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Ochratoxin formation in *Aspergillus ochraceus* with particular reference to spoilage of coffee

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Abstract

Production of ochratoxin on media by eight isolates of *Aspergillus ochraceus* from coffee or its processing environment in India, Indonesia, Kenya, and Brazil, and seven Brazilian isolates from other commodities, has been compared with yields in shaken fermentation on shredded wheat and coffee (*Coffea arabica*). Shredded wheat most consistently allowed expression of biosynthesis of ochratoxins A and B in yields up to 3.5% of the dry product. Culture on artificial media was an unreliable predictor of ochratoxin yield on both shredded wheat and coffee. Coffee was a relatively poor substrate for ochratoxin production particularly when sterilised. Notably, two Asian coffee isolates produced 400 mg kg^{-1} ochratoxin A on unsterilised ground green coffee, showing this to be a preferred substrate for further experimentation. The study focused on isolates of *A. ochraceus*, which from evidence of culture on media would not be expected to be suitable fungi for future studies to establish both the fact of spoilage of coffee by *A. ochraceus* and the dynamics of ochratoxin formation by isolates of this species.

Keywords: *Aspergillus ochraceus*; Ochratoxin A; Ochratoxin B; Shredded wheat; Coffee

1. Introduction

Recognition of the occurrence of small amounts of ochratoxin A in coffee, through sensitive analytical methodologies, has caused the coffee industry to address the problem partly by analytical quality control and partly by focusing on good practice in bean separation, drying, storage and transportation procedures (Frank, 1999), applicable in principle to all agricultural food commodities subject to post harvest spoilage by fungi. However, there is poor understanding of the dynamics of the occurrence of ochratoxin A in commercial coffee, and also even of the particular fungi involved. As a tropical product the most likely fungi are yellow spored *Aspergillus* species typified by *A. ochraceus*, and the black Aspergilli *A. niger* and *A. carbonarius*. According to the extent of formation of ochratoxin in pure laboratory culture on artificial media (Heenan et al., 1998), the latter species would not appear to be significant sources of ochratoxin A in the field, but this remains to be demonstrated. Literature on *A. ochraceus*

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implies a wide variation in ability of particular isolates to produce ochratoxin A in the laboratory, with absence of production by many isolates (Mantle, 1998). Nothing has been published on the extent to which *A. ochraceus* isolates can produce ochratoxin A in coffee, but the idea that some trace amounts might arise by uptake of the toxin direct into the plant from soil has recently received experimental support (Mantle, 2000). Consequently, the present pilot study concerning direct formation of ochratoxin A in coffee has sought first to explore comparative productivity on shredded wheat of some recently isolated *A. ochraceus* from Brazil, obtained originally for purposes other than studying coffee. Isolates selected from these were then compared with other isolates obtained during a recent broad investigation of ochratoxin A in coffee in Brazil and several other coffee producing countries worldwide (Frank, 1999). Comparison has been made primarily in the shaken shredded wheat fermentation developed for maximum production of ochratoxins by *A. ochraceus* (Harris, 1996). Subsequently, for further study of the coffee isolates, the substrate used was whole or ground green coffee. Shaken solid substrate fermentation was chosen because it prevents superficial mycelial growth on the substrate. Superficial mould growth is likely to produce off-flavours in the coffee, so that in commercial practice a commodity with this spoilage would be rejected at tasting before marketing. However, as the most important practical aspect of ochratoxin biosynthesis in coffee concerns hidden growth of *A. ochraceus* within the bean, it is more important to study the physiology and biochemistry of the fungus–bean interaction.

2. Materials and methods

2.1. Isolates of Aspergillus ochraceus

The following fungi were used in the study: Australian D2306, originally used by Tapia and Seawright (1984) and since by Harris (1996) and Stoev et al. (2000); Brazilian NPMM 1123, 1124, 1157, 1158, 1159 (from black pepper), 1068 (from dried sausage), 1170 (from black beans) (Department of Veterinary Microbiology, Rural Federal University of Rio de Janeiro, SP, Brazil); Brazilian 189, 467, Indian 17, 66, Indonesian 169, 174, 180 and Kenyan 79 (Department of Microbiology, University of Surrey, Guildford, UK).

2.2. Culture on artificial media and in shaken solid substrate fermentation

Cultures were maintained on potato dextrose agar (Difco, Detroit, MI, USA) in Petri dishes at 25°C. Czapek Dox broth (CDYE; Difco) supplemented with yeast extract (0.5% w/v) (Y-4000, Sigma, St. Louis, MO, USA) was dispensed (50 ml) in 500-ml Erlenmeyer flasks and inoculated with dry spores by sedimentation onto the surface. Flasks were incubated at 25°C for 2 weeks.

Shredded wheat breakfast cereal biscuits (Cereal Partners UK, Welwyn, UK) were crushed coarsely and dispensed (40 g) into 500-ml Erlenmeyer flasks. *Coffea arabica* beans (Brazil) or ground beans (Zaire), with an ochratoxin A content of 0.5 $\mu g\ kg^{-1}$ (close to the limit of analysis) were dispensed (40 g) with distilled water (8 ml) into 500-ml Erlenmeyer flasks and placed on a rotary shaker (200 rev./min; 10 cm eccentric throw) for several hours to imbibe the water evenly. Except where stated, flasks were then sterilised at 120°C for 20 min.

