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Study on toxigenic potential of *Aspergillus* species from coffee

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Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced in coffee by three specis of *Aspergillus viz, Aspergillus carbonarius, A. niger* and *A. ochraceus*. In the present study, the ochratoxigenic potential of various *Aspergillus* species isolated from coffee bean were evaluated in yeast extract sucrose (YES) and coffee meal extract agar (CMEA) medium using HPLC technique. Of the 255 isolates obtained, the most common *Aspergillus* species found was *A. niger* (219 isolates) followed by *A. ochraceus* (36 isolates) of which 31% and 83% of *A. niger and A. ochraceus* isolates capable of producing OTA respectively. The OTA production by the *A. ochraceus* isolates was in the range of 1.9 to 122 μg kg^{-1} and 0.75 to 42.4 μg kg^{-1} in YES and CMEA media respectively. While, the *A. niger* isolates could produce OTA in the range of 0.8 to 12 μg kg^{-1} and 0.5 to 6.15 μg kg^{-1} in YES and CMEA media respectively. A close examination the data revealed that a higher percentage of mould infection and also high incidence of ochratoxigenic moulds was observed in robusta samples (27 *A. ochraceus* and 159 *A. niger* isolates) compared to arabica (9 *A. ochraceus* and 60 *A. niger* 60 isolates) indicating robusta type was more susceptible to mycotoxigenic mould infection than arabica type.

Key words : Coffee, *Aspergillus*, ochratoxin A, toxigenic potential, HPLC

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic mycotoxin (Elling *et al.*, 1985) produced by *Aspergillus ochraceus* (van der Merwe *et al.,* 1965) and *Penicillium verrucosum* (Pitt, 1987). It is generally assumed that in temperate regions, OTA is primarily produced by *P. verrucosum* whereas in tropical and sub-tropical areas, *Aspergillus ochraceus* is responsible for OTA accumulation (Pitt and Hocking, 1997). Until the first description of OTA production by *A. niger* var. *niger* (Abarca *et al.*, 1994) and *A. carbonarius* (Horie, 1995), it was believed OTA is produced only by *Aspergillus ochraceus* and related species belonging to section *Circumdati* (Hesseltine *et al.*, 1972 ; Varga *et al.*, 1996) and *P. verrusosum*. The natural occurrence of OTA has been reported in a wide variety of foods including cereals (Chelkowski *et al.*, 1983), cocoa (Matissek and Raters, 2000), spices (Thirumala *et al.*, 2000), liquorice (Majerus *et al.*, 2000) and coffee (Gopinandhan *et al.*, 2007). The first report on the occurrence of OTA in coffee was reported by Levi *et al.*,1974 ; Studer-Rohr *et al.*, 1995). Before the discovery of ochratoxigenic ability of *A. carbonarius* and *A. niger, Aspergillus ochraceus* has frequently been proposed as the mojor cause of OTA in coffee bean (Frank, 1999). *A. carbonarius* and *A. niger* capable of producing OTA on coffee bean have been reported by several authors (Nakajima *et al.*, 1997 ; Teren *et al.*, 1997; Taniwaki *et al.*, 1999 ; Joosten *et al.*, 2001 ; Urbani *et al.*, 2001 ; Suarez- Quiroz *et al.*, 2004). Joosten *et al.*, (2001) demonstrated *A. carbonarius* strain (M333) isolated from Thailand coffee produced significant amount of OTA (930 µg kg^{-1}) on coffee bean.

