Biological Resource Centres and the Use of Microbes

Edited by

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Mycotoxins – the experience and expertise of Micoteca da Universidade do Minho

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Summary

A review is made of the work on mycotoxins of current researchers at Microteca da Universidade do Minho. Existing standard methods of screening for total secondary mycotoxins are recommended. The effect of preservation on detection is discussed. Ochratoxin A (OTA) and patulin were detected from fungi from grapes. The influences of vineyard region are discussed. *Aspergillus* biodegradation products of OTA were OTα and other compounds. The same fungus strain produced and degraded OTA. Removal of OTA from liquid to solid occurred during *in vitro* studies on OTA-spiked and pulped grapes. The novel original reports of (a) metabolites detected from dried herbarium samples, (b) the isoepoxydon dehydrogenase gene probe for the patulin metabolic pathway, (c) patulin from *Penicillium brevianamide*, (d) bioactivity of brevianamides against insects, (e) indications that 2-deoxyglucose increases patulin production, and (f) mycotoxin production in water are referred to. Priorities for culture collections are suggested.

Keywords: Mycotoxins; Fungi; Culture Collections

Introduction

One of the most important areas of mycology is mycotoxins in food, drink, air and feedstuff. Mycotoxins are fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness, or death in man or animals. Exposure to mycotoxins can produce acute toxicities. They may be carcinogenic, mutagenic, teratogenic, and immunosuppressive. Mycotoxins are of international importance due to the economic losses associated with

their impact on human health, animal productivity and domestic and worldwide trade.

It is advantageous to consider the field of mycotoxicology as a "systems approach" (Coker, 1999). The Commodity System involves the production, marketing, and utilisation of the commodity. The Spoilage System: is the net result of the numerous interacting spoilage agents which can be described as biological, chemical, physical, macro- and micro-environmental. The Mycotoxin System (Table 1) may be considered as five interacting subsystems: toxicology, metabolism, health, productivity and wealth. The Control System involves: (1) prevention, (2) identification and segregation of contaminated materials, and (3) detoxification. A Hazard Analysis Critical Control Point (HACCP) approach is required to identify optimal points of control.

Fungi produce other toxic compounds of importance in commodities, including those not often considered as within the mycotoxin system (*e.g.* Kelley *et al.*, 2003). Toxigenic fungi (*e.g. Metarizium anisopliae*) are being proposed to control crop pests, and so are potential sources of fungal toxin(s) contamination of crops. Pharmaceutical preparations may be susceptible. Fermentation processes such as brewing and malt production can be seriously affected. Finally, staff in scientific organisations who work with fungi (*e.g.* fungal culture collections (CC)) are at risk from a greater mycotoxin load than the general public.

Table 1. Main mycotoxins

Fungi	**Mycotoxins**
Aspergillus parasiticus	Aflatoxins B1, B2, G1, G2, M1
A. flavus	Aflatoxins B1, B2, M1
Penicillium expansum + others	Patulin
P. verrucosum, A. ochraceous,	
A. niger, A. carbonarius	Ochratoxin A
Fusarium sporotrichioides	T-2 toxin
F. graminearum	Deoxynivalenol
F. moniliforme	Fumonisin B1

Fungal CC play an important role in mycotoxicology. Maintenance of the main mycotoxin producing fungi is the most important function. The potential value of these fungi can be appreciated with the current price of aflatoxin M_1 being € 5,000,000 g^{-1} (Sigma, Portugal). Fungi which produce other mycotoxins need to be preserved, as do those which interact with toxin producers in the environment. They may be required for commercial production, physiology/molecular biology, ecological studies, and/or taxonomy. A collection of non-toxigenic fungi which do not contaminate commodities are required as "controls". MUM follows the criteria established for major culture collections (Santos and Lima, 2001) especially for penicillia and aspergilli (Table 2). It has access to equipment for most aspects of mycotoxicology, and is involved in the following mycotoxin/commodity systems:

