Taxonomy: the key to mycotoxin identification in food and feedstuffs

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Abstract

The literature on the toxicity and chemistry of mycotoxins is considerable but most papers fail to formally identify the fungal isolate or isolates involved.

Members of the genera *Aspergillus* and *Penicillium* produce a large number of mycotoxins. Some are specific, while others are shared by more that one species in a genus, or by species from both genera.

Toxicological and chemical studies usually relate to toxins produced by isolates grown on artificial media under laboratory conditions. However, the mycotoxins or metabolites so produced, frequently do not reflect the spectrum produced when the species in question occurs naturally on food products. This has given rise to confusion as to which mycotoxins are a health risk to humans and livestock.

Introduction

In the developed world, recognition that fungi and their products can cause disease and ill health in man and animals through ingestion of mouldy food and feed, has led to improved methods of cultivation and storage of grain. In general, food is of high quality and fungal contamination low. Unfortunately, poverty and food shortages in many areas of the developing world still cause people to consume food of any quality available.

Members of the genus *Aspergillus* and its close relative *Penicillium* are the dominant fungal contaminants of stored products, foods and feedstuffs. Both genera produce harmful mycotoxins so correct identification at the species level is of paramount importance.

Literature on mycotoxins is vast, with emphasis being placed on toxicology and chemistry rather than identification of toxin-producing species. A common mistake which has resulted, is that where fungal isolations and mycotoxin analyses are made on a product, the fungi isolated therein are assumed to be responsible for the mycotoxins recovered. For example, sterigmatocystin production has been attributed variously to *Aspergillus versicolor,* members of the Section *Nidulantes* (true toxin producers), *Aspergillus ustus, Eurotium chevalieri, E.repens,* and *E.rubrum* (not known to produce the toxin). Additionally, where fungal names have been used, very few such identifications have been sent for expert verification.

Much of the literature on mycotoxin production stems from laboratory studies. *Aspergillus* and *Penicillium* produce a range of toxins *in vitro,* but how many of these mycotoxins occur in the human and animal food supply? It is essential to establish which mycotoxins occur in practice, and with what frequency and in what amounts. Furthermore, a comprehensive multidisciplinary approach is required to firmly establish the correlation between *Aspergillus* and *Penicillium* species and the toxins that they produce (Bridge et al. 1992).

Fortunately, the last decade has seen a wealth of new approaches, combining traditional morphological taxonomy with newer techniques such as isoenzyme patterns (Cruickshank and Pitt 1987a,b, 1990; Sugiyama and Yamatoya 1990; Yamatoya et al. 1990;), molecular techniques (Mullaney and Klich 1990; Logrieco et al. 1990; Croft et al. 1990; Varga et al. 1993), secondary metabolites (Frisvad and Filtenborg 1983, 1989; Kozakiewicz et al. 1993; Paterson and Buddie 1991; Schubert and Kreisel 1991), latex agglutination tests (Stynen et al. 1992) and scanning electron microscopy (Udagawa and Takada 1985; Kozakiewicz 1986, 1989, 1991).

In addition, an international working group, 'The International Commission on *Penicillium* and *Aspergillus*' (ICPA), established in 1986, is undertaking studies of these two important genera. Participants include taxonomists using traditional morphological characters, together with specialists from the fields of physiology and biochemistry.

However, despite such advances, misidentifications and mistakes still occur, usually because the researchers involved have been inadequately trained in the field of taxonomy or mycotoxicology or both.

Penicillium and *Aspergillus* Toxins

Certain mycotoxins are unique to a particular species, e.g. PR-toxin production by *P. roquefortii* and islanditoxin produced by *P. islandicum*, whereas other mycotoxins can be produced by species from both genera. For example, patulin may be produced by *P. expansum, P. griseofulvum, A. terreus* and *A. clavatus*, and citrinin by *P. citrinum, P. expansum, P. verrucosum* and *A. terreus*. Some species produce only a single mycotoxin, e.g. *P. citrinum* and *Eupenicillium ochrosalmoneum* producing citrinin and citreoviridin, respectively, whilst others produce three or four toxins each. *P.viridicatum* produces xanthomegnin, vioxanthin, viomellein and viridamine, and *A.flavus* aflatoxin B_1, and B_2, aflatrem, cyclopiazonic acid (CPA), kojic acid, maltoryzin and 3-nitropropionic acid.