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Similarly, Suarez-Quiroz *et al.*, (2004) isolated *A. ochraceus* and *A. niger* from Mexicon coffee and reported *A. ochraceus* could produce OTA ranging from 0.3 to 679 µg kg-1 and 1 to 697 µg kg-1 on rice medium and coffee bean respectively. While *A. niger* could produce OTA ranging from 5.7 to 114 µg kg-1 and 2.2 to 9.8 µg kg-1 on rice medium and coffee bean respectively. In a recent survey by Taniwaki *et al.*, (2003) reported on the distribution of these three ochratoxigenic species in 408 coffee samples from four coffee producing regions of Brazil. Of the 872 isolates obtained, the most common species found was *A. niger* (549 isolates) followed by *A. ochraceus* (269 isolates) and *A. carboanrius* (54 isolates) with 3%, 75% and 77% of the *A. niger*, *A. ochraceus* and *A. carboanrius* isolates capable of producing OTA respectively. These findings led to the conclusion that three *Aspergilli*, *A. carbonarius*, *A. niger* and *Aspergillus ochraceus* are responsible for OTA in coffee. Reports relating to ochratoxigenic moulds and OTA contamination in Indian coffee is rather limited. There are only few published works exist relating to micro-biota associated with different coffee varieties (Velmourougane *et al.*, 2000), incidence of toxigenic moulds in raw & cured coffee beans (Panneerselvan *et al.*, 2000), coffee fruits (Panneerselvam *et al.*, 2002), OTA toxin contamination in coffee bean (Ramesh and Vasanthi, 2005 ; Gopinandhan *et al.*, 2007) and coffee by-products (Gopinandhan *et al.*, 2006). To our knowledge nothing has been published hitherto on the toxigenic potential of ochratoxigenic moulds from Indian coffee samples. With this objective in mind the present study was undertaken to assess the toxigenic potential of various ochratoxigenic moulds isolated from coffee bean.

MATERIALS AND METHODS

A total of 80 coffee bean samples (each weighing 1 kg) pertaining to 2006-07) harvest seasons were collected from various coffee curing factories located in major coffee growing regions of Chikmagalur, Coorg and Hassan districts in Karnataka, Waynad and Palkad districts in Kerala and Salem and Pattiveeranpatti districts in Tamilnadu states. From these 80 samples, two samples (one arabica [*Coffea arabica*] and one robusta [*Coffea canephora*] sample were randomly selected from each district for isolating ochratoxigenic moulds.

Mycological analysis

Mycological analysis of coffee beans was done as described by Taniwaki *et al.*, (2003). Sub sample of coffee beans were surface disinfected with 0.4% chlorine solution for 1 min (Pitt *et al.*, 1997) and rinsing in sterile distilled water followed by drying on sterile filter paper (Arnold *et al.* 2001). Fifty coffee beans were placed directly (10 beans per plate) onto Dichloran 18% Glycerol Agar (Hocking and Pitt, 1980). The plates were incubated at 25ºC for 5 to 7 days and then inspected for colony growth visually and with the aid of a stereomicroscope. The percentage infection of coffee beans by moulds (number of beans on which mould infection was present/total number of beans examined) was recorded. Putative colonies of *Aspergillus* species were grown on standard identification media Czapek yeast extract agar (CYA) and identified according to Klich and Pitt (1998). Numbers of isolates identified as *Aspergillus ochraceus* and *A. niger* were counted for each sample. The individual isolates of *Aspergillus ochraceus* and *A. niger* were codified serially indicating region, coffee variety and species name and maintained in Czapek agar medium. Although other species of moulds (*Yeast, Penicillum, Fusarium, Cladoporium, Alternaria, Wallemia* and *Aureobasidium*) were also frequently present, no attempt was made to characterize them.

Test for OTA production by A. ochraceus and A. niver

Isolates of *A. ochraceus* and *A. niger* were three point inoculated into yeast extract 15% sucrose agar mdeium (Samson *et al.*, 2000). The isolates were also inoculated onto coffee meal extract agar (CMEA) medium to assess the real fungal ability to produce OTA on a natural substrate. The CMEA medium was prepared as described by Pardo *et al.*, (2005). CMEA medium was made by boiling 30 g of ground green coffee beans in 1 liter of water for 30 minutes. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1 liter and 20 g of agar was added. Autoclaved medium was aseptically poured into sterile Petri-plates. Isolates were three point inoculated into YES and CMEA medium at 25ºC for 7 days and evaluated for tghe production of OTA by the agar plug technique (Bragulat *et al.*, 2001). Briefly, three agar

plugs were removed from the central area of the colony and introduced into a pre-weighed sterile eppendorf plastic tube (1.5 ml capacity) and weighed again. The agar plugs were extracted with 0.5 ml of methanol/formic acid (25:1) for 1 h and filtered through 0.4 micron 13 mm disc nylon membrane. The OTA in the filtrate was detected and quantified by high performance liquid chromatography. A known toxigenic strain of *A. ochraceus* (NRRL 3519, obtained from the culture collection of Nestle Research Center, Lausanne, Switzerland) was also cultured in a similar manner as a control on both YES and CMEA medium.

