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Survey of Vietnamese coffee beans for the presence of ochratoxigenic Aspergilli

Z. Ilic[1], T. Bui[1], N. Tran-Dinh[1,4], M.H.V. Dang[3], I. Kennedy[2] & D. Carter[1]
[1]*School of Molecular and Microbial Biosciences, University of Sydney, NSW, 2006, Australia;* [2]*Department of Agricultural Chemistry and Soil Science, University of Sydney, NSW, 2006, Australia;* [3]*Post-Harvest Technology Institute, Ho Chi Minh City, Vietnam;* [4] *Present Address: Food Science Australia, CSIRO, North Ryde, Sydney, Australia*

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Abstract

Vietnamese coffee beans were investigated for the presence of ochratoxigenic Aspergilli. Ninety-three percent of the coffee samples studied were positive for *A. niger*. No other ochratoxigenic species were present. HPLC analysis determined that 8.7% of the *A. niger* strains were positive for ochratoxin A (OA) production. There was no significant difference in the level of contamination or incidence of toxigenic strains in samples that had been rejected by manual sorting and those that were destined for human consumption. No OA-producing fungi were uncovered in a fresh coffee bean sample analysed, suggesting that the OA problem most likely occurs post-harvest.

Key words: *Aspergillus* species, black Aspergilli, coffee, ochratoxin A, Vietnam

Introduction

Ochratoxin A (OA) is an emerging problem in coffee production worldwide. In the past decade, importer coffee companies and researchers have taken a greater interest in the mycological quality of green coffee beans to estimate the risk to humans posed by this mycotoxin. Low levels of OA in foods are not considered to pose a risk to health, however, at high levels OA is considered to be a potential carcinogen [1–3]. Low levels of OA have been found in coffee produced from a wide range of different countries [4, 5], and some level of human exposure appears inevitable.

Coffee is grown in the wide tropical belt surrounding the equator between the tropics of Cancer and Capricorn [6], and in the past decade, Vietnam has become a major coffee producer. Vietnamese coffee is exported world-wide, especially to the USA, and EU countries such as Germany, Great Britain, Sweden and France [7]. Coffee and coffee products are also exported to Vietnam's Asian neighbours, and are regularly consumed by the Vietnamese population.

Traditionally, OA production was believed to be restricted to *Penicillium verrucosum* [8, 9] and *Aspergillus ochraceus* [10, 11], with *P. verrucosum* predominating in temperate regions, and *A. ochraceus* producing OA in warmer areas [1]. It now appears that a number of additional *Aspergillus* species can make OA, particularly those belonging to *Aspergillus* Section *Nigri*: *A. awamori*, *A. foetidus*, *A. niger*, *A. carbonarius*, *A. lacticoffeatus* and *A. sclerotioniger* [12–19], as well as those belonging to *Aspergillus* section *Circumdati: A. cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. steynii*, *A. sulphurous and A. westerdijkiae* [20]. The black Aspergilli are found in many ecological niches within the food supply, both as mild pathogens on fresh fruit and vegetables and in

stored foods [9]. *A. carbonarius* and *A. niger* are able to grow across a wide temperature range (6–47°C) [21], and have a relatively low water activity limit for growth (*A. carbonarius* = 0.88 a_w [22] and *A. niger* is 0.78 a_w [23]) making them particularly important contaminants on dried food stuffs [24].

The potential for certain agricultural commodities to be infected by mycotoxigenic fungi before and after harvest is a well recognised problem [25, 26]. It is not currently known at which point during coffee growth, harvest and processing most mycotoxin is produced, however, it is likely that levels increase when drying and storage are inadequate. Urbano et al. [27] suggested that the fungal contamination found in Brazilian coffee and an associated OA problem was due to faults in harvesting and storage practices. As a tropical country, it is likely that environmental conditions in Vietnam are frequently conducive to the production of mycotoxins, including OA. A recent study of coffee samples from a range of producing countries found Vietnamese coffee to have the highest level of OA contamination, along with the highest percentage of defective beans [28]. The current study aimed to survey Vietnamese coffee beans for infection by ochratoxigenic *Aspergillus* species, in particular the black Aspergilli.

Materials and methods

Sampling of coffee beans from Vietnam

The coffee bean samples used in this study are listed in Table 1. The field survey was conducted between 1st March and 8th March 2000. Samples were collected from the Central Highlands region of Vietnam, with the majority originating from Dac Lac Province, in particular Buon Ma Thuot and surrounding areas. One dried coffee bean sample was collected from Gia Lai Province, bordering Dac Lac in the north.

The majority of the coffee beans were collected from farms and had been harvested in the previous growing season. Coffee samples were also acquired from markets, stores and homes, and suppliers provided details on the area from which they had sourced the beans. All coffee samples were stored in plastic zip-lock bags or in paper bags at 4°C. In some cases the coffee beans had been sorted by the producer and were categorised as ''clean'': no visible mould; approved for human consumption and ''rejected'': visible mould or insect damage apparent; not destined for human consumption.

