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Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries

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Abstract

Robusta coffee cherries collected before and during sun drying from two coffee farms in Thailand were examined for moulds producing ochratoxin A (OA). *Aspergillus ochraceus* was only detected in one sample, whereas *Aspergillus carbonarius* was isolated from 7 out of 14 samples. On γ-irradiated coffee cherries, each of the six tested *A. carbonarius* strains produced OA. More than 4800 μg kg^{-1} of toxin were detected under optimal conditions (25°C, a_w 0.99). OA production was strongly reduced (230 μg kg^{-1}) at an a_w of 0.94. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Coffee; Ochratoxin A; *Aspergillus carbonarius*; Drying

1. Introduction

Ochratoxin A (OA) is a nephrotoxic and nephrocarcinogenic mycotoxin that has been detected in a variety of food products, including cereals, nuts, green coffee beans, cocoa beans, dried fruits, spices, wine and beer, as well as in kidneys from pigs fed rations with OA. The natural occurrence of OA in green coffee beans has been reported by several authors in concentrations ranging between 0.2 and 360 μg kg^{-1}, but considerable inconsistencies are found in the literature regarding the influence of the roasting and brewing processes on the OA content of coffee (Pittet et al., 1996). A recent study has shown that more than 80% of the OA originally present in green coffee beans is destroyed during soluble coffee manufacture under industrial conditions (Blanc et al., 1998). It is estimated that one cup of soluble coffee contains on average 2.5 ng OA (Stegen et al., 1997), which means that consumption of 28 cups per week by a 60-kg person would correspond to ca. 1% of the Provisional Tolerable Weekly Intake established by the WHO/FAO Joint Expert Committee on Food Additives (JECFA) (WHO, 1996).

Very little is known about the origin of OA contamination in green coffee (Mantle, 1998). Recent field and experimental data collected in Thailand demonstrated that OA is mainly produced during Robusta coffee cherry drying (Bucheli et al.,

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2000), and not during industrial Robusta green coffee storage under tropical conditions (Bucheli et al., 1998). In order to protect coffee cherries from OA formation, it is paramount to identify the moulds that are capable of producing this mycotoxin. For years it was assumed that *Aspergillus ochraceus* was the main culprit. It was indeed demonstrated that this species is capable of producing limited amounts of OA in green coffee (Levi et al., 1974; Studer-Rohr et al., 1995; Taniwaki et al., 2000), but as *A. ochraceus* is not very frequently encountered in the tropics (Pitt et al., 1993, 1994), its true significance is questionable.

Recently, it was suggested that *Aspergillus niger* and closely related species could be more relevant in this respect, because they are more prevalent and because OA production is a rather common trait for this group, particularly for *Aspergillus carbonarius* (Téren et al., 1996; Heenan et al., 1998).

To examine this possibility, we investigated the mycoflora of fresh and drying coffee cherries from two farms in the south of Thailand. Isolated *A. carbonarius* strains were screened for OA production on coconut cream agar (Dyer and McCammon, 1994; Heenan et al., 1998) and challenge tests were performed with selected strains on γ-irradiated coffee cherries to investigate the influence of several environmental parameters on OA production.

2. Materials and methods

2.1. Samples

Coffee cherries (*Coffea canephora* var. *robusta*) were collected before and during sun drying (after 5 and 10 days) in 200-g aliquots from two farms located ca. 70 km from each other in the Chumphon province the south of Thailand (Khaoyao district, Ampor Muang, and Khaothalu district, Ampor Sawi, respectively). The samples were frozen before shipment to the Nestlé Research Center (NRC) in Switzerland for mycological analysis, challenge tests and OA analysis.