The HPLC system consisted of a Shimadzu-LC 10A model set at 330 nm excitation adn 470 nm emission. The HPLC was fitted with Spherisorb ODS II (5 micron 250 mm × 4.6 mm internal diameter) connected to a pre-column ODS Hypersil (5 micron 25 mm × 4.6 mm internal diameter). The mobile phase consisted of 45% acetonitrile-55% 4 mM sodium acetate/acetic acid (19:1). The separation was performed at ambient temperature and the flow rate was 1 ml/min. OTA (Sigma grade) calibration curve (1, 5, 10, 20, 40, 80, 100 μg kg^{-1} was constructed by plotting the peak area of the standard against the corresponding amount of analyte in an injection volume of 20 μl. The calibration curve was linear (r^2 = 0.999) over this range. When the peak area of the samples exceeds the peak area of the highest concentration of OTA calibration standard, the sample was diluted appropriately and re-injected. The results were expressed in ng of OTA g^{-1} of culture.

RESULTS

Ten coffee bean samples from 2006-07 harvest seasons were examined for total mould infection by direct plating method. The data in Table 1 revealed that all the ten samples analyzed had mould infection and notably higher percentage of mould infection in robusta (78-84%) compared to arabica (62-76%) samples. Samples of arabica showed a low incidence of *A. ochraceus* (9 isolates and *A. niger* (60 isolates) infection compared to robusta samples which had 27 and 159 isolates of *A. ochraceus* and *A. niger* respectively. In total, 255 Aspergilli isolates were obtained from 10 different coffee bean samples and of which 36 were *ochraceus* and the remaining (219) were *niger* isolates. It is clear from the results shown in Table 2 that 30 (83%) out of 36 *ochraceus* isolates tested were found to be toxigenic and 11 (31%) of 35 *niger* isolates showed positive for OTA production.

Table 1 : Per cent infection of coffee bean by moulds and frequency occurrence of *Aspergillus* species

Region	Variety	% infection*	Total number of colonies	
			A.ochracues	*A. niver*
Chikmagalur	Arabica	68	04	15
	Robusta	78	07	32
Hassan	Arabica	62	02	12
	Robusta	82	07	29
Coorg	Arabica	76	01	12
	Robusta	82	05	35
Waynad	Robusta	84	05	36
Palkad	Robusta	84	03	27
Dindigul	Arabica	66	01	12
Salem	Arabica	66	01	09
		Total	36	219

*A total of 50 beans were examined for each sample.

The data in Table 3 indicated that the varying level of ochratoxin A production by *Aspergilli* isolates (*niger* and *ochraceus*) in two different media. In all isolates, the toxin production was comparatively higher in synthetic media (YES medium) than the natural substrate (CMEA medium). Among the thiety *ochraceus* isolates screened, the *ochraceus* obtained from a robusta coffee sampled at Waynad district in Kerala state (WAYROB,Och.St.5) showed the highest toxin production of 122 and 41 μg kg^{-1} in YES and CMEA medium respectively. Similarly, the *niger* isolate obtained from arabica coffee in Salem region of Tamilnadu state (SALARA.Nig.St.2) presented the maximum production of 12 and 6.2 μg kg^{-1} of OTA in YES and CMEA medium respectively. Only 5 out of 30 *ochraceus* isolates (1 from arabica and 4 from robusta coffees) showed OTA production above 40 μg kg^{-1} and only one *niger* isolate exhibited OTA production above 10 μg kg^{-1} level.

DISCUSSION

It was apparent from the result shown in Table 1 that robusta samples recorded higher mould infection (74-84%) than those observed in arabica (62-76%) samples. This observation agree with the findings of Panneerselavm *et al.* (2000) and Ngabirano *et al.*, (2001) who reported similar results. Further, the types of moulds recorded in coffee beans analyzed under this study (apart from *Aspergillus* species)

Table 2 : Percentage of isolates of *Aspergillus* species with ochratoxigenic potential.