Important *Penicillium* Mycotoxins and Their Occurrence

The relationship between *Penicillium* species and their associated toxins is complex. Cyclopiazonic acid is produced by *P. griseofulvum, P. camembertii* and *P. commune*, three species which are closely related in a single section of a single subgenus (Frisvad and Filtenborg, 1989). In contrast, penitrem A is produced by two closely related species, *P. crustosum* and *P. glandicola* (*P. granulatum*), and by an unrelated species *P. janczewskii* (Frisvad and Filtenborg, 1989), whereas toxins produced by species in the subgenus

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Biverticillium such as *P. islandicum* and *P. purpurogenum* are very different from those produced in other subgenera (Pitt and Leistner 1991). In addition, most species in *Eupenicillium* and *Talaromyces*, if toxigenic, are not considered important health hazards because they are soil inhabitants.

Most *Penicillium* toxins can be placed into one of two groups: those which are nephrotoxins and those which are neurotoxins. Only *Penicillium* toxins which are significant to human and animal health will be discussed here (see Table 1).

Table 1. Important mycotoxins produced by *Penicillium* species

Species	Mycotoxin
P. aurantiogriseum	penicillic acid, terrestric acids, toxic glycopeptides, verrucosidin
P. cyclopium	penicillic acid, viomellein, vioxanthin, xanthomegnin
P. brevicompactum	brevianamide A and B, mycophenolic acids, Raistrick phenols
P. camembertii	Cyclopiazonic acid
P. chrysogenum	meleagrin, penicillic acid, roquefortine C
P. citreonigrum	citreoviridin
P .citrinum	citrinin
P. commune	cyclopiazonic acid
P .crustosum	penitrems, roquefortine C
P. expansum	citrinin, patulin, roquefortine C
P. glandicola	patulin, penitrem A
P. griseofulvum	cyclopiazonic acid, griseofulvins, patulin, roquefortine C
P. hordei	roquefortine C, terrestric acid
P. islandicum	erythroskyrin, islanditoxin, luteoskyrin, skyrin
P. janczewskii	griseofulvin, penicillic acid, penitrem A
P. neoechinulatum	penicillic acid
P. oxalicum	oxaline, roquefortine C, secalonic acid
P. roquefortii	roquefortine A, B, C and D mycophenolic acid, PR-toxin
P. simplicissimum	xanthomegnin
P. solitum	viridicatins
P. verrucosum	citrinin, ochratoxin A and B, oxalic acid
P. viridicatum	viridic acid, viomellein, vioxanthin, xanthomegnin

Citreoviridin

Citreoviridin a neurotoxin, is the causal agent of the human disease, cardiac beriberi, or 'yellow rice' disease, in Japan. Since 1910, when the sale of yellow rice was banned, the disease has virtually disappeared in Japan, but may well be present in other parts of Asia. Citreoviridin is produced by *P. citreonigrum* (Uraguchi 1969; Ueno and Ueno 1972). Fortunately, apart from rice, it is uncommon on cereals and rare in other foods. *P. citreonigrum* growth in rice appears to occur at low temperatures and short day lengths (Pitt 1991). However, the toxin is also produced by *Eupenicillium ochrosalmoneum*, a species now known to be common in standing maize (Wicklow and Cole, 1984). Symptoms of citreoviridin poisoning include vomiting, convulsions, respiratory arrest and increasing paralysis. These symptoms are similar in both animals and humans. To my knowledge there are no known studies to determine the incidence of citreoviridin in developing world rice crops, or its implication in human or animal ill health.

Citrinin

Citrinin, a renal toxin, is produced by *P. citrinum*, *P. expansum* and *P. verrucosum*. It has been reported to be produced by at least 20 other penicillia (Pitt and Leistner 1991), but these claims require further investigation.

P. viridicatum was reported as the main producer of citrinin (Friis et al. 1969; Krogh et al. 1973), but these were misidentifications of *P. verrucosum* (Pitt 1987).