2.2. Fungi

Thirty strains of *A. carbonarius* were isolated from coffee cherries during this study (Table 1). In addition *A. carbonarius* CBS 110.49 and CBS 127.49 (isolated from coffee and air, respectively) were obtained from the Centraalbureau voor Schim-

Table 1
Growth and OA production of *Aspergilli* on coconut cream agar and coffee cherries

Species	Strain	Source	Coconut cream agar		Coffee cherries	
			Growth[a]	Fluorescence[b]	Growth[a]	OA (μg kg^{-1})
A. ochraceus	M75	Coffee, Brazil	++	++	+	nd[c]
A. ochraceus	M337[d]	Coffee, Thailand	++	−	nt[e]	nt[e]
A. carbonarius	M323[d]	Coffee, Thailand	++	+	++	280
A. carbonarius	M324[d]	Coffee, Thailand	++	+	++	650
A. carbonarius	M325[d]	Apples	++	−	−	nd
A. carbonarius	M326[d]	Tomatoes	++	−	++	115
A. carbonarius	CBS 110.49	Coffee	+/−	−	+	3
A. carbonarius	CBS 127.49	Air	++	−	++	410
A. carbonarius	M333	Coffee, Thailand	++	+	++	930
A. carbonarius	M334	Coffee, Thailand	++	+	++	180
A. carbonarius	M335	Coffee, Thailand	++	+	++	680
A. carbonarius	M336	Coffee, Thailand	++	+	++	920

[a] ++: good growth, +: moderate growth, +/−: slight growth, −: no growth.
[b] ++: strong fluorescence, +: moderate fluorescence, −: no fluorescence.
[c] nd: not detected (< 2 μg kg^{-1}).
[d] Identity confirmed by Centraalbureau voor Schimmelcultures, Netherlands.
[e] nt: not tested.

melcultures in Baarn, Netherlands. *A. carbonarius* M325 and M326 (isolated from apples and tomatoes, respectively) and *A. ochraceus* M75 (isolated from green coffee from Brazil) came from the Nestlé Research Centre culture collection.

2.3. Mycoflora determination

Coffee cherries collected before and during sun drying (20 g) were suspended in 180 ml diluent (0.85% sodium chloride + 0.1% tryptone) and homogenized with a Waring blender for 1 min. Dilutions were plated on DG18 agar (Oxoid CM 729). After incubation (5–7 days at 25°C), predominant moulds were identified according to Pitt and Hocking (1997).

Colonies recognised as belonging to *Aspergillus* section *Nigri* were isolated on Malt Extract Agar (Oxoid CM 59) and identified according to Raper and Fennel (1965). To confirm the identity of presumptive *A. carbonarius* and *A. ochraceus* strains, some isolates were sent to the Centraalbureau voor Schimmelcultures, Baarn, Netherlands for determination.

2.4. Screening of fungi for ability to produce ochratoxin

Selected strains were single point inoculated on coconut cream agar (CCA) (Dyer and McCammon, 1994). After incubation for 7 days at 30°C, the plates were examined for OA production according to Heenan et al. (1998).

2.5. Ochratoxin A production on coffee cherries

2.5.1. Gamma sterilization of the coffee cherries and adjustment of the water activity

Ripe coffee cherries, originating from one of the coffee farms in Thailand, were sterilized by means of γ-irradiation (10 kGy) using a ^{60}Co source (Swiss Federal Institute of Technology, Physical Chemistry Department, Lausanne, Switzerland). To adjust the water activity the sterilized cherries were transferred to desiccators in which the relative humidity (RH) was kept constant by the presence of a beaker containing demineralised water or saturated salt solutions. The following salts were used: KNO_3 (corresponding to ca. 94% RH), $BaCl_2$ (ca. 90% RH) and KCl (ca. 84% RH). To follow the equilibration process, the a_w of the coffee was determined every week with an Aqualab Decagon CX-2T water activity meter (Decagon, Pullman, WA).

2.5.2. Inoculation and incubation

Fungal strains were grown for 14 days at 25°C on Malt Extract Agar (MEA), after which mycelium and conidia were collected from the agar surface with a sterile brush and spread over the surface of 15-g portions of coffee cherries, which had equilibrated to the desired water activity as described above. The inoculated material was subsequently incubated at different temperature and water activity combinations for 2 weeks.