Ochratoxin A (OTA) is classified as "possibly carcinogenic to humans" (Group 2B) by the International Agency for Research in Cancer. The EU has established limits in various commodities (*e.g.* cereals, dried vine fruits) and limits for other commodities are under evaluation (*e.g.* coffee, cacao, beer and wine). There is concern about the presence of OTA in wine (Zimmerli and Dick, 1995), and MUM is involved in an EU project in this area. Patulin is a contaminant of fruit and vegetables, and new EU levels have been proposed (Jones and Toal, 2003). It can affect detrimentally malt production. Portuguese apples appear susceptible (Martins *et al.*, 2002). A UK project on reducing patulin in apple juice was completed recently (Paterson *et al.*, 2003), and MUM has research interests in this toxin. The significance of fungi in US water distribution systems was investigated (Kelley *et al.*, 2003). MUM is engaged in EU projects on fungal contamination of drinking water distribution system, and bottled drinking water.

A review of mycotoxin research with which scientists at MUM have experience, and the relevance of fungal CC are presented here.

Material and Methods

Standard procedures were employed for the growth and maintenance of fungi as described in the references. Secondary metabolite databases were established on TLC, HPLC, and/or UV spectrum characteristics of over 100 purified compounds (Paterson and Bridge, 1994). A variety

Table 2. Penicillia and aspergilli in MUM

***Aspergillus* species**	***Penicillium* species**
Aspergillus aculeatus Iizuka	*Penicillium aurantiogriseum* Dierckx
Aspergillus auricomus (Guég.) Saito	*Penicillium bilaiae* Chalabuda
Aspergillus candidus Link: Fr.	*Penicillium brevicompactum* Dierckx
Aspergillus carbonarius (Bainier) Thom	*Penicillium camemberti* Thom
Aspergillus cervinus Massee	*Penicillium chrysogenum* Thom
Aspergillus clavatus Desm.	*Penicillium commune* Thom
Aspergillus ellipticus Raper & Fennell	*Penicillium corylophilum* Dierckx
Aspergillus flavus Link: Fr.	*Penicillium crustosum* Thom
Aspergillus fumigatus Fresen.	*Penicillium digitatum* (Pers.: Fr.) Sacc.
Aspergillus funiculosus G. Sm.	*Penicillium expansum* Link
Aspergillus japonicus Saito	*Penicillium fellutanum* Biourge
Aspergillus niger Tiegh. nom. cons.	*Penicillium funiculosum* Thom
Aspergillus ochraceus G. Wilh.	*Penicillium glabrum* (Wehmer) Westling
Aspergillus oryzae (Ahlb.) E. Cohn	*Penicillium griseofulvum* Dierckx
Aspergillus ostianus Wehmer	*Penicillium implicatum* Biourge
Aspergillus parasiticus Speare	*Penicillium italicum* Wehmer
Aspergillus phoenicis (Corda) Thom & Currie	*Penicillium megasporum* Orpurt & Fennell
Aspergillus pulvinus Kwon-Chung & Fennell	*Penicillium minioluteum* Dierckx
Aspergillus restrictus G. Sm.	*Penicillium neoechinulatum* (Frisvad, Filt. & Wicklow) Frisvad & Samson
Aspergillus tamarii Kita	
Aspergillus terreus var. *africanus* Fennell & Raper	*Penicillium oxalicum* Currie & Thom
Aspergillus terreus var. *terreus* Thom	*Penicillium pinophilum* Hedgc. apud Thom
Aspergillus ustus (Bainier) Thom & Church	*Penicillium purpurogenum* Stoll
Aspergillus versicolor (Vuill.) Tirab.	*Penicillium roqueforti* Thom
	Penicillium solitum Westling
	Penicillium spinulosum Thom
	Penicillium thomii Maire
	Penicillium variabile Sopp
	Penicillium verrucosum Dierckx
	Penicillium vulpinum (Cooke & Massee) Seifert & Samson