Citrinin affects domestic animals, including dogs (Carlton et al. 1974) and pigs, where it causes porcine nephropathy (Rosa et al. 1985). Kidney degeneration is the cause, with similar kidney damage implicated in humans. *P. citrinum* is another producer of citrinin. It is also a ubiquitous species and is therefore very commonly isolated from numerous foods, including cereals, milled grain and flour (Frisvad 1988).

In cereals, *P. verrucosum* is the main producer of citrinin and ochratoxin A (OA). Citrinin and OA often co-occur, but it is OA which is isolated more frequently.

Cyclochlorotine and Islanditoxin

Produced by *P. islandicum*, cyclochlorotine and islanditoxin are two chlorine containing cyclic peptides. Both are highly toxic and cause liver dysfunctions (Scott 1977). *P. islandicum* is a common contaminant of rice, but again information on animal diseases caused by ingestion of contaminated feed are rare.

Erythroskyrin and Luteoskyrin

These two skyrins (dimeric anthraquinones), are produced by *P. islandicum*. Although less toxic than cyclochlorotine, they are liver and kidney toxins. Luteoskyrin is also carcinogenic. There are few reports of animal diseases (Pitt and Leistner, 1991). They are usually contaminants of rice, but there is little data to determine their real importance to animal health.

Ochratoxins

Krogh and Hassenlager (1968) originally identified ochratoxin A (OA), an acute nephrotoxin, as being produced by *P. viridicatum*. However, at this time, not all isolates tested produced OA (Stack et al. 1977). This was because isolates identified then as *P. viridicatum* represented a complex of species. Further studies divided it into separate sub-groups (Ciegler et al. 1973, 1981). Scanning electron micrographs (SEM) of these same isolates (Fig. 1) indicated at least three different species were involved. Of these it is now accepted that only *P. verrucosum sensu stricto* (Fig. 1*c*) produces OA and citrinin (Pitt 1987).

P. verrucosum is a widespread contaminant of cereals, notably in temperate zones (Pitt and Leistner 1991), and is particularly associated with Scandinavian barley (Frisvad and Viuf 1986). In a recent survey in Sweden (Olsen et al. 1993) high levels of OA were recorded in rye, bran products and brown kidney beans. In the U.K. OA has been found in soya beans, soya flour, maize, cornflour, nuts and cocoa beans (MAFF 1980).

OA plays a major role in nephritis of Scandinavian pigs (Krogh and Hasselager 1968). Being fat soluble it is not readily excreted, and therefore accumulates in fatty tissue. It is possible that OA may pose a serious threat to human health,