To compare the OA production among different *A. carbonarius* strains, coffee cherries were used, which had not been previously equilibrated to a determined a_w, and the incubation was carried out in Petri dishes sealed with parafilm.

Each of these experiments was performed in duplicate.

2.6. Ochratoxin A analyses

To determine the OA concentration in coffee cherries, test portions were extracted by shaking overnight with 100 ml of methanol: 3% aqueous sodium hydrogen carbonate (50:50). The suspensions were filtered through a Whatman GF/B glass microfibre filter under reduced pressure, and aliquots of filtrate were evaporated to dryness under a stream of nitrogen at 40°C. Dried residues were re-dissolved in 150 μl of a mixture consisting of 45% acetonitrile: 55% 4 mM sodium acetate/acetic acid (19:1), and injected onto a reversed phase HPLC system which consisted of a Waters 600E quaternary gradient pump, a Waters 717 autosampler, and a Waters 474 scanning fluorescence detector (Millipore, Marlborough, USA). All separations were performed under conditions described by Pittet et al. (1996).

3. Results

3.1. Mycoflora and ochratoxin A content of coffee cherries

Table 2 presents the results of the mycological examination of 14 samples of coffee cherries from

Table 2
Mycological examination of Robusta coffee from Thailand

Farm	Viable mould count (CFU g^{-1})	*A. carbonarius* estimated count (CFU g^{-1})	Other moulds encountered (in order of decreasing prevalence)
A	7.6×10^3	10^1–10^2	*Cladosporium* spp., *Aspergillus* section *Nigri*
A	3.6×10^3	nd[a]	*Cladosporium* spp., *Aspergillus* section *Nigri*
A	5.0×10^3	10^1–10^2	*Cladosporium* spp.
A	6.6×10^4	nd	*Cladosporium* spp., *Aspergillus* section *Nigri*
A	6.6×10^5	10^3	*Aspergillus* section *Nigri*, *Fusarium* spp., *Cladosporium* spp., *Penicillium* spp.
A	4.4×10^4	nd	*Aspergillus* section *Nigri*, *Cladosporium* spp., *Aspergillus* section *Flavi*
A	8.8×10^4	10^2–10^3	*Aspergillus* section *Nigri*, *Cladosporium* spp., *Aspergillus* section *Flavi*
B	1.3×10^4	nd	*Aspergillus* section *Nigri*, *Cladosporium* spp., *Penicillium* spp., *Aspergillus* spp., *Aspergillus* section *Circumdati*
B	4.2×10^3	nd	*Cladosporium* spp., *Aspergillus* section *Nigri*, *Fusarium* spp.
B	2.2×10^3	10^2	*Cladosporium* spp., *Aspergillus* section *Nigri*, *Aspergillus* spp.,
B	2.6×10^4	nd	*Cladosporium* spp., *Aspergillus* section *Nigri*, *Aspergillus* section *Flavi*
B	5.2×10^4	10^2–10^3	*Aspergillus* section *Nigri*, *Cladosporium* spp., *Aspergillus* section *Flavi*
B	1.0×10^3	nd	*Aspergillus* section *Nigri*, *Wallemia sebi*, *Cladosporium* spp., *Aspergillus* section *Flavi*, *Eurotium* spp.
B	3.8×10^4	10^2–10^3	*Aspergillus* section *Nigri*, *Penicillium* spp., *Cladosporium* spp., *Aspergillus* section *Flavi*, *Wallemia sebi*

[a] nd: not detected.

two farms in Thailand. Mould counts ranged from 1×10^3 to 6.6×10^5 CFU g^{-1}. In most samples, the mycoflora was dominated by *Cladosporium* spp. and representatives of *Aspergillus* section *Nigri*. Among these, *A. carbonarius* was rather frequently found: strains belonging to this species were isolated from seven samples (50%). Other commonly isolated moulds were *Fusarium* spp., *Penicillium* spp. and *Wallemia sebi.*

Only in one sample a strain belonging to the *Aspergillus* section *Circumdati* was detected, which was identified as *A. ochraceus.*

No OA was detected in the fresh cherries, whereas the samples taken after 5 and 10 days of drying contained 1.7 and 6.9 μg kg^{-1}, respectively.