Isolation and identification of black Aspergilli from coffee beans

Isolation of *A. carbonarius* and *A. niger* from coffee bean samples was performed by direct plating onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar [9, 29], after surface disinfection. Twenty coffee beans were examined from each coffee sample. Coffee beans were surface disinfected by washing in 8% bleach for 2 min, followed by double distilled water rinsing. Strains of *A. carbonarius* and *A. niger* were recognised by their distinct dark brown to black colouration of conidia [9, 30].

Coffee beans were also plated onto Dichloran 18% Glycerol (DG18) agar [31] to determine if *A. ochraceus* was present. *Aspergillus ochraceus* colonies are not densely sporulating and grow on DG18 as pale to light yellow or amber yellow colonies [30]. Strains were identified macroscopically and microscopically following subculturing on Czapek Yeast extract Agar (CYA) media and incubation at 30°C for 7 days [30].

Extraction and screening for OA by HPLC

Analysis of *A. niger* isolates for OA by HPLC was based on the method of Bragulat et al. [32]. All isolates identified as *Aspergillus* were subcultured onto Yeast Extract Sucrose (YES) agar. Plates were incubated for 14 days at 25°C. Three agar plugs (1 cm^3) were removed from the central area of the colony, placed in a small vial. Methanol (0.5 ml) was added, and the vial was shaken vigorously and incubated at room temperature for 60 min. Extracts were filtered (Millex HV 13 mm, Millipore) and a total volume of 100 μl of each extract was analysed, using an LKB 2248 HPLC Pump with a LKB LCC 2252 Controller (Optimize Technologies, Oregon City, USA), equipped with a fluorescence detector, (model RF-10AXL, Shimadzu, Japan) (excitation 340 nm, emission 470 nm) and a C18 column (SphereClone ODS 2250 X 4.6 mm, 5 μm; Phenomenex, Torrance, USA). The mobile phase was pumped at 1.0 ml/

Table 1. Number of ochratoxin A producing strains among total number of *A. niger* strains isolated from coffee beans sampled from various coffee-growing regions of Vietnam

Coffee sample	No. *A. niger* strains isolated	No. OA-producing strains	Region	Description
V1	3	2	Dac Lac, Cu M'Ga	Dried beans
V3	3	0	Dac Lac, Cu M'Ga	Dried beans
V5	15	3	Dac Lac, Cu M'Ga	Dried beans
V6	5	1	Dac Lac, Cu M'Ga	Dried beans
V9	6	0	Dac Lac, BMT	Clean beans
V10	4	1	Dac Lac, BMT	Sorted rejected sample
V13	5	0	Dac Lac, BMT	Dried beans
V15	1	0	Dac Lac, Krong Pak	Dried beans
V16	0	0	Dac Lac, Krong Pak	Freshly picked beans
V17	10	1	Dac Lac, Krong Pak	Dried beans
V19	6	0	Dac Lac, Krong Pak	Dried beans
V21	7	0	Dac Lac, BMT	Sorted rejected sample
V22	5	0	Dac Lac, Hiep Phuc	Sorted clean sample
V23	19	0	Dac Lac, Dakmil	Sorted clean sample
V25	3	0	Gia Lai	Dried beans

min and consisted of an isocratic program of 57% acetonitrile, 41% water and 2% acetic acid.

Statistical analysis of OA production data

Fisher's Exact test and the χ^2-test were used to assess whether significant differences occurred among the different coffee samples. These were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, USA,). Spearman's correlation, calculated by MiniTab, version 11.21, was used to determine if there was a relationship between the number of strains isolated and the number of toxigenic strains isolated among the different samples.

Results and discussion

Vietnamese coffee beans were surveyed in an effort to determine the cause of OA contamination that has been reported in Vietnamese coffee [28]. Ochratoxigenic *Aspergillus* species found to date on coffee samples include *A. ochraceus*, *A. niger*, *A. carbonarius*, *A. lacticoffeatus* and *A. sclerotioniger*, which are distinguishable by morphological techniques [19]. *Aspergillus niger* was the only ochratoxigenic species found in the current study, and it was present in 14 out of the 15 (93.3%) coffee bean samples analysed. This differs from other studies, where *A. niger* has only ever been reported to co-occur in coffee with the more commonly encountered OA-producing *Aspergillus* species, *A. ochraceus* and *A. carbonarius* [6, 27, 33], although the small sample size might be responsible for the failure to detect other species. There are few published data on the mycoflora associated with food crops in Vietnam, and it is not known if *A. ochraceus* and *A. carbonarius* are commonly encountered on other food products. *Aspergillus* species produce prolific asexual conidia and are often regarded as ubiquitous and cosmopolitan, however geographic partitioning of some species has been found. For example, an extensive survey of peanuts, corn and associated soils from Vietnam found that although *A. flavus* was present at high levels, *A. parasiticus*, which is common in peanuts and soils from the USA and Australia, was entirely absent [34]. Local soil, resident microbiota and climatic conditions may all influence colonisation and persistence by fungal species and may restrict or prevent the growth of certain fungal species.

