

RECOMMENDATIONS CONCERNING THE CHRONIC PROBLEM OF MISIDENTIFICATION OF MYCOTOXIGENIC FUNGI ASSOCIATED WITH FOODS AND FEEDS

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1. INTRODUCTION

Since the aflatoxins were first reported in 1961 from *Aspergillus flavus*, mycotoxins have often been named after the fungus which was first found to produce them. A long list of connections between fungal species and mycotoxins and antibiotics has been reported, but unfortunately many of the identifications, and hence the connection between mycotoxin name and the source of the toxin, are incorrect (Frisvad, 1989). The most famous example of such incorrect connections was Alexander Fleming's identification of the original penicillin producer as *Penicillium rubrum*. Fortunately, in this example, the substance was named after the genus *Penicillium*, rather than the species, as K. B. Raper re-identified the strain as *P. notatum*, which was subsequently determined to be a synonym of *P. chrysogenum* (Pitt, 1979b). Later, penicillin was found in other strains of *P. chrysogenum* (Raper and Thom, 1949).

The early aflatoxin literature is plagued with wrong reports of aflatoxin production by *Penicillium puberulum* (Hodges et al., 1964),

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Aspergillus ostianus (Scott et al., 1967), *Rhizopus* sp. (Kulik and Holaday, 1966), the bacterium *Streptomyces* (Mishra and Murthy, 1968) and several other taxa. The most famous of these reports was the paper of El-Hag and Morse (1976). They reported that *Aspergillus oryzae*, the domesticated species used in the manufacture of soy sauce and other Oriental fermented foods, produced aflatoxin. However, the culture of *A. oryzae* they used was quickly shown to be contaminated by an aflatoxin producing *A. parasiticus* (Fennell, 1976). Immediate correction of this error did not prevent Adebajo et al. (1992), El-Kady et al. (1994), Atalla et al. (2003) or Drusch and Ragab (2003) reporting that *A. oryzae* produces aflatoxin.

Often, publications reporting mycotoxin production are reviewed by people who have little or no understanding of mycological taxonomy. For example, “*P. patulinum*” and “*P. clavatus*” are mentioned in Drusch and Ragab (2003). In Bhatnagar et al. (2002), “*P. niger*” is mentioned as producing ochratoxin A. Each of these names is an incorrect combination of genus and species. Bhatnagar et al. (2002) give *P. viridicatum* as producing ochratoxin A in a table, while using *P. verruculosum* as the species name in the text, confusing it with *P. verrucosum*, the correct name for the producer of this toxin.

Such mistakes could have been avoided. This paper provides a set of recommendations to be followed to ensure correct reports of connections between mycotoxin production and fungal species.

2. EXAMPLES OF INCORRECT CITATIONS OF SOME FUNGI PRODUCING WELL KNOWN MYCOTOXINS

2.1. Aflatoxin

The known producers of aflatoxin are given in a separate paper in these Proceedings (Frisvad et al., 2006). The list of other species that have been (incorrectly) reported to produce aflatoxins includes *Aspergillus flavo-fuscus*, *A. glaucus*, *A. niger*, *A. oryzae*, *A. ostianus*, *A. sulphureus*, *A. tamarii*, *A. terreus*, *A. terricola*, *A. wentii*, *Emericella nidulans* (as *A. nidulans*), *Emer. rugulosa* (as *A. rugulosus*), *Eurotium chevalieri*, *Eur. repens*, *Eur. rubrum*, *Mucor mucedo*, *Penicillium citrinum*, *P. citromyces*, *P. digitatum*, *P. frequentans*, *P. expansum*, *P. glaucum*, *P. puberulum*, *P. variable*, *Rhizopus* sp. and the bacterium *Streptomyces* sp. None of

these species produce aflatoxins, and many of these names are not accepted as valid species in any case.

2.2. Sterigmatocystin

Fungi known to produce sterigmatocystin include *Aspergillus versicolor*, *Emericella nidulans*, several other *Emericella* species and some *Chaetomium* species. Although sterigmatocystin is a precursor of aflatoxins (Frisvad, 1989), only *Aspergillus ochraceoroseus* (Frisvad et al., 1999; Klich et al., 2000), and some *Emericella* species accumulate both sterigmatocystin and aflatoxin (Frisvad et al., 2004a; Frisvad and Samson, 2004a). Species in *Aspergillus* section *Flavi*, which includes the major aflatoxin producers, efficiently convert sterigmatocystin into 3-methoxysterigmatocystin and then into aflatoxins (Frisvad et al., 1999).