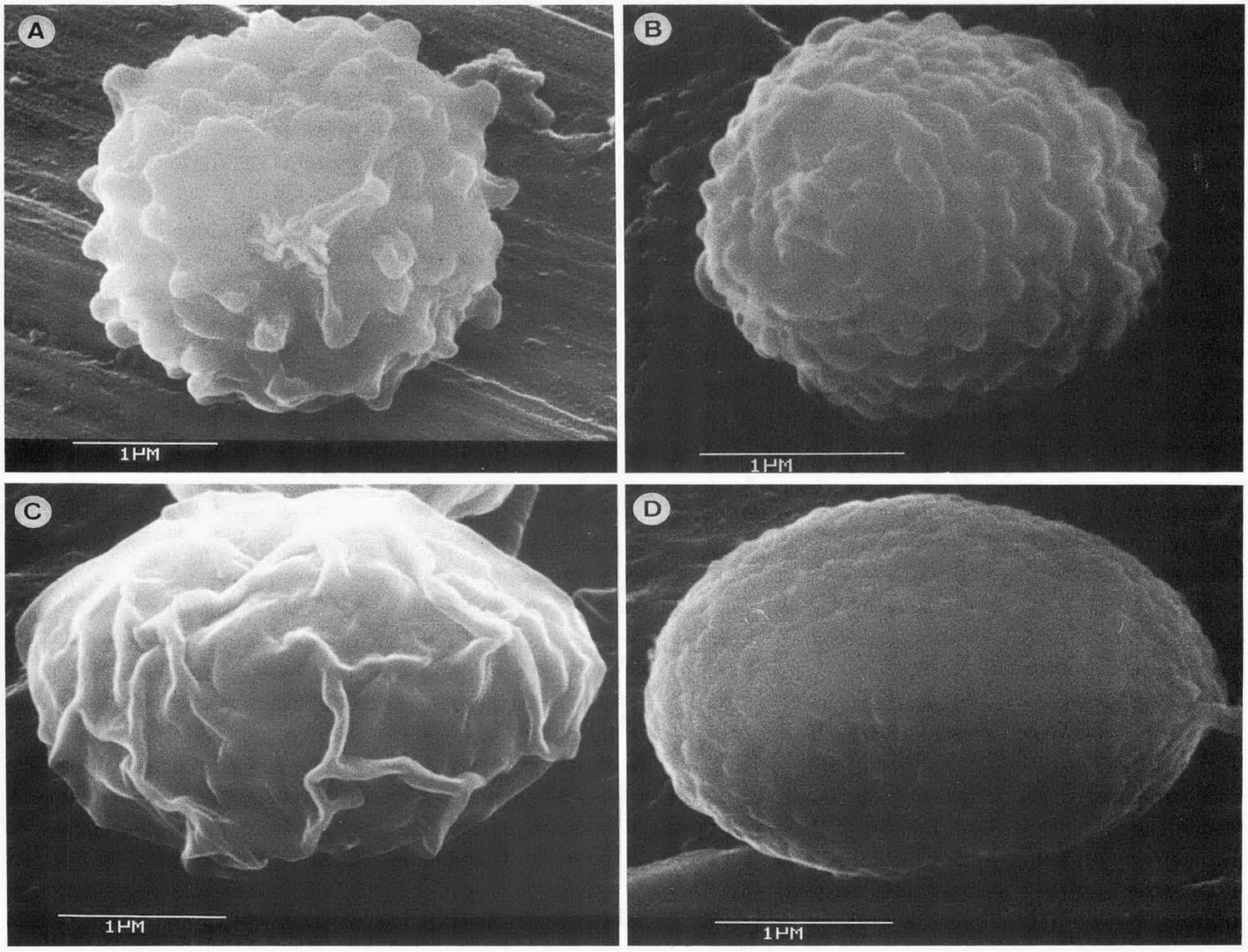

Fig. 1. SEM micrographs illustrating four spore ornamentations found amongst isolates labelled as *Penicillium viridicatum*. (A) *P. viridicatum*; (B) *P. viridicatum* (deteriorated strain); (C) *P. verrucosum*; (D) *P. aurantiogriseum*.

particularly in other large pork consuming countries such as Germany and eastern Europe. Indeed, *P. verrucosum* has been frequently isolated from meat products in Germany (Pitt and Hocking 1985).

OA has also been isolated recently from cow's milk in Sweden (Breitholtz-Emanuelsson et al. 1993), and pig products in the U.K. (MAFF 1993). In addition, OA has been implicated in Balkan endemic nephropathy, a kidney disease prevalent in Rumania, Bulgaria and the former Yugoslavia.

Patulin

The main producer of patulin is *P. expansum*, a species pathogenic on pomaceous fruits. The usual source of patulin in human consumption is apple and pear juice made from poor quality fruit. Recently, in the U.K., patulin was considered carcinogenic, and considerable quantities of these products were removed from retail store shelves. However, the Ministry of Agriculture rejected its carcinogenic significance on grounds that dosages through the oral route are microscopic.

Patulin is also produced by *P. roquefortii*, the cheese mould, though it is not formed under cheese-making conditions. Because of its ability to grow at low oxygen levels, *P.roquefortii* is very common in silage and airtight storage (Ceynowa 1986; Moreau 1979). It is a potential problem so far not investigated.

Penitrem A

The production of penitrem A was ascribed to *P. aurantiogriseum* by Wilson et al. (1968), to *P. palitans* by Ciegler (1969), and to *P. puberulum, P. martensii* and *P. crustosum* by Ciegler and Pitt (1970). However, Pitt (1979) concluded that only *P. crustosum* produces penitrem A in any large quantity and that all other attributions are based on misidentifications.

P. janczewskii, P. clavigerum and *P. glandicola* (= *P.granulatum*) also produce the toxin, but at low levels only (Frisvad and Lund 1993).