Petri dish cultures were flooded with sterile distilled water containing Tween 80 (0.01%), agitated to give a spore suspension and the suspension used to inoculate the solid substrates to a total of 16 ml added water. Shaken solid substrate fermentations lasted for 2 weeks at 29°C.

2.3. Ochratoxin analysis

After fermentation, coffee beans were dried to constant weight at 80°C before milling for extraction. Ground coffee and shredded wheat was sampled directly from the mobile substrate of the shaken flask contents, and in some cases was dried as above. Samples (5 g) were treated with ethyl acetate:0.01 M H_3PO_4 (9:1, 100 ml) overnight in a closed vessel. Solvent was decanted, filtered and 50 ml extracted with 3% $NaHCO_3$ (2×0.5 volume). The combined extract was adjusted to pH 5 with HCl and extracted with ethyl acetate (2×0.5 volume). The combined extract was taken to dryness in vacuo, the residue dissolved in methanol (1 ml) and 20 μl analysed by reversed phase (C_{18}) HPLC with a diode-array

detector (Hewlett Packard 1040 M, Waldbronn, Germany) in acetonitrile:water:acetic acid (40:60:1). UV spectra of peaks attributed to ochratoxins A and B confirmed identity, and ochratoxins were quantified from the integrated signals by comparison with calibration for ochratoxin A.

Cultures on CDYE broth were treated with 3% $NaHCO_3$ (100 ml), homogenised and filtered. Filtrate (20 ml) was acidified with HCl and extracted with ethyl acetate as above. Further analysis followed the above procedure.

3. Results

3.1. Shredded wheat as a substrate for ochratoxin production

Putative indication of production of ochratoxins was shown by a few of the *A. ochraceus* isolates on potato dextrose agar, grown at 25°C for 7–10 days, by the bright blue fluorescence of the medium under UV_{350} irradiation (Table 1). As expected, the Australian isolate D2306 was positive in this respect, as were three from Brazil.

Shaken solid substrate fermentations on shredded wheat supported high yields of ochratoxin A by isolate D2306, but by only two of the seven Brazilian isolates. Yields given in Table 1 were measured at 14 days but isolates D2306 and 1068 had already reached this value within 5 days. Fluorescence of ochratoxins was useful in monitoring efficiency of solvent partition steps in extraction. However, there was poor correlation between fluorescence in potato dextrose agar under UV light and ochratoxin formation in the solid substrate. In isolates 1159 and 1170 fluorescence persisted in the bicarbonate extract against subsequent partition with ethyl acetate and only a trace of ochratoxin A was detected. Ratios of ochratoxin A to ochratoxin B varied (Table 1). Isolate 1123, which also accumulated the most ochratoxin B, gave a fermentation product 3.5% of which was ochratoxins A and B on a dry weight basis. Water content of the chocolate coloured granular product was in the range 47 to 60%. In contrast, six of eight of the coffee isolates produced ochratoxin A on shredded wheat, though in

Table 1
Comparison of ochratoxin production in shredded wheat and coffee by isolates of *Aspergillus ochraceus* in shaken solid substrate fermentation and artificial media[a]

Isolate	Fluorescence on PDA	Sterile shredded wheat		Coffee				CDYE liquid Ochratoxin A	
		Ochratoxin A (mg kg^{-1})	Ratio A:B	Ochratoxin A (mg kg^{-1})					
				Sterile		Nonsterile		Fluorescence	(mg per 50 ml)
				Beans	Ground	Beans	Ground		
D 2306	+ +	7085	7:1	0.6	15			+ +	3.3
1123 Black pepper	+ +	9700	2:1	Trace	1.5			+ +	8.4
1068 Dried sausage	±	1100	11:1	13.5	12.2				
1124 Black pepper	±	Trace							
1157 Black pepper	−	Trace							
1158 Black pepper	−	Trace							
1159 Black pepper	+ +	Trace							
1170 Black beans	+ +	Trace							
169 Bean, Indonesia	−	1400	22:1	0.9	15	8	400	Trace	Trace
66 Air, India	−	700	34:1	Trace	3.7	1.4	460	Trace	Trace
17 Bean, India	−	450	14:1	3.4	30.5			Trace	0.39
180 Bean, Indonesia	−	240	7:1	6.5	Trace			Trace	0.39
189 Bean, Brazil	−	70	22:1	13	26	Trace	3.3	Trace	Trace
79 Air, Kenya	−	4	12:1	Trace	130	Trace	170	Trace	0.12
467 Bean, Brazil	−	Trace		0.9	Trace			Trace	0
174 Bean, Indonesia	−	Trace		1.3	Trace			Trace	0

[a] PDA, potato dextrose agar; CDYE, Czapek Dox yeast extract broth. Trace means < 0.1 mg per 50 ml CDYE broth or < 1 mg kg^{-1} solid substrate.

a wide range of yields (Table 1). The trace amount recorded for the other two coffee isolates was close to the limit of the assay (1 mg kg^{-1}).