Many *Aspergillus* species have been reported to produce sterigmatocystin, incorrectly except for those cited above. Sterigmatocystin production by *Penicillium* species has not been reported, apart from an obscure reference to *Penicillium luteum* (Dean, 1963). However, Wilson et al. (2002) claimed that *P. camemberti*, *P. commune* and *P. griseofulvum* produce sterigmatocystin. Perhaps they mistook sterigmatocystin for cyclopiazonic acid. Three *Eurotium* species have been claimed to produce sterigmatocystin (Schroeder and Kelton, 1975), but this was based only on unconfirmed TLC assays. Unfortunately the strains used were not placed in a culture collection.

2.3. Ochratoxin A

Ochratoxin A is produced by four main species, *Aspergillus carbonarius*, *A. ochraceus*, *Petromyces alliaceus*, *Penicillium verrucosum*, and a few other related species as detailed elsewhere (Frisvad and Samson, 2004b; Samson and Frisvad, 2004; Frisvad et al., 2006). A very large number of species have been claimed to produce ochratoxin A, but not all will be detailed here. However, some of the names frequently cited in reviews will be mentioned. Of the *Penicillia*, *P. viridicatum* was the name cited for many years as the major ochratoxin A producer, but it was shown that *P. verrucosum* was the correct name for this fungus, the only species that produces ochratoxin A in cereals in Europe (Frisvad and Filtenborg, 1983; Frisvad, 1985; Pitt, 1987). The closely related *P. nordicum*, which occurs on dried meat in Europe, was mentioned as producing ochratoxin A by Frisvad and Filtenborg (1983) and Land and Hult (1987), but not accepted as a separate species until the publication of Larsen et al. (2001).

P. verrucosum has been correctly cited as the main *Penicillium* species producing ochratoxin A for a number of years now, but in a series of recent reviews and papers *P. viridicatum* and *P. verruculosum* (no doubt mistaken for *P. verrucosum*) have been mentioned again (Mantle and McHugh, 1993; Bhatnagar et al., 2002; Czerwiecki et al., 2002a, b). In the latter two papers *P. chrysogenum*, *P. cyclopium*, *P. griseofulvum*, *P. solitum*, *Aspergillus flavus*, *A. versicolor* and *Eurotium glaucum* were listed as ochratoxin A producers. The strain of *P. solitum* reported by Mantle and McHugh (1993) to produce ochratoxin A were assigned more recently to *P. polonicum*, but neither species produces ochratoxin A (Lund and Frisvad, 1994; 2003). These isolates were contaminated by *P. verrucosum*. The reports by Czerwiecki et al. (2002 a, b) are more problematic in that the fungi have been discarded, so it will never be possible to check the results.

The following species were listed as ochratoxin A producers by Varga et al. (2001): *Aspergillus auricomus*, *A. fumigatus*, *A. glaucus*, *A. melleus*, *A. ostianus*, *A. petrakii*, *A. repens*, *A. sydowii*, *A. terreus*, *A. ustus*, *A. versicolor*, *A. wentii*, *Penicillium aurantiogriseum*, *P. canescens*, *P. chrysogenum*, *P. commune*, *P. corylophilum*, *P. cyaneum*, *P. expansum*, *P. fuscum*, *P. hirayamae*, *P. implicatum*, *P. janczewskii*, *P. melinii*, *P. miczynskii*, *P. montanense*, *P. purpurescens*, *P. purpurogenum*, *P. raistrickii*, *P. sclerotiorum*, *P. spinulosum*, *P. simplicissimum*, *P. variable* and *P. verruculosum*. None of these species produces ochratoxin A, and it seems clear that the authors have uncritically accepted lists from earlier reviews. In the recent *Handbook of Fungal Secondary Metabolites* (Cole and Schweikert, 2003a, b; Cole et al., 2003), only two of the species cited as producing ochratoxin A are correct: *A. ochraceus* and *A. sulphureus*. The others mentioned are not.

2.4. Citrinin

Citrinin is produced by a number of species in *Penicillium* and *Aspergillus*, notably *P. citrinum*, *P. expansum*, *P. verrucosum*, *A. carneus*, *A. niveus* and an *Aspergillus* species resembling *A. terreus* (Frisvad, 1989; Frisvad et al., 2004b), but not by *Aspergillus oryzae* or *P. camemberti*, as claimed by Bennett and Klich (2003). Critical checking of the original reports clearly did not occur. Many other species have been claimed to produce citrinin, including *A. ochraceus* (Mantle and McHugh, 1993), *A. wentii* (Abu-Seidah, 2002) and *Eurotium pseudoglaucum* (El-Kady et al., 1994), but either fungus or mycotoxin may have been misidentified in these cases.