Penicillic acid

This toxin is a causal agent of the disease known as 'blue - eye' in maize in mid-western USA. The practice of shelling the maize while moist, followed by refrigeration before drying, will produce this disease (Kurtzman and Ciegler 1970).

In a study of the disease these authors isolated three penicillic acid producers, namely *P. puberulum, P. martensii* and *P.*

palitans. All three would today be identified as *P. aurantiogriseum* (a good producer of the toxin), or at least as members of the *P. aurantiogriseum* complex.

Other penicillic acid producers include *P. verrucosum* and *P. hordei* (Frisvad and Lund 1993). Since the toxin is inactivated by SH-groups (cysteine and glutathione) it is considered unlikely that penicillic acid would occur in wheat, barley, rye and oats (Frisvad and Lund 1993), a conclusion with which I concur.

Penicillic acid is produced under conditions of low temperature.

Roquefortine

Roquefortine C produced by *P. griseofulvum* is usually found in cereals stored in subtropical and tropical climates (Frisvad and Lund 1993). *P. griseofulvum* has also been isolated from cattle, poultry and pigfeed in Denmark, Norway, Australia and USA (Frisvad and Filtenborg 1989). I am unaware of any chemical studies involving this toxin.

Secalonic acid

Secalonic acid D produced by *P. oxalicum* is another mycotoxin classically found in cereals stored in subtropical and tropical areas (Frisvad and Lund 1993). It has also been found in southern USA on maize (Reddy and Reddy 1991), and in maize dust (Ehrlich et al. 1982) at levels of up to 4.5 mg/kg.

Viomellein, Vioxanthin and Xanthomegnin

These are naphthoquinones produced by both *Aspergillus* and *Penicillium* species. *Penicillium* producers include *P. viridicatum* and members of the *P. aurantiogriseum* complex (Frisvad and Lund 1993). Viomellein has been reported in barley (Hald et al. 1983), and studies in the United Kingdom have shown that these metabolites can occur at high levels in poorly stored cereals (Scudamore et al. 1986a,b). However, specific analytical methods for these metabolites are still insensitive. When more reliable methodology has been designed it is likely that compounds such as viomellein, xanthomegnin and vioxanthin will be recorded with more frequency from stored cereals.

They have been implicated in photosensitisation, and kidney and liver damage (Scudamore 1993).

Important *Aspergillus* Toxins and Their Occurrence

The taxonomy of *Aspergillus* is more clear-cut than that of *Penicillium*, with identification problems confined to a few groups. One such area is the Section *Flavi* where morphological differences between closely related species are minute (Kozakiewicz 1982, 1989). Correct identification in this section is of paramount importance since some of the species produce mycotoxins, whilst others are used in the food fermentation industry (Kozakiewicz 1984).

Most *Aspergillus* toxins can be placed into one of three groups: those which are carcinogenic, those which are nephrotoxins and those which are neurotoxins. Only a limited number is currently considered to pose a serious threat to human and animal health. A list of important *Aspergillus* species and their toxins is provided (see Table 2).

Table 2. Important mycotoxins produced by *Aspergillus* species and their teleomorphs

Species	Mycotoxins
A. clavatus	cytochalasin E, patulin, tryptoquivalins
A. fumigatus	fumigaclavines, fumigatin, fumitoxins, fumitremorgins, gliotoxin, tryptoquivalins, verruculogen
A. restrictus	mitogillin
Eurotium amstelodami	physions
E. chevalieri	physions
E. repens	physions
E. rubrum	physions
A. terreus	citreoviridin, citrinin, patulin, territrems
A. versicolor	nidulotoxin, sterigmatocystin
Emericella nidulans	nidultoxin, sterigmatocystin
A. candidus	terphenyllin, xanthoascin
A. flavus	aflatoxin B_1, B_2, aflatrem, kojic acid, maltoryzin, 3-nitropropionic acid
A. nomius	aflatoxin B_1, B_2, G_1, G_2, kojic acid
A. parasiticus	aflatoxin B_1, B_2, G_1, G_2, kojic acid, 3-nitropropionic acid
A. tamarii	cyclopiazonic acid, fumigaclavine A, kojic acid
A. niger	malformins, naphthopyrones
A. ochraceus	ochratoxin, penicillic acid, secalonic acid A, xanthomegnin, viomellein

Aflatoxins

Aflatoxins are common on produce high in oils, such as groundnuts, Brazil nuts, pistachio, almonds, walnuts and other edible nuts, cotton seed, palm kernels, copra, figs, spices and cereals such as maize, rice bran, sorghum and millet from warmer climates (Moss 1991; Scudamore 1993). Aflatoxin is not found in significant quantities on soybeans (Moss 1991).