of other HPLC methods were employed for specific mycotoxins (*i.e.* OTA (Abrunhosa *et al.*, 2002; Serra *et al.*, 2003) and patulin (Kazi *et al.*, 1997; Paterson *et al.*, 2003). Fungal cultures were often analysed by the agar plug method which involves removing small plugs from agar cultures and placing onto the origin of TLC plates before development. *P. expansum* strains were isolated from Portuguese grapes and inoculated onto yeast extract sucrose agar (YES) and grape-juice agar (GJ), and tested for mycotoxin production (Abrunhosa *et al.*, 2001). Immature and mature grapes were collected and fungi were isolated and identified. The fungi were analysed by HPLC for OTA (Bragulat *et al.*, 2001). Penicillia were isolated from apple production systems in UK, and subjected to patulin analysis by TLC. The presence of the isoepoxydon-dehydrogenase (IDH) patulin gene was determined by standard PCR based methods. Two-dimensional TLC and HPLC analysis were used for patulin confirmation (Paterson *et al.*, 2003). The IDH probe was tested on various environmental samples, such as soil, twigs bark, apples, from an organic orchard in the UK (Paterson *et al.*, 2000).

Small pieces were cut from dried herbarium specimens (DHS) of various *Pencillium* species which were suspended in solvent, and analysed by TLC. Living specimens were compared (see Paterson and Bridge, 1994). The effects of preservation techniques on mycotoxin production of live cultures were investigated (Santos *et al.*, 2002).

Nuts and peppers were subjected to multi-mycotoxin analysis and results compared to the data-bases (Friere *et al.*, 1999; 2000). Water was extracted for analysis with chloroform and the solvent removed by rotary evaporation. They were resuspended in methanol or water and analysed by gradient HPLC. In some cases sterile distilled water was inoculated with fungi to test for metabolite production. Aflatoxin and zearalenone immuno-affinity columns (IAC) were used occasionally for the analysis of water (Kelley *et al.*, 2003).

Crude extracts in solvents were made of penicillia and analysed by TLC and tested against insect pests (see Paterson and Bridge, 1994). Pure compounds were also bioassayed against insect pests. Active strains were analysed by HPLC using alkylphenone retention indices. Brevianamide A was purified from cultures, and tested against insect pests.

The biological control enhancer 2-deoxyglucose (DOG) was filter sterilised and added to YES agar (Abo-Dahab *et al.*, 1996). The cultures were analysed by the agar plug method. Apples were sprayed with DOG, extracted with ethyl acetate, and analysed for patulin (Kazi *et al.*, 1997). *Aspergillus* strains isolated from grapes were grown on a YES medium containing OTA. The conversion of OTA was monitored by extraction of the culture fluid and analysis by HPLC (Abrunhosa *et al.*, 2002). The reduction of OTA in pulped grapes was determined using IAC and HPLC (Fernandes *et al.*, 2003).

Results

Most of the fungal secondary metabolites analysed had distinctive TLC characteristics, UV spectra, and HPLC retention indices. The metabolites could be detected from, *inter alia,* fungi (Paterson and Bridge, 1994), water (Fig. 1) (Kelley *et al.*, 2003) and nuts and pepper (Table 3) (Friere *et al.*, 1999; 2000).