2.5. Patulin

A number of species in different genera, notably *Penicillium*, *Aspergillus* and *Byssoschlamys*, produce patulin. Among the most efficient producers of patulin are *Aspergillus clavatus*, *A. giganteus*, *A. terreus*, *Byssoschlamys nivea*, *P. carneum*, *P. dipodomyicola*, *Penicillium expansum*, *P. griseofulvum*, *P. marinum*, *P. paneum* and several dung associated *Penicillia* (Frisvad, 1989; Frisvad et al., 2004b). It is not, however, produced by species in all of the 42 genera listed by Steiman et al. (1989) and Okele et al. (1993). These papers include erroneous statements that *Alternaria alternata*, *Fusarium culmorum*, *Mucor hiemalis*, *Trichothecium roseum* and many others produce patulin. The production of patulin by *Alternaria alternata* was later reported by Laidou et al. (2001), and mentioned in a review by Drusch and Ragab (2003). However patulin was not found in hundreds of analyses of *Alternaria* extracts (Montemurro and Visconti, 1992), or in extracts from more than 200 *Alternaria* cultures tested by us at the Technical University of Denmark (B. Andersen, personal communication).

2.6. Penitrem A

Many species have been claimed to produce penitrem A, but most have been misidentifications of *Penicillium crustosum* (Pitt, 1979; Frisvad, 1989). Names given to isolates that were in fact *P. crustosum* include *P. cyclopium*, *P. verrucosum* var. *cyclopium*, *P. verrucosum* var. *melanochlorum*, *P. viridicatum*, *P. commune*, *P. lanosum*, *P. lanosocoeruleum*, *P. granulatum*, *P. griseum*, *P. martensii*, *P. palitans* and *P. piceum* (Frisvad, 1989). Other species which do produce penitrem A include *P. carneum*, *P. melanoconidium*, *P. tulipae*, *P. janczewskii*, *P. glandicola* and *P. clavigerum* (Frisvad et al., 2004b). Only the first three of these species are likely to occur in foods.

2.7. Cyclopiazonic Acid

Cyclopiazonic acid is produced by *Aspergillus flavus*, *A. oryzae*, *A. tamarii*, *A. pseudotamarii*, *Penicillium camemberti*, *P. commune*, *P. dipodomyicola*, *P. griseofulvum* and *P. palitans* (Goto et al., 1996; Huang et al., 1994; Pitt et al., 1986; Polonelli et al., 1987; Frisvad et al., 2004b). Cyclopiazonic acid was originally isolated from and named after *P. cyclopium* CSIR 1082, but this fungus was reidentified as *P. griseofulvum* (Hermansen et al., 1984; Frisvad, 1989). Despite this, most reviews still cite *P. cyclopium* or *P. aurantiogriseum* [of which

Pitt (1979) considered *P. cyclopium* to be a synonym] as producers (Scott, 1994; Bhatnagar, 2002; Bennett and Klich, 2003). Scott (1994) drew an incorrect conclusion

“ α -cyclopiazonic acid is a metabolite of several *Penicillium* and *Aspergillus* species and is of Canadian interest from two viewpoints. First, one of the important producers (*P. aurantiogriseum*, formerly *P. cyclopium*, Pitt et al., 1986), commonly occurs in stored Canadian grains...”

Although *P. aurantiogriseum* no doubt occurs in cereal grains, it is not a producer of cyclopiazonic acid.

Another example of an error being cited repeatedly is the claimed production of cyclopiazonic acid by *Aspergillus versicolor* (Ohmomo et al., 1973; cited by Bhatnagar et al., 2002) even though Domsch et al. (1980) and Frisvad (1989) had stated that the isolate described by Ohmomo et al. (1973) was correctly identified as *A. oryzae*, a well-known producer of cyclopiazonic acid (Orth, 1977). *Penicillium hirsutum*, *P. viridicatum*, *P. chrysogenum*, *P. nalgiovense*, *Aspergillus nidulans* and *A. wentii* have also wrongly been claimed to produce cyclopiazonic acid (Cole et al., 2003; Abu-Seidah, 2003).

2.8. Xanthomegnin, Viomellein and Vioxanthin

Xanthomegnin, viomellein and vioxanthin are nephrotoxins produced by all members of *Aspergillus* section *Circumdati* (Frisvad and Samson, 2000), *Penicillium cyclopium*, *P. freii*, *P. melanoconidium*, *P. tricolor* and *P. viridicatum* (Lund and Frisvad, 1994), and by *P. janthinellum* and some other genera and species which do not occur in foods. Some of these *Penicillium* species occur in cereals, so these toxins have been found occurring naturally (Scudamore et al., 1986). These toxins are not produced, however, by *P. crustosum* as reported by Hald et al. (1983), by *P. oxalicum* as reported by Lee and Skau (1981) or by *A. nidulans*, *A. flavus*, *A. oryzae* or *A. terreus* as reported by Abu-Seidah (2003).