The aflatoxin producers, *Aspergillus flavus* and *A. parasiticus*, are probably the most important of all mycotoxigenic species, since aflatoxin is carcinogenic. Isolates of these two species are maintained in all the major world collections and are used extensively for reference and as verified isolates for mycotoxin research. The integrity of isolate labels associated with such collections is rarely questioned. Kozakiewicz (1982) showed that the veracity of such labels was questionnable.

A. parasiticus was originally described from material isolated from sugarcane in Hawaii (Speare 1912). Subsequently, his original isolate was cultured and distributed worldwide. Scanning electron micrographs of all extant isolates revealed conidia with two distinct ornamentations or morphs (Fig. 2a,b). Isolates always consisted of one or other of these two morphs, but never mixtures of both (Kozakiewicz 1982). When these morphs were attributed to their respective cultures, a sharp dichotomy was revealed. One form occurs in the isolate derived from the type of *A. parasiticus* (Fig.2a), whilst the other has been established as that of *A. flavus* (Fig.2b) (Kozakiewicz 1982). In other words, cultures for the type of *A. parasiticus* held at three world collections are in fact *A. flavus*. There has been no confirmation that this mistake has been corrected other than at the International Mycological Institute, Egham, Surrey, England. Misidentifications of these two species are prevalent in cultures held in many collections. In routine examinations of IMI cultures 10 isolates have been re-identified to date.