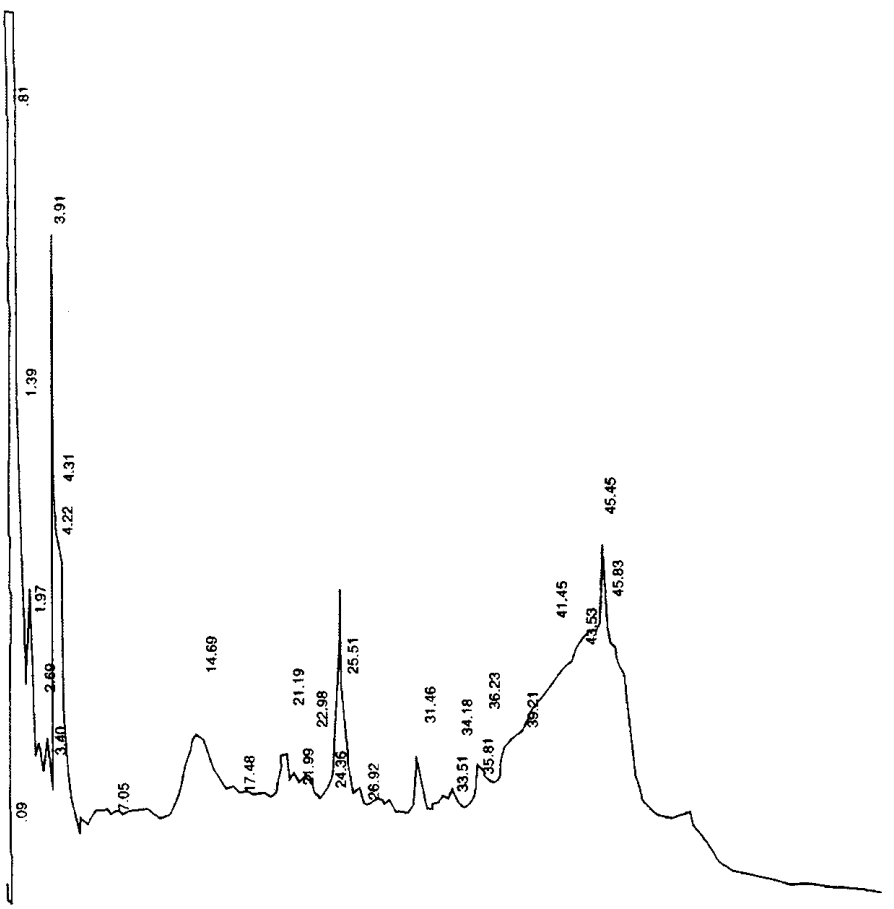

Figure. 1. HPLC chromatogram of water inoculated with *Fusarium graminaearum.*

Table 3. HPLC data from cashew kernels

Healthy	Infected	RI	Identity
0.5 (2.7)	0.5 (0.7)		
0.7 (3.0)	0.7 (17.2)		
0.9 (3.3)		587	
1.0 (1.2)	1.0 (9.2)		
1.1 (0.9)	1.1 (2.3)		
1.2 (4.1)	1.3 (3.8)		
	1.4 (7.2)	596	
	1.5 (12.3)	598	
	1.7 (0.3)	602	
	4.6 (0.8)	654	
	5.8 (0.7)	676	Nivalenol
	6.2 (0.5)	684	Deoxynivalenol
	7.3 (0.5)	704	Austdiol
	7.7 (0.6)	711	Penicillic acid
	8.2 (0.3)	720	Orsellinic acid
	8.6 (0.2)	723	Neosolaniol
	9.0 (2.2)	734	Gibberellic acid
	9.7 (0.8)	747	Ferulic acid
	10.1 (0.5)	755	Aurantioclavine
	10.6 (0.1)	764	Festuclavine
	10.9 (0.3)	769	Aflatoxin G_2
11.5 (0.7)	11.3 (0.1)		
11.6 (0.4)	11.7 (0.3)		
	12.7 (0.2)	802	"PR-1635"
12.9 (0.6)	13.0 (0.6)		
	13.1 (0.4)	809	2,4-dihydroxy-6-(2-oxopropyl) benzoic acid, lactol
	13.6 (0.3)	818	Aflatoxin M_1
14.3 (0.8)	14.3 (6.3)		
		833	Cyclopaldic acid
		834	Aflatoxin G_2
14.5 (1.2)	14.6 (0.4)		
	15.9 (0.1)	860	Aflatoxin G_1
	16.4 (6.3)	869	Aflatoxin B_2
	17.0 (0.3)	880	Isochromantoxin
	17.6 (0.1)	891	Prechinulin
	18.0 (0.3)	898	Lapidosin

Table 3. HPLC data from cashew kernels (*cont.*)

Healthy	Infected	RI	Identity
18.3 (0.1)	18.4 (0.3)		
		906	Desacetylpebrolide
18.6 (0.1)		912	Brefeldin A
21.7 (0.3)		984	Cladosporin
	26.8 (11.0)	1111	Desertorin C
	29.5 (2.4)	1186	Fumitremorgen B
31.3 (0.6)	31.3 (0.9)		
	31.7 (0.5)	1263	Luteoskyrin
32.1 (6.7)	32.3 (0.7)		
32.5 (2.1)		1293	Paxilline
34.0 (0.9)	34.3 (1.2)		
35.6 (18.9)	35.5 (1.3)		
36.6 (0.3)		1449	Trichorzianines B VII
37.9 (11.3)	37.5 (0.3)		
	38.0 (0.6)	1506	Aflatrem
39.7 (9.8)	39.8 (0.4)		
41.0 (6.5)	41.1 (0.8)		
42.0 (5.5)	42.0 (0.5)		
42.7 (4.4)	42.8 (0.4)		
43.4 (3.5)	43.4 (0.3)		
43.9 (3.0)	43.9 (0.3)		
44.4 (6.7)		1816	
	46.77 (0.7)	1940	Stigmasterol

