	0.00	0.71	70.83	7.48	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
12	0.00	0.71	0.00	0.71	64.28	8.01	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	25.00	4.38	10.72	2.66	0.00	0.71	3.57	1.49	0.00	0.71
13	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
14	0.00	0.71	0.00	0.71	10.00	2.12	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	15.00	2.48	0.00	0.71
15	0.00	0.71	0.00	0.71	83.33	9.16	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	16.70	4.15	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
16	0.00	0.71	13.44	2.97	48.21	6.19	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	13.39	2.96	0.00	0.71
17	0.00	0.71	0.00	0.71	37.50	4.64	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	37.50	4.64	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
18	0.00	0.71	3.57	1.49	88.69	9.42	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	7.75	2.35	0.00	0.71
19	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
20	0.00	0.71	0.00	0.71	91.67	9.59	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	8.35	2.43	0.00	0.71
21	0.00	0.71	0.00	0.71	95.83	9.81	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71
22	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
23	0.00	0.71	0.00	0.71	60.00	7.65	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	27.50	4.07	12.50	2.31	0.00	0.71
24	0.00	0.71	0.00	0.71	62.50	7.82	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	37.50	5.49	0.00	0.71
25	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
26	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
27	0.00	0.71	0.00	0.71	48.21	6.19	0.00	0.71	0.00	0.71	0.00	0.71	23.21	4.26	0.00	0.71	0.00	0.71	0.00	0.71	3.57	1.49	0.00	0.71
28	0.00	0.71	0.00	0.71	70.83	7.48	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71
29	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
30	0.00	0.71	0.00	0.71	42.86	5.85	16.07	3.09	16.07	3.09	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
31	0.00	0.71	0.00	0.71	76.19	8.69	0.00	0.71	0.00	0.71	23.82	4.34	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
32	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
33	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
34	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
35	0.00	0.71	0.00	0.71	95.83	9.81	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	11.32	2.74	0.00	0.71
36	0.00	0.71	0.00	0.71	0.00	0.71	50.00	5.37	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
37	0.00	0.71	0.00	0.71	38.69	4.76	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	11.32	2.74	0.00	0.71
38	75.00	7.70	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
39	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
40	0.00	0.71	0.00	0.71	95.83	9.81	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	4.18	1.57	0.00	0.71
41	0.00	0.71	0.00	0.71	45.00	5.10	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	5.00	1.66	0.00	0.71
42	0.00	0.71	0.00	0.71	41.67	4.93	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	8.35	2.43	0.00	0.71
43	0.00	0.71	0.00	0.71	100.00	10.03	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
44	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	75.00	7.70	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71
45	0.00	0.71	0.00	0.71	75.00	8.57	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	0.00	0.71	25.00	3.91	0.00	0.71

F1-F12= *A. alternate*, *A. f. columnaris*, *A. niger*, *A. ochraceus*, *A. terreus*, *Nigrospora* sp., *P. brevicompactum*, *P. corylophilum*, *P. variabile*, *P. suchlasporia*, *R. stolonifer* and *T. flavus* respectively.
Freq. = Frequency of fungal isolation.
Trans. = transformed data into root square of % values + 0.5.

Table 4 - Correlation among frequencies of fungi isolated from coffee beans.

Fungi	1	2	3	4	5	6	7	8	9	10	11	12
1. *A. alternata*	1	0.365*	-0.427**	-0.058	-0.042	-0.042	-0.048	-0.075	-0.042	-0.042	-0.133	-0.042
2. *A. f.columnaris*		1	-0.293	-0.084	-0.062	-0.062	-0.107	-0.109	-0.062	-0.062	-0.003	-0.062
3. *A. niger*			1	-0.306*	-0.060	0.078	-0.310*	0.007	0.045	0.027	-0.005	-0.172
4. *A. ochraceus*				1	0.439**	-0.031	-0.053	-0.055	-0.031	-0.031	-0.144	-0.031
5. *A. terreus*					1	-0.023	-0.039	-0.040	-0.023	-0.023	-0.106	-0.023
6. *Nigrospora* sp.						1	-0.039	-0.040	-0.023	-0.023	-0.106	-0.023
7. *P. brevicompactum*							1	-0.069	-0.039	-0.039	-0.108	-0.039
8. *P. corylophilum*								1	0.562**	-0.040	-0.119	-0.040
9. *P. variabile*									1	-0.023	0.010	-0.023
10. *P. suchlasporia*										1	0.130	-0.023
11. *R. stolonifer*											1	0.116
12. *T.flavus*												1