3.2. Ochratoxin A production on coffee

Shaken cultures of the fungi on moist coffee beans allowed no obvious superficial growth but, at the end of the fermentation, a few *A. ochraceus* conidiophores were found in each bean, protected in the crease from abrasion during shaken culture. Ground green beans, though having a significant lipid content which might have caused the powder to coalesce, conveniently retained a loose powder consistency during shaken fermentation, but only if the added moisture did not exceed 40%. There was no macroscopic evidence of superficial fungal growth. Only the colour of the ground coffee became darker over the fermentation period, and condensed water in the neck of the culture flask was evidence of respiration attributable to *A. ochraceus*.

Ochratoxin production by all isolates was very poor on sterilised coffee beans, the most extreme contrast with yield on shredded wheat being with the three isolates which gave the highest yields on that substrate. Yields on ground coffee were, for some coffee isolates, a little higher but the Kenyan isolate, which produced the least ochratoxin A on shredded wheat, produced the most on sterile ground coffee.

As sterilisation by wet heat at 120°C might have had deleterious effects on the substrate as a source of fungal nutrition, four coffee isolates from different regions were cultured on unsterilised beans and ground coffee (Table 1). The most notable new finding was a much higher ochratoxin A yield on ground unsterilised coffee by the two Asian isolates, to an order close to that achieved by these isolates on shredded wheat.

3.3. Culture on Czapek Dox/yeast extract broth as an indicator of potential for ochratoxin formation

The Australian isolate D2306 and the best Brazilian isolate 1123 both yielded several milligrams of ochratoxin A during stationary culture on 50 ml CDYE broth (Table 1), a yield roughly comparable, relative to available nutrients, to that on shredded wheat. As expected, the broth was highly fluorescent. Only three of the coffee isolates tested gave a significant yield of ochratoxin A on this rich medium but the yield was not matched by any obvious fluorescence in the broth. Notably, the two coffee isolates giving the best yields on shredded wheat did not produce significantly on CDYE broth. Ochratoxin A was not detected by HPLC analysis of extracts from the two other isolates, Brazilian 467 and Indonesian 174.

4. Discussion

In spite of the limited scope of the present pilot study, several principles become clearer and new considerations arise.

Shaken solid substrate fermentation, using sterilised shredded wheat, seems to allow more reliable expression of ochratoxin formation by isolates of *A. ochraceus* than the other liquid media or solid substrates used. Fluorescence of cultures on agar media can be misleading, as found by Mantle and McHugh (1993) for some isolates from beans for human food grown in the USA.

Green coffee, whether as whole beans or ground to provide an increased surface area to volume ratio, is generally a poor substrate for *A. ochraceus* in conditions of shaken fermentation designed to limit superficial moulding and force the fungus to penetrate into the tissue so as to mimic moulding restricted to moist bean endosperm.

Unsterilised ground coffee seemed to be a better substrate than coffee sterilised by standard autoclave procedures. It is possible that, although there could be no overt evidence of other fungi during the unsterile shaken fermentation, the very common *Fusarium stilboides* component of mycobiota within coffee beans (Frank, 1999) may be a facilitating factor. The refractory carbohydrate of the coffee bean's endosperm presents more of a challenge to fungi than does, for example, the starch of wheat and therefore a complex mycobiota may be necessary for significant toxigenic spoilage by *A. ochraceus*. This possibility requires further study because the general nature of the mycobiota in coffee could be a predisposing or facilitating factor in ochratoxin formation by particular Aspergilli.

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References

Frank, M., 1999. HACCP and its mycotoxin control potential: an evaluation of ochratoxin A in coffee production. In: Paper presented at 3rd FAO/WHO/UNEP International Conference on Mycotoxins, Tunis, Algeria.

Harris, J.P., 1996. The Biosynthesis of Ochratoxin A and other Structurally Related Polyketides by *Aspergillus ochraceus*, University of London, London, Ph.D. Thesis.

Heenan, C.N., Shaw, K.J., Pitt, J.I., 1998. Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. J. Food Mycol. 1, 67–72.

Mantle, P.G., 1998. Ochratoxin A in coffee. J. Food Mycol. 1, 63–65.

Mantle, P.G., 2000. Uptake of radiolabelled ochratoxin A from soil by coffee plants. Phytochemistry 53, 377–378.

Mantle, P.G., McHugh, K.M., 1993. Nephrotoxic fungi in foods from nephropathy households in Bulgaria. Mycol. Res. 97, 205–212.

Stoev, S.D., Goundasheva, D., Mirtcheva, T., Mantle, P.G., 2000. Susceptibility to secondary bacterial infections in growing pigs as an early response in ochratoxicosis. Exp. Toxicol. Pathol., in press.

Tapia, M.O., Seawright, A.A., 1984. Experimental ochratoxicosis in pigs. Aust. Vet. J. 61, 219–222.