Detoxification of ochratoxin A was observed by treatment with 51 of 76 fungi tested. *Aspergillus clavatus, A. ochraceus, A. versicolor,* and *A. wentii* degraded OTA. The black aspergilli degraded OTA to much larger amounts of the products represented by retention times (Rt) of 2.37 (= OTα) and 3.34 min than *A. ochraceus* and *A. wentii.* However, products at Rt of 5.07 and 5.76 appeared for *A. ochraceus* and *A. wentii* which were not observed (1) from the black aspergilli, (2) by acid hydrolysis or, (3) by carboxypeptidase treatment (Fig. 2). Reduction of OTA in fermented and pulped grapes which were spiked with OTA occurred during the physical solid/liquid separation phase of vinification.

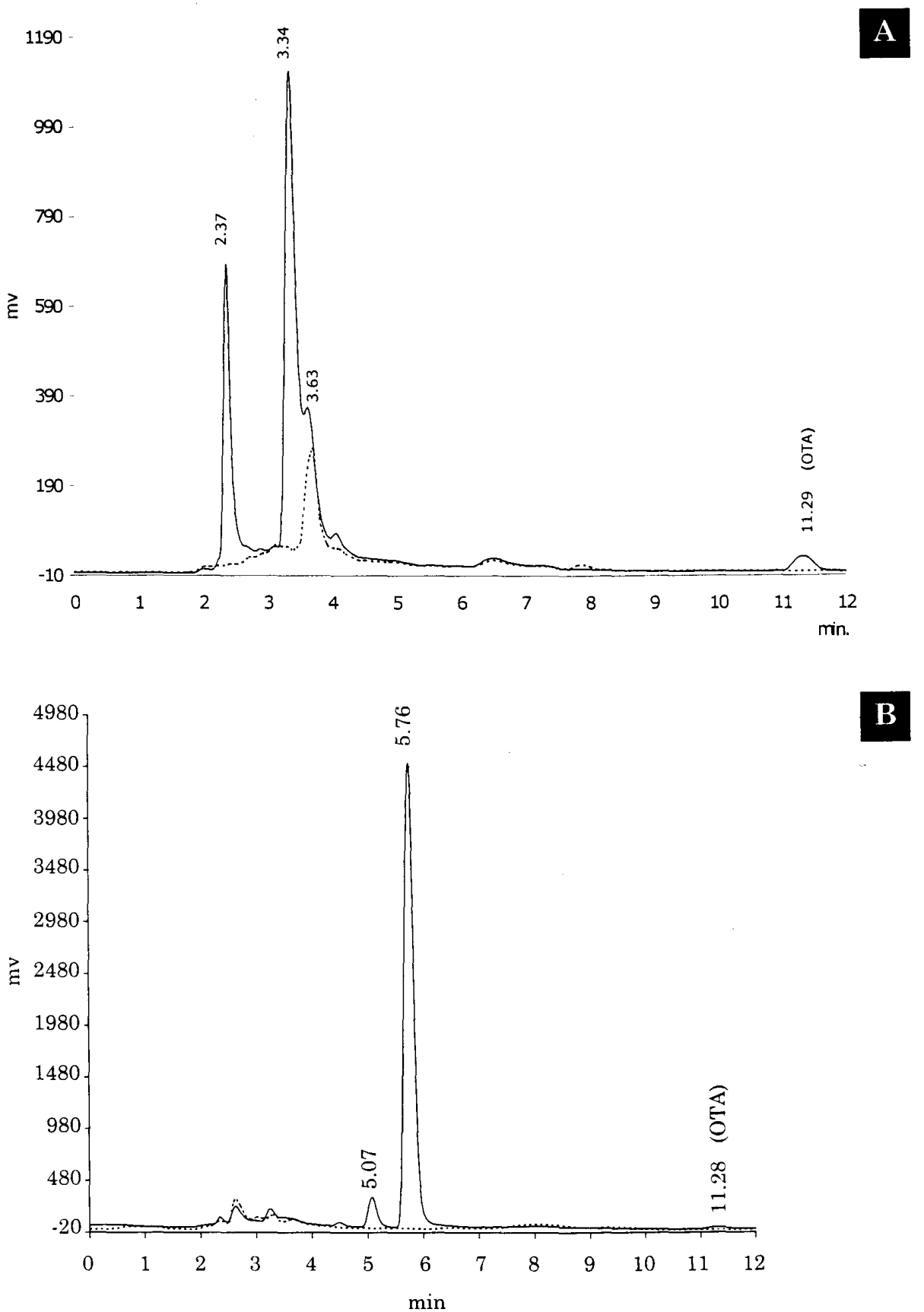

Figure 2. Breakdown products from ochratoxin A produced by a black *Aspergillus* strain (A) and a *Aspergilus ochraceus* strain (B).

A. carbonarius was the most frequently OTA producers from grapes (Serra *et al.*, 2003), with *A. niger* being occasionally isolated. OTA producing fungi isolated from grapes occurred in Portuguese vineyards with a hotter/dryer climate at a higher incidence than those from other regions. The percentage of colonised berries with *A. carbonarius* / *A. niger* increased with maturation time, and designated producer strains were not observed from immature grapes (Table 4).

Table 4. Ochratoxigenic aspergilli from grapes

Region	Vineyard	% of grapes			Mean / average (%)
		Green (immature)	Veraisson*	Ripe (harvest)	
Vinhos verdes (temperate)	1	nd	nd	nd	0.7
	2	nd	nd	2 % *A. carbonarius*	
	3	nd	nd	nd	
Douro (warmer)	4	nd	nd	8 % *A. carbonarius*	10.0
	5	nd	nd	20 % *A. niger*	
	6	nd	nd	2 % *A. niger*	
Ribatejo (hot)	7	nd	2 % *A. carbonarius*	12 % *A. carbonarius*	6.7
	8	nd	nd	2 % *A. carbonarius*	
	9	nd	nd	nd	
Alentejo (hottest/dry)	10	nd	nd	38 % *A. carbonarius* 2 % *A. niger*	21.0
	11	nd	nd	2 % *A. carbonarius*	

Nd – not detected
*Varaisson (French) means a grape beginning to change colour to red and ripen.