Species	Variety	No. of Samples*	Total No. of isolates recorded	Total No. of isolated tested	% of toxigenic isolates
Aspergillus ochraceus	Arabica	5	9	9	78
	Robusta	5	27	27	85
	Total	36			
*Aspergillus niger***	Arabica	5	60	17	24
	Robusta	5	159	18	39
	Total		219		

*A total of 50 beans were examined for each sample.

** A total of 219 isolates of *A. niger* were recorded. In view of the large number of isolates, only 17 isolates from arabica and 18 isolates from robusta varieties representing all the districts were tested for their toxigenic potential.

were *Yeast*, *Penicillum*, *Fusarium*, *Cladoporium*, *Alternaria*, *Wallemia* and *Aureobasidium* (data were not shown) and this data is in good agreement with the previous reports by Mislivec *et. al.* (1983) ; Micco *et al.* (1989) and Pardo *et al.* (2004) who recorded similar types of moulds in gree coffee from other sources. Of the 255 Aspergilli isolates obtained from 10 samples, 86% (219 isolates) were *niger* and 14% (36 isolates) were *ochraceus*.

Taniwaki *et. al.* (2003) isolated 872 Aspergilli strains from a total of 408 Brazilian coffee samples and of which 63% (549 strains) were *niger* and 31% (269 isolates) were *ochraceus*. The high occurrence of *niger* (86%) isolates as evident in Table 1 could probably due to widespread occurrence of *niger* in tropical climate (prevailing in a country like India) both in fields and in stored foods and also by the fact that their black colored spores apparently give protection from sun light and UV light providing a

Table 3 : Comparison of OTA production in different media by isolates of *Aspergillus* species

Isolates	OTA Production (ng g^{-1})		Isolates	OTA Production (ng g^{-1} of culture)	
	YES Medium	CMEA Medium		YES Medium	CMEA Medium
Reference *A.ochraceus* strain (NRRL 3519)122.0 ± 3.0	36 ± 1.33				
I. *Aspergillus ochraceus*					
CHKARA.Och.St.1	2.55 ± 0.35	0.75 ± 0.21	WAYROB.Och.St.1	38.1 ± 0.56	18.95 ± 0.91
CHKARA.Och.St.3	5.25 ± 0.49	1.80 ± 0.42	WAYROB.Och.St.2	16 ± 0.56	10.50 ± 0.98
CHKARA.Och.St.4	7.10 ± 0.28	2.70 ± 0.21	WAYROB.Och.St.3	90.5 ± 0.98	42.4 ± 1.13
HASARA.Och.St.1	46.15 ± 0.77	15.0 ± 0.56	WAYROB.Och.St.4	5.7 ± 0.56	2.20 ± 0.42
HASARA.Och.St.2	8.75 ± 0.49	5.30 ± 1.13	WAYROB.Och.St.5	121.9 ± 0.98	40.50 ± 2.12
DINARA.Och.St.1	6.95 ± 0.77	3.50 ± 0.56	PALROB.Och.St.1	16.85 ± 0.63	4.90 ± 0.56
SALARA.Och.St.1	9.60 ± 0.42	5.05 ± 0.49	PALROB.Och.St.2	21.95 ± 0.91	11.75 ± 0.91
CHKROB.Och.St.1	12.75 ± 0.49	7.10 ± 0.56	PALROB.Och.St.3	76.35 ± 1.06	32.30 ± 1.27
CHKROB.Och.St.3	5.80 ± 0.50	2.90 ± 0.42			
CHKROB.Och.St.4	7.20 ± 0.42	4.10 ± 0.56	II. *Aspergillus niger*		
CHKROB.Och.St.5	65.2 ± 0.84	24.90 ± 1.13	CHKARA.Nig.St.1	1.40 ± 0.42	0.65 ± 0.21
CHKROB.Och.St.7	32.60 ± 0.71	13.30 ± 0.56	HASARA.Nig.St.2	4.30 ± 0.85	1.70 ± 0.28
HASROB.Och.St.1	9.05 ± 0.49	4.15 ± 0.77	DINARA.Nig.St.11	0.8 ± 0.42	0.55 ± 0.21
HASROB.Och.St.2	12.65 ± 0.63	6.20 ± 0.84	SALARA.Nig.St.2	12.0 ± 0.98	6.15 ± 1.06
HASROB.Och.St.3	9.35 ± 0.63	5.20 ± 0.56			
HASROB.Och.St.4	16.15 ± 0.49	7.85 ± 0.63	CHKROB.Nig.St.12	1.40 ± 0.28	0.65 ± 0.35
HASROB.Och.St.6	16.15 ± 0.77	6.0 ± 0.56	HASROB.Nig.St.23	6.15 ± 0.91	1.90 ± 0.57
HASROB.Och.St.7	12.95 ± 0.49	7.03 ± 0.84	COOROB.Nig.St.19	1.50 ± 0.57	0.80 ± 0.42
COOROB.Och.St.1	1.9 ± 0.56	1.10 ± 0.28	WAYROB.Nig.St.15	4.05 ± 0.92	1.10 ± 0.28
COOROB.Och.St.2	3.75 ± 0.77	1.05 ± 0.35	WAYROB.Nig.St.19	8.00 ± 0.57	3.05 ± 0.78
COOROB.Och.St.3	14.95 ± 0.49	6.10 ± 0.56	WAYROB.Nig.St.23	1.85 ± 0.64	1.20 ± 0.42
COOROB.Och.St.4	21.75 ± 0.77	15.0 ± 0.98	PALROB.Nig.St.27	2.35 ± 0.64	0.85 ± 0.35