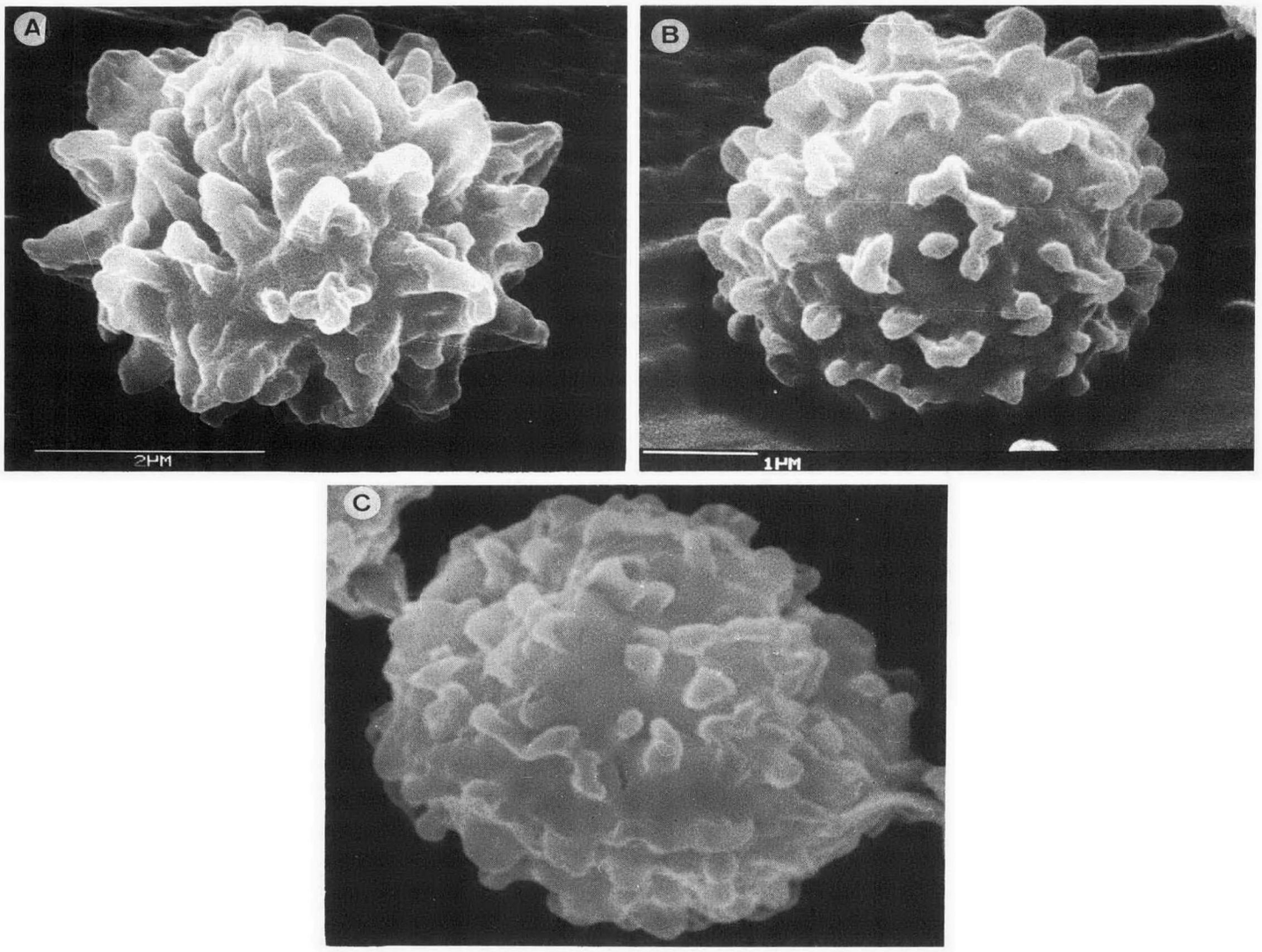

Fig. 2. SEM micrographs illustrating spore ornamentions in members of the Section *Flavi*. (A) *Aspergillus parasiticus*; (B) *A .flavus*; (C) *A. nomius*.

Such mistakes must lead to invalid conclusions and confusion concerning research into mycotoxin production. Further, a more serious situation must operate within geographic regions of taxonomic deficiency where not only will the level of competent identification be lower but sadly such inaccuracies may lead to total disassociation with the importance of the species as a biological unit.

The recent description of a new species, *Aspergillus nomius*, has further complicated the situation (Kurtzman et al. 1987). Morphologically it resembles *A. flavus* (compare Fig.2b and 2c), but differs by producing smaller more elongate sclerotia, those in *A. flavus* being more globose, and by the production of aflatoxin B_1, B_2, G_1, G_2, and an unique metabolite, nominine (Klich and Pitt 1988; Liljegren et al. 1988; Samson and Frisvad 1991). *A. flavus* produces only aflatoxin B_1 and B_2. An examination of *A. flavus* cultures in IMI showed one to be attributable to *A. nomius*. Others must exist.

The secondary metabolites which distinguish *A. flavus* and each of its related species are listed in Table 3. Included are *A. oryzae, A. sojae* and *A. tamarii*, species used in the food fermentation industry and considered to be 'safe' in that they do not produce aflatoxins. *A. oryzae* is thought to be a 'domesticated' form of *A. flavus*, and *A. sojae* a 'domesticated' form of *A. parasiticus*, in that they do not produce the corresponding aflatoxins (Kozakiewicz 1985). *A. tamarii* is a distinct species which produces kojic and cyclopiazonic acids, but additionally produces aspirochlorin, canadensolide and fumiclavine A (Frisvad and Samson 1991).

Cyclopiazonic acid (CPA)

The ability to produce CPA has been attributed to *Aspergillus versicolor* (Ohmomo et al. 1973). However, Domsch et al. (1980) showed that Ohmomo's isolate was a misidentification. According to Domsch et al. (1980) the correct identification was *Aspergillus oryzae*, a known producer of CPA. Unfortunately, the original mistake continues to be cited in the literature, even in substantial texts (Davis and Diener 1987; Golinski 1991; Smith and Ross 1991).