Recognised OTA producing penicillia were not isolated from Portuguese grapes (Abrunhosa *et al.*, 2001; Serra *et al.*, 2003). The most relevant mycotoxin producing species was *Penicillium expansum*. These strains could be divided into 4 groups depending on patulin

and/or citrinin production on YES and GJ. Patulin was not detected on YES and GY from 80 % and 35 % of strains respectively. *P. brevi-compactum* was also isolated. *P. expansum* was isolated frequently from throughout the apple production system in UK orchards. The isolates (*ca* 97 %) produced patulin on YES, and contained the IDH gene. Isolates of *P. roqueforti* had both traits as did a small number of other isolates of other species. Some strains of *P. brevicompactum* were positive for the gene product, and patulin production was confirmed (Paterson *et al.*, 2003). The PCR product of the IDH gene was detected from orchard samples such as soil, twigs, bark, etc. (Paterson *et al.*, 2000).

Insecticidal extracts from the penicillia indicated that many compounds were present. Most of the purified compounds were active. Brevianamide A was determined to be active against the insects in a dose response manner. Activity and high activity were observed from brevianamide D and OTA respectively (see Paterson and Bridge, 1994).

DOG increased the production of patulin in agar culture (Abo-Dahab *et al.*, 1996). There appeared to be a linear increase in patulin production from apples treated with DOG. However, when the analysis was repeated the increases observed were not statistically significant (Kazi *et al.*, 1997).

Mycotoxins and secondary metabolites were detected from DHS, some of which had been preserved for decades (Paterson and Bridge, 1994). Variation in patulin and citrinin production appeared to be strain specific, rather than depending on preservation technique used (Santos *et al.*, 2002). Citrinin production was stable throughout preservation, unlike that of patulin. Patulin was detected more consistently using silica-gel storage or freeze drying, and on GJ compared to YES growth media.