Each value is mean ± stgandard deviation of three determinations.

competitive advantage in such habitats (Pitt and Hocking 1997). No *Aspergillus carbonarius* was found in the coffee samples tested in the present study.

As could be seen from the results shown in Table 2 that 78% and 85% of *ochraceus* and 24% & 39% of *niger* isolates from arabica and robusta coffees respectively were ochratoxigenic indicating *ochraceus* isolates are the principal OTA producing species in coffee. In the present study, the overall percentage of *ochraceus* isolates producing OTA (83%) is slightly higher compared to the reported frequency of 75% by Taniwaki *et al.* (2003). However, the observed percentage of ochratoxigenic *niger* isolates recorded in this study is much higher (31%) than previously reported levels of 7% by Nakajima *et al.* (1997) and 3% by Taniwaki *et al.* (2003).The OTA positive isolates were evaluated for their toxigenic potential in two different medium viz, YES and CMEA and the results (Table 3) evidently represented that OTA production was significantly more in case of synthetic medium compared to natural substrate. This could be due to the physical and chemical nature of the medium. The extrapolation of results from synthetic medium to natural substrate may not be appropriate and hence isolates were parallely tested with coffee based medium.

The range of OTA production by the *ochraceus* isolates from robusta coffee was wider from 1.9 to 122 μg kg^{-1} and this data do not corroborate with earlier findings of the Mantle and Chow (2000) who reported a much higher level of OTA production of 3.7 and 30.5 mg kg^{-1} by two Indian *ochraceus* strains from air and coffee bean respectively when grown on coffee based medium. Similarly, the range of OTA production by *niger* isolates from arabica coffee were slightly higher starting from 0.8 to 12 μg kg^{-1} & 0.5 to 6.15 μg kg^{-1} compared to 1.4 to 8 μg kg^{-1} & 0.65 to 3 μg kg^{-1} of OTA by those *niger* isolates from robusta coffee in YES and CMEA media respectively. Nakajima *et al.*, (1997) reported 2 out of 30 *niger* isolates produced OTA ranging from 0.08 to 0.56 μg kg^{-1} and Suarez-Quiroz *et al.*, (2004) demonstrated *niger* from Mexicon coffee showed OTA production ranging from 2.2 to 9.8 μg kg^{-1} on coffee based medium. A comparison of data on toxigenic levels reported from *ochraceus* and *niger* species in the literature and also from this study indicated there is a tremendous intraspecific variation in OTA production by the Aspergilli species. As with aflatoxins, this variation could be a combination of phenotypic plasticity and genetic variation. Though the toxigenic potential is mainly determined by inherent ability of the organism to produce OTA, the organism is also dependent on the interplay of several environmental conditions such as moisture level. relative humidity, temperature and types of substrate for the maximum toxin production.

The *in-vitro* studies on the influence of environmental conditions such as moisture level, relative humidity, temperature, pH and types of growth medium on OTA production by the virulent ochratoxigenic isolates from this study will be taken up as the future line of works.

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