3.2. Growth and ochratoxin A production on coconut cream agar and coffee cherries

Twelve *Aspergillus* strains, including six *A. carbonarius* and one *A. ochraceus* strain isolated from Thai coffee cherries, were screened for presumptive OA production on CCA. All strains showed good growth, with the exception of *A. carbonarius* CBS 110.49 (Table 1). Seven strains produced fluorescent green blue compounds when examined under UV light, in particular *A. ochraceus* M75, suggesting that OA was formed by most strains.

Growth of the different isolates on coffee cherries was rather variable. *A. ochraceus* M75 showed poor growth and did not produce any detectable OA, but all *A. carbonarius* strains isolated from Thai coffee grew abundantly and produced a significant amount of OA, with the maximum being attained by strain

Table 3
Influence of temperature and water activity on growth and ochratoxin A production by *A. carbonarius* M333 on coffee cherries

Temperature (°C)	a_w	Growth[a]	OA (μg kg^{-1})
25	0.99	++	4810
25	0.94	++	230
25	0.90	+/−	nd[b]
25	0.85	−	nd
20	0.99	++	3380
27	0.99	++	4490
30	0.99	++	2790
35	0.99	+	7
37	0.99	+	nd

[a] ++: good growth, +: moderate growth, +/−: slight growth, −: no growth.
[b] nd: not detected (limit of detection, 2 μg kg^{-1}).

M333 (930 μg kg^{-1}). This strain was therefore used for subsequent experiments in which the influence of temperature and a_w on OA production was investigated (Table 3). It was found that far greater quantities of OA could be produced in coffee cherries previously equilibrated at 100% RH. At the optimal temperature (25°C), 4810 μg kg^{-1} was detected after 2 weeks of incubation. At 20°C and 30°C, OA accumulation was still considerable (3380 and 2790 μg kg^{-1}, respectively), but at 35°C, only 7 μg kg^{-1} was produced. OA production on coffee cherries was much lower at reduced water activities. At 0.94 a_w, 230 μg kg^{-1} was found after 2 weeks, although growth was apparently not inhibited under these conditions. OA production was not detected at 0.90 a_w.

4. Discussion

Although several publications exist on the composition of the mycoflora of coffee (Levi et al., 1974; Betancourt and Frank, 1983; Mislivec et al., 1983), the isolation of *A. carbonarius* from this commodity had not been reported previously. Only in a very recent publication of Taniwaki et al. (2000) the presence of this species on coffee from Brazil was confirmed, but its prevalence in this survey appeared to be low. However, our findings suggest that it may be a rather common contaminant of Thai coffee cherries. It is conceivable that this apparent contradiction could be due to the fact that in most of the previous studies green coffee was examined, which may have a different mycoflora than coffee cherries. An alternative explanation is that *A. carbonarius* is also common in green coffee, but that representatives of this species have previously been identified as *A. niger*. This ubiquitous species is quite similar in appearance, and discrimination between the two species may not have been considered as very relevant until recently when it was demonstrated that OA production is much more common in *A. carbonarius* than in *A. niger* (Heenan et al., 1998).

Our data also show that many of the *A. carbonarius* strains isolated from coffee cherries were able to produce substantial amounts of OA on this substrate and underline the significance of water activity in this respect. At 0.94 a_w, OA production was much less than on cherries kept under an atmosphere saturated with water. For prevention of OA accumulation in coffee cherries this means that the first days of drying are probably critical, which is in agreement with observations made by Bucheli et al. (2000). The temperature also plays a major role. Assuming that the strain with which the experiments were carried out is representative, it would indicate that a temperature of 35°C or higher would reduce OA production during drying.

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