OTA was not detected from the grapes from which OTA producing fungi were isolated (unpublished results). Metabolite production, including patulin, was detected from fungi isolated from US water distribution systems. Zearalenone and 3-acetyldeoxynivalenol and were detected from water inoculated with *Fusarium graminearum*. Zearalenone (7.3 ng) production from the fungus was confirmed quantitatively from 500 ml of DTW. Additional peaks were observed from the eluate which corresponded to other known mycotoxins and compounds from this fungus (Fig. 1). Aflatoxin B_2 (0.2 $\mu g\ l^{-1}$) and G_2 (0.1 $\mu g\ l^{-1}$) were detected

from storage tank water. Zearalenone, or derivative, was detected at 0.5 ng l^{-1}. A trace amount of aflatoxin B_1 (less than 0.2 ng) was detected from 20 l of UK domestic tap water (Kelley *et al.*, 2003). Many metabolites were detected by the mycotoxin analysis of Brazilian nuts and pepper (Table 3). In general, more were detected from the deteriorated samples than those which were in better condition visually (Friere *et al.*, 1999; 2000).

Discussion

The TLC and HPLC methods described in Paterson and Bridge (1994), with emphasis on the Frisvad references therein, are recommended for fungal secondary metabolite profiling, given the theoretical background and quantity of data that have been produced by them. They can identify fungi and indicate if they are productive. For example, the profiles from *P. expansum* and *P. roqueforti* were distinctive and different from each other, although they were positive for the IDH gene (Fig. 3) and production (Paterson *et al.*, 2003).

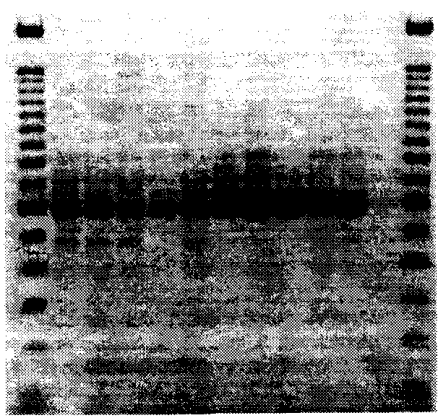

Figure 3. Isoexpoxydon dehydrogenase gene fragment from *Penicillium expansum* (the first five tracks) and *P. roqueforti* (the remaining five tracks). The base pair markers are demonstrated at the extreme ends of the gel.

The hypothesis that *A. carbonarius* is the main producer of OTA in grapes and that *A. niger* was occasionally implicated was supported by Serra *et al.* (2003). The results indicate that hot/dry climates increase the incidence of these fungi. OTA was not detected from grapes from which producing fungi were isolated. This was may be due to the low incidence of ochratoxigenic fungi in these grapes compared to other reports (unpublished data). It is well known that quantification of fungi as a means of determining how many fungi are in a commodity is

imprecise. More *in vitro* and *in vivo* studies are required on mycotoxigenic fungi isolated from, and the presence of mycotoxins in commodities, to assess if the counts can predict mycotoxin contamination. There was no quantitative relationship between the amount of *Fusarium* DNA and the concentration of relevant mycotoxins in grain (Xu *et al.*, 2003).

It is interesting that a fungus (*e.g. A. ochraceus*) can produce and degrade OTA. So removal of the fungus may not lead to reductions in OTA in a commodity. More work on the physiology of mycotoxin production/degradation is required. It is suggested that research is undertaken on establishing a "biodynamic ratio" of OTA to OTα or other product, when analysing samples from production systems. It may be possible to determine if a sample was in a state of overall accumulation or degradation of OTA. Further work is required on the effect of DOG on patulin production to verify it is safe for use on fruit. The importance of the interrelationship between the production and control systems for mycotoxins is demonstrated by the observation that there is an increase of OTA until the maceration of grapes, and a decrease after, leading to a lower concentration in the final wine product (Fernandes *et al.*, 2003).