CPA is also produced by *A. flavus* and most strains of *A. tamarii* (Table 1) (Frisvad and Samson 1991). It has been detected in nuts, spices, meat and eggs (Frisvad 1988), and naturally contaminated agricultural products, where it has been noted to co-occur with aflatoxin (Smith and Ross 1991). Indeed, a survey conducted in Georgia, USA, on 50 samples of peanuts and 45 samples of maize, found that over 90% of peanuts and 50% of the maize contained CPA at levels similar to those found for aflatoxin (Takashi et al. 1992).

The toxicity of CPA has been demonstrated in many animals. Symptoms include severe gastrointestinal upset and neurological disorders after ingestion of CPA contaminated feed (Smith and Ross 1991). Affected organs of the digestive tract show degenerative changes and necrosis. Birds affected exhibit an unusual ducking movement which has been shown to be characteristic of CPA toxication (Cole 1986).

Ochratoxins

Ochratoxin A is produced by *Aspergillus ochraceus* mainly in hot climates and by *P. verrucosum* in temperate climates.

Ochratoxins, most commonly ochratoxin A, have been isolated worldwide from many products including cereals (maize, barley, oats, rye and sorghum), animal feedstuffs (Smith and Ross 1991), breakfast cereals, Japanese rice (Yoshizawa 1991) and European lagers (Payen et al. 1983).

Sterigmatocystin

Sterigmatocystin, produced mainly by *A. versicolor* and *Emericella nidulans*, is found in small grains such as wheat, barley, and rice, in animal feedstuffs and wheat and oat-based breakfast cereals (Yoshizawa 1991), spices and acid treated bread (Frisvad 1988).

It is a metabolite closely related to aflatoxin, and therefore acutely toxic, although at dose levels much higher than those necessary for aflatoxin poisoning (Cole and Cox 1981). Sterigmatocystin is highly carcinogenic, attacking the livers of test animals, causing hepatocellular carcinomas, and causing a wide range of tumour growths at the sites of application (Smith and Ross 1991).

Conclusions

Contamination of food and feedstuffs by *Aspergillus* and *Penicillium* species and their toxic metabolites is a serious problem worldwide. They have adverse effects on animal and human health and cause economic problems for international trade, in particular that of developing countries. Despite the wealth of literature on this topic, much more basic survey and research work needs to be done in order to determine the true extent of fungal and mycotoxin contamination and the associated clinical effects.

Therein lies the problem. Such research programs require adequately trained personnel, which are in short supply. A few specialists in taxonomy and systematics are to be found in Europe, North America, and Australia, but not in developing countries where many of the mycotoxin problems occur. In order that misidentifications on the scale discussed in this paper do not continue to occur in the future, and properly supported clinical studies can be undertaken, the following is proposed :

- Surveys conducted in order to assess species-toxin relationships for a wide variety of commodities.
- The methodology for identification and enumeration of mycotoxigenic fungi in foods and feedstuffs to be standardised.
- Fungal isolates used in taxonomic, biochemical and toxicological studies to be deposited in recognised culture collections, where they can be kept under optimal conditions.
- The identification of such isolates to be verified by a specialist.
- ICPA to regularly publish a list of misidentifications taken from the literature.
- Information on isolates and their properties to be stored in databases.
- Isolates of commercial value to be safety-deposited.
- The establishment of small research collections in developing countries, suitable to the local needs and resources.
- Adequate funding to be available to maintain such collections where they already exist, so that important isolates are not lost.
- Personnel running such collections to be properly trained in curation and identification.
- Communication between laboratories in developing and developed countries to be improved.

The International Commission on *Penicillium* and *Aspergillus* (ICPA) is already carrying out collaborative taxonomic studies and revising culture collection names. In addition, it is collating a set of reference cultures for *Aspergillus* and *Penicillium* toxigenic species with morphological and secondary metabolite profile descriptions. Such publications will be of use to all mycologists, chemists and toxicologists who need to accurately identify species within these two important genera.

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Table 3. Metabolites produced by *A.flavus* and related species

Species	Aflatoxin B_1, B_2	Aflatoxin G_1, G_2	Cyclopiazonic acid	Kojic acid	Nominine
A.flavus	+	-	+	+	-
A.parasiticus	+	+	-	+	-
A.nomius	+	+	-	+	+
A.oryzae	-	-	(+)	+	-
A.sojae	-	-	-	+	-
A.tamarii	-	-	+	+	-

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