RESULTS AND DISCUSSION

The mycological examination of the coffee bean samples (Table 1) revealed the presence of 12 fungal species that belonged to 5 genera (SILVA *et al.*, 2008; BATISTA *et al.*, 2009). The predominance of *A. niger* in the tested coffee bean samples was in agreement with previously reported data (URBANO *et al.*, 2001; NEHAD et al., 2007; NOONIM *et al.* 2008). The main mycotoxigenic fungal genera, i.e. *Aspergillus* and *Penicillium*, have frequently been associated with coffee beans (JOOSTEN *et al.*, 2001; PARDO *et al.*, 2004; BOKHARI, 2007; LEONG *et al.*, 2007).

ANOVA indicated that the effects of fungus, sample, and fungus × sample interaction were highly significant sources of variation in the fungal isolation frequencies. Compared to the other sources, fungus was the most important source of variation (Table 2).

The isolation frequencies varied according to the tested samples. For example, *Alternaria alternata* and *A. flavus* var. *columnaris* showed equal isolation frequencies in sample No. 5, but the former was significantly more frequently isolated from sample No. 7 than the latter. Although *A. flavus* var. *columnaris* was isolated from samples No. 9 and 18 at equal frequencies, the frequency at which *A. niger* was isolated from these 2 samples varied significantly. Whereas *Penicillium brevicompactum* and *Rhizopus stolonifer* were isolated from sample No. 4 at equal frequencies, *P. brevicompactum* was significantly more frequently isolated than *R. stolonifer* (Table 3). However, the contamination of coffee beans with mycotoxigenic fungal genera (NOONIM *et al.*, 2008; VILELA *et al.*, 2010) may start in the field and subsequently extend to storages and markets, in particular under conducive conditions (JESTOI *et al.*, 2004; JUAN *et al.*, 2008; PATERSON and LIMA, 2010).

Correlation analysis showed that the fungal isolation frequencies were positively and/or negatively correlated with each other (Table 4). Some correlations between isolated fungal species and their isolation frequencies were significant or highly significant. Highly significant positive correlations were noted among *Asper-*