The *P. brevicompactum* from grapes may be able to produce patulin (Paterson *et al.*, 2003). The situation for *P. expansum* from Portuguese grapes and UK apples appears different. Almost all the UK *P. expansum* produced detectable patulin on YES whereas few did from Portuguese grapes. The non-producers require to be tested at higher sample concentration and by the IDH gene probe. Very high levels of patulin and lower levels of citrinin, were detected from Portuguese apples (Martins *et al.*, 2002). The IDH gene probe is different from most other mycotoxin gene probes in that it is specific for a single mycotoxin (patulin). It is thought that this gene probe is unique in this respect. Probes to the polyketide synthetase gene exist and the gene is common to all polyketide producers. There are methods for classes of compound (*e.g.* trichothecenes (Nicholson *et al.*, 2003)). The detection of the gene fragment in environmental samples could be from other fungi apart from penicillia (Paterson, 2003), and/or a non-fungal source. Patulin production may occur on the twigs, bark and soil, etc. of an apple tree and be translocated to the fruit through sap without the need for infection *per se*, and similarly for OTA in coffee (Paterson *et al.*, 2000). The utility of the IDH gene probe was confirmed by Varga

et al. (2003) for *Aspergillus,* in which was the first report of patulin from *A. longivesica.*

The large brevianamide A HPLC peak observed from an anti-insect *P. brevicompactum* strain (see Paterson and Bridge, 1989) prompted further investigation of the compound. It is possible that brevianamide A may have evolved as an anti-insect compound within the conidiophore of the fungus as a form of chemical defence. Activity from brevianamide A and D was the first report of activity from these compounds. MUM will extend the research to toxicological effects on protozoa. These microorganisms are useful models which can act as alternatives to animal experiments in toxicological studies (Dias *et al.*, 2003b) and as biosensor to detect mycotoxins (Benitez *et al.*, 1994). Protozoa counting can now be increased ×100 by image analysis methodology (Dias *et al.*, 2003a).

The detection of mycotoxins from DHS was the first such report. The cultures were intentionally killed but contained detectable mycotoxins after decades of storage. DHS of original cultures should be utilised in chemotaxonomic studies, and to confirm specific metabolite production, to avoid variation in preserved living cultures. Gene probing of BHS for the IDH gene may be possible. The effects of preservation techniques on patulin and citrinin production from *P. expansum* were difficult to define, as variation appeared to be strain specific, rather than dependant on the preservation technique used (Santos *et al.*. 2002).

Investigators into the effects of preservation should be aware that fungi might loose the capacity for secondary metabolism despite compounds being detected from cultures. Also, the difference between a mycotoxin being detected and not, could be a small quantitative one, and of little significance. The effect of preservation on the physiology of metabolite/mycotoxin production over a growth/production period of, for example, 0 to 10 days requires investigation in systems appropriate to these purposes (*e.g.* bioreactors). The effect on mycotoxin production of repeated sub-culturing of strains revived from preservation requires investigation, as the use of a freeze-dried or cryo-preserved culture for each experiment may not be practicable.

All the reports of metabolites from isolated fungi and mycotoxins/ metabolites in water are the first such reports (Kelley *et al.*, 2003). Zearalenone is known to have oestrogenic properties and reports of

this activity from water is causing concern due to associated hormonal effects. The large number of metabolites detected from nuts and peppers may have reflected the large number of fungi which were isolated, although the samples were separate from each other in space and time. It is possible that the spots and peaks may not be fungal in origin, but it was an indication of fungal metabolite contamination. Metabolite detection from commodities is an "archaeological record" of fungi which were present, as well as indicating the identity of current occupiers.

In conclusion, the role of fungal CC need not be restricted to maintenance of fungi as laudable as is this objective. A wide range of activities can be investigated given the resources and the foundation of well-preserved fungi.

Priorities

- Confirmation of the production of the main mycotoxins from fungi with preservations studies directed towards them.
- Other mycotoxins should be similarly researched.
- Collections of complete environmental samples and extracts for analytical and fungal isolation purposes from production systems.

Safety

Adhere to national laboratory safety procedures for dangerous chemicals. Make solutions of mycotoxins as soon as possible to avoid dust. Treat toxigenic fungi with same care as powdered mycotoxins. Do not breath in fungi. Additional measures required for mass production and spraying of fungi. Implement Castegnaro *et al.* (1991).

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