2.9. Penicillic Acid

Penicillic acid is associated with *Penicillium* series *Viridicata* and *Aspergillus* section *Circumdati* (Lund and Frisvad, 1994; Frisvad and Samson, 2000; Frisvad et al., 2004). Production reported by *P. roqueforti* (Moubasher et al., 1978; Olivigni and Bullman, 1978) is now considered to be due to the similar species *P. carneum* (Boysen et al., 1996).

2.10. Rubratoxins

Rubratoxins are hepatotoxic mycotoxins known to be produced only by the rare species *Penicillium crateriforme* (Frisvad, 1989). Rubratoxins are not produced by *P. rubrum*, *P. purpurogenum* or *Aspergillus ochraceus* as reported by Moss et al. (1968), Natori et al. (1970) and Abu-Seibah (2003).

2.11. Trichothecenes

Trichothecenes are especially troublesome as it is only after the introduction of capillary gas chromatography coupled to mass spectrometry (MS) and more recently the introduction of liquid chromatography combined with atmospheric ionization MS that reliable methods have been available for these mycotoxins. Because immunochemical methods have been improved in recent years they also can now be considered valid. However results from TLC and HPLC based methods are dubious, unless combined with immunoaffinity cleanup, as many authors have neglected very time consuming but crucial clean-up steps.

Trichothecene have been reported to be produced by several *Fusarium* species as detailed elsewhere in these proceedings (Frisvad et al., 2006). Marasas et al. (1984) showed that *Fusarium nivale*, which gave nivalenol its name, does not produce trichothecenes. However, under its newer, correct name, *Microdochium nivale* was still incorrectly cited as a trichothecene producer in a recent review (Bhatnagar et al., 2002). It has even been claimed recently that *Aspergillus* species (*A. oryzae*, *A. terreus*, *A. parasiticus* and *A. versicolor*) produce nivalenol, deoxynivalenol and T-2 toxin (Atilla et al., 2003). *A. parasiticus* was claimed to produce very high amounts of deoxynivalenol and T-2 toxin after growth on wheat held at 80% relative humidity for 1-2 months. These data are totally implausible. Possibly the wheat was already contaminated with trichothecenes before use, but the high levels indicate that there may have been false positives as well.

3. RECOMMENDATIONS

To avoid incorrect reporting of fungal species producing particular mycotoxins, we recommend the following rules when working with mycotoxin producing fungi and the reporting of the results:

3.1. Ensure correct identification and purity of fungal isolates

- Fungal isolates from the particular substrate should be checked with the literature on the mycobiota of foods, e.g. Filtenborg et al. (1996), Pitt and Hocking (1997) or Samson et al. (2004), which correlate particular fungal species with particular food types or substrates. Unusual findings especially should be carefully checked. For example, *Aspergillus oryzae* is the domesticated form of *A. flavus* and *A. sojae* is the domesticated form of *A. parasiticus*, and these fungi are not expected to be isolated other than from production plants used for making Oriental foods or enzymes.
- Use typical cultures as reference for comparison, both for identification and mycotoxin production. Frisvad et al. (2000), lists typical cultures for each species of common foodborne *Penicillium* subgenus *Penicillium* species. Some effective mycotoxin producing cultures are listed in Table 1.
- Check the purity of cultures, as contaminated cultures are a very common problem. Check for contaminants by growing cultures on standard media such as CYA (Pitt and Hocking, 1997). Especially when fungi are grown on cereals or liquid cultures it is very difficult to assess if the culture is pure, and it necessary to streak them out on agar substrates where it is much easier to see if the culture is pure.

Table 1. Reference cultures for the production of the more common *Aspergillus* and *Penicillium* mycotoxins