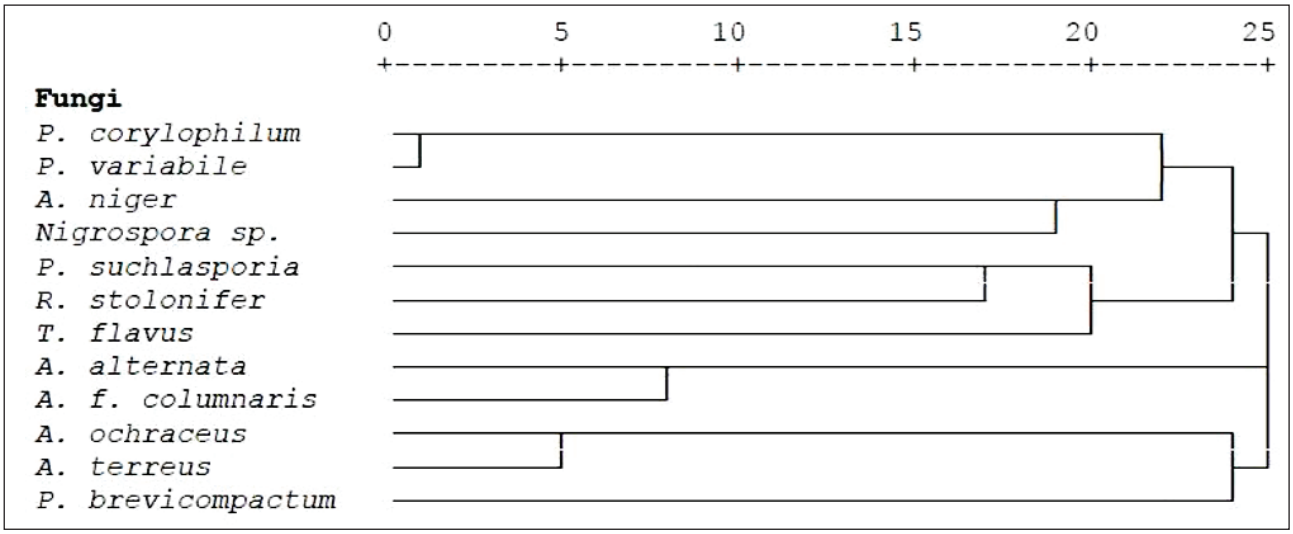

Fig. 1 - Phenogram based on average linkage cluster analysis of frequencies of fungi recovered from coffee beans.

Table 5 - Production of oxalic acid by *A. niger* isolates.

A. niger isolates	Oxalic acid (ppm)
***A.n.*1**	00.00
***A.n.*2**	200.00
***A.n.*3**	150.00
***A.n.*4**	00.00
***A.n.*5**	00.00
***A.n.*6**	00.00
***A.n.*7**	00.00
***A.n.*8**	550.00
***A.n.*9**	100.00
***A.n.*10**	90.00

gillus ochraceus and *Aspergillus terreus* as well as among *Penicillium corylophilum* and *Penicillium variabile*. Furthermore, a highly significant negative correlation was found between *A. alternata* and *A. niger*. The highly significant positive correlations between some isolated fungi found in this study imply that similar colonizing conditions are provided by the coffee beans for those fungi; the opposite conclusion can be drawn for negatively correlated fungi (YASSIN *et al.,* 2013).

The phenogram (Fig. 1) illustrates the cluster analysis of fungal isolation frequencies (%) on the basis of their distribution patterns using all samples. Three distinct groups of isolated fungi are shown; each of them is divided into subgroups. Strongly and positively associated fungi were grouped in the same cluster. The grouping pattern of the isolated fungi in the cluster analysis suggests the potential existence of sample- (environment-) related fungal groups (YASSIN *et al.,* 2011).

With regard to the mycotoxigenicity, 50% of the tested *A. niger* isolates were able to produce oxalic acid. Other tested isolates failed to produce any detectable amounts of oxalic acid (Table 5).

Aspergillus species are well known to be responsible for the secretion of different toxic metabolites (AL-ABDALALL, 2009; YASSIN *et al.,* 2010; EL-SAMAWATY *et al.,* 2011). Moreover, the organotoxic metabolite oxalic acid has also been shown to be produced by *A. niger* isolated from commoditized agricultural products (BAHKALI *et al.*, 2013; YASSIN *et al.,* 2013). Accumulation of such compounds produced by mycotoxigenic fungi could affect the quality of coffee (ARRUSA *et al.*, 2005; KUMAR *et al.*, 2008) and harm human consumers (PALANEE *et al.*, 2001; DAVID *et al.*, 2005).

CONCLUSIONS

The coffee beans that were examined in this study were found to be contaminated with many fungal genera that might affect the quality of coffee. *A. niger* was the most widely distributed and most frequently isolated fungus (86.67%). Furthermore, 50% of the tested *A. niger* isolates were mycotoxigenic. They were able to produce oxalic acid in the range of 90–550 ppm. Because *A. niger* was the predominant and most widely distributed fungus in the examined coffee bean samples, more efforts should be directed to minimize the risk of oxalic acid contamination of commoditized coffee beans in KSA.

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