Qualitative analysis for OA production by HPLC revealed eight of the 92 (8.7%) *A. niger* strains to be ochratoxigenic, and these occurred in five of the 15 coffee bean samples (Table 1). The eight OA-producing *A. niger* strains had much smaller chromatogram peaks but had identical HPLC retention times (8 min 15 s) compared to the OA standard. Although the incidence of OA-producing *A. niger* in Vietnamese coffee bean samples appeared low, the fact that they were the only species of ochratoxigenic fungi isolated, together with reports of OA in some Vietnamese coffee

samples [28], suggests that under the right conditions they might cause OA contamination. A variety of factors can contribute to the production of OA by a given strain. The nature of the substrate is an important consideration, along with temperature variation and drought stress, and a strain may not necessarily produce the mycotoxin very abundantly on laboratory media compared to production in the coffee bean in the field or in storage.

The high incidence of *A. niger* contamination and lack of other OA-producing species could be indicative of prevailing climatic conditions at the time of sampling and may be subject to seasonal variation. In addition, environmental variation throughout the year and from year to year may change the levels of OA contamination by the resident fungi. Substantial variation between years was seen in Brazilian coffee beans, where there was an increase in OA levels detected in 1999 compared to 1998, especially in beans collected in the North of Parana [27]. This coincided with a rainy period during sampling, which may have allowed greater infection and development of fungi, with consequently higher levels of OA produced. Ongoing sampling will help assess how vulnerable Vietnamese coffee beans are to OA contamination throughout seasonal and annual fluctuations.

Statistical analysis revealed no significant correlation between the number of strains infecting a coffee sample and the number of toxigenic strains isolated ($P > 0.05$). Further, no significant difference in infection levels was found between the regions of Cu M'Ga, Buon Ma Thuot, and Krong Pak ($P = 0.791$), and likewise there was no significant difference in levels of OA-producing *A. niger* between these regions ($P = 0.086$). Therefore, a relatively high infection rate for a particular coffee bean sample might not necessarily correlate with a high incidence of OA-producing species. Urbano et al. [27] also found this lack of correlation with Brazilian coffee samples. The overall incidence of OA-producing species of *A. niger* found in Vietnamese coffee beans (8.7%) is consistent with other studies, which found 6.6% and 11.5% of *A. niger* strains contaminating coffee beans to be OA-producers [27, 35]. Studies of *A. niger* strains derived from other sources vary far more markedly, however, with the proportion of strains reported to produce OA ranging from 1.7% in Australian dried vine fruits [16], to 41.3% in Argentinian wine grapes [36] and 52% in animal feed [37]. This variability could be attributed to host commodity, geographic distribution, or the sensitivity of toxin assays employed.

Statistical analysis showed no significant difference between *A. niger* levels in sorted "clean" samples and "rejected" samples ($P > 0.05$) (Table 1). In fact, coffee bean sample V23, showing highest *A. niger* contamination, was rated to be "clean" and would have been destined for human consumption. Coffee bean sample V16, which was the only freshly picked sample used in this study, had no fungal contamination of any kind. Consistent with this, low levels of OA and ochratoxigenic fungi have been found in Brazilian coffee cherries and green coffee samples, indicating that the OA problem develops post-harvest during processing and storage [27, 38]. It would be interesting to sample future fresh coffee beans to determine if they are normally free of contamination; if so the high incidence of *A. niger* observed in dried Vietnamese coffee beans indicates a need for improvements to processing and storage.

Although the levels detected are low, OA-contaminated coffee is a growing concern in Vietnam for both health and economic reasons. Exposure of the local population to OA through the consumption of OA-contaminated coffee is a potential health risk, and restrictions on the exportation of OA-contaminated coffee could affect the economy of this coffee-producing nation. This is important in light of the recent move by the European Commission (October, 2004) to establish a maximum level for OA in roasted and ground coffee beans of 5 μg/kg and in soluble coffee of 10 μg/kg. This survey provides strong evidence implicating *A. niger* in the OA contamination of Vietnamese coffee. Detailed investigation of a larger number of coffee bean samples over a number of seasons, together with a quantitative analysis of OA-producing strains are required to support this finding. Vietnamese coffee may face an increased risk of mycotoxin contamination as current practices during harvest and post-harvest do not prevent mould growth.

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Address for correspondence: D. Carter, School of Molecular and Microbial Biosciences, University of Sydney, NSW, 2006, Australia
Phone: +61-2-93515383; Fax: +61-2-93514571
E-mail: d.carter@mmb.usyd.edu.au