Mycotoxin	Producing species and reference culture
Aflatoxins B ₁ and B ₂	<i>Aspergillus parasiticus</i> CBS ^a 100926 <i>Aspergillus flavus</i> CBS 573.65
Aflatoxins G ₁ and G ₂	<i>Aspergillus parasiticus</i> CBS 100926
Sterigmatocystin	<i>Aspergillus versicolor</i> CBS 563.90
Ochratoxin A	<i>Petromyces alliaceus</i> CBS 110.26 <i>Penicillium verrucosum</i> CBS 223.71
Patulin	<i>Aspergillus clavatus</i> CBS 104.45 <i>Penicillium griseofulvum</i> CBS 295.97
Cyclopiazonic acid	<i>Penicillium griseofulvum</i> CBS 295.97
Roquefortine C	<i>Penicillium griseofulvum</i> CBS 295.97
Citrinin	<i>Penicillium citrinum</i> CBS 252.55 <i>Penicillium verrucosum</i> CBS 223.71
Penicillic acid	<i>Penicillium cyclopium</i> CBS 144.45
Penitrem A	<i>Penicillium crustosum</i> CBS 181.89
Verrucosidin	<i>Penicillium polonicum</i> CBS 101479
Xanthomegnin	<i>Penicillium cyclopium</i> CBS 144.45
Rubratoxin B	<i>Penicillium crateriforme</i> CBS 113161

^aCBS = Culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands

- If unusual producers are found, check them carefully for purity and correct identity using the references cited above. A specialist taxonomist may be consulted.

3.2. Ensure that cultures are deposited in a recognised culture collection

- Deposit all interesting strains producing mycotoxins in international culture collections, and cite the culture collection numbers in any publications regarding the strains. This procedure should be mandatory for all microbial, biochemical and chemical journals.

3.3. Ensure substrate is sterile and not already contaminated with mycotoxins

- If natural substrates, such as cereals, are used for mycotoxin production, they should be sterilised before use (e.g. by autoclaving or by gamma-irradiation). Control assays should be carried out for all mycotoxins being studied on the material intended for use as the substrate. This will ensure false positives are not reported.
- Also check for interfering peaks. Natural substrates such as grains may contain interfering compounds, and the chemical composition of these matrices may change during fungal growth. In such matrices, highly selective cleanup procedures should be used and combined with highly selective analytical methods.

3.4. Ensure optimal conditions for mycotoxin production are used

- Use several media and growth conditions to ensure that the fungus can actually produce the mycotoxins. Four good media for mycotoxin production are listed in Table 2.

3.5. Ensure appropriate analytical and confirmatory procedures for mycotoxin extraction and identification

- Sample preparation methods are important and should be validated. Sample preparation is specific for the food matrix it is designed for. Use only validated analytical methods.

Table 2. Efficient media for mycotoxin production

Czapek Yeast Autolysate agar (CYA) (Pitt, 1979; Pitt and Hocking, 1997)		Yeast Extract Sucrose agar (YES) (Frisvad and Filtenborg, 1983)	
NaNO ₃	3 g	Yeast extract (Difco)	20 g
K ₂ HPO ₄	1 g	Sucrose	150 g
KCl	0.5 g	MgSO ₄ • 7H ₂ O	0.5 g
MgSO ₄ • 7H ₂ O	0.5 g	ZnSO ₄ • 7H ₂ O	0.01 g
FeSO ₄ • 7H ₂ O	0.01 g	CuSO ₄ • 5H ₂ O	0.005 g
ZnSO ₄ • 7H ₂ O	0.01 g	Agar	20 g
CuSO ₄ • 5H ₂ O	0.005 g	Distilled water	1 litre
Yeast extract (Difco)	5 g		
Sucrose	30 g		
Agar	20 g		
Distilled water	1 litre		
Rice powder Corn steep agar (RC) (Bullerman, 1974)		Mercks Malt Extract (MME) agar (El-Banna and Leistner, 1988)	
Rice powder	50 g	Malt extract	30 g
Corn steep liquid	40 g	Soy peptone	3 g
ZnSO ₄ • 7H ₂ O	0.01 g	ZnSO ₄ • 7H ₂ O	0.01 g
CuSO ₄ • 5H ₂ O	0.005 g	CuSO ₄ • 5H ₂ O	0.005 g
Agar	20 g	Agar	20 g
Distilled water	1 litre	Distilled water	1 litre
		pH 5.6	

- Use efficient extraction techniques, for example, fumonisins are very polar and penitrem A is very apolar. Extractions should be validated by recovery experiments.
- Use authenticated standards of the mycotoxins for comparison, ideally as internal and external standards.
- More than one separation technique should be use, combined with selective detection principles. Single UV, refractive index, evaporative light scattering, or flame ionisation detection are non-specific. Fluorescence and full UV spectra are specific to some compounds, while mass spectrometry and especially tandem mass spectrometry is very selective for most compounds when monitoring several ions. Generally four identification points should give a very specific detection, e.g. obtained by LC-MS/MS monitoring two fragmentation reactions.
- Use more than one discretionary test to secure correct identification of the mycotoxin, Combined these with derivatization or alternative clean-up procedures when finding unexpected results.

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