CHAPTER 1.

Fungi producing significant mycotoxins

Summary

Mycotoxins are secondary metabolites of microfungi that are known to cause sickness or death in humans or animals. Although many such toxic metabolites are known, it is generally agreed that only a few are significant in causing disease: aflatoxins, fumonisins, ochratoxin A, deoxynivalenol, zearalenone, and ergot alkaloids. These toxins are produced by just a few species from the common genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Claviceps*. All *Aspergillus* and *Penicillium* species either are commensals, growing in crops without obvious signs of pathogenicity, or invade crops after harvest and produce toxins during drying and storage. In contrast, the important *Fusarium* and *Claviceps* species infect crops before harvest. The most important *Aspergillus* species, occurring in warmer climates, are *A. flavus* and *A. parasiticus*, which produce aflatoxins in maize, groundnuts, tree nuts, and, less frequently, other commodities. The main ochratoxin A producers, *A. ochraceus* and *A. carbonarius*, commonly occur in grapes, dried vine fruits, wine, and coffee. *Penicillium verrucosum* also produces ochratoxin A but occurs only in cool temperate climates, where it infects small grains. *F. verticillioides* is ubiquitous in maize, with an endophytic nature, and produces fumonisins, which are generally more prevalent when crops are under drought stress or suffer excessive insect damage. It has recently been shown that *Aspergillus niger* also produces fumonisins, and several commodities may be affected. *F. graminearum*, which is the major producer of deoxynivalenol and zearalenone, is pathogenic on maize, wheat, and barley and produces these toxins whenever it infects these grains before harvest. Also included is a short section on *Claviceps purpurea*, which produces sclerotia among the seeds in grasses, including wheat, barley, and triticale. The main thrust of the chapter contains information on the identification of these fungi and their morphological characteristics, as well as factors influencing their growth and the various susceptible commodities that are contaminated. Finally, decision trees are included to assist the user in making informed choices about the likely mycotoxins present in the various crops.

1. Introduction

Mycotoxins have been defined as "fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man or animals, including birds" (Pitt, 1996). This definition is widely accepted, although interpretation of "animals" is still under discussion in some quarters. The definition as it stands may be taken as including lower (invertebrate) animals; as it seems likely that some mycotoxins are indeed aimed at insects, such as the mites that prey on fungi, this interpretation is reasonable. However, most mycotoxicologists consider that mycotoxins are of relevance only when they affect humans and domestic animals, and this much narrower definition is used here.

It has been known for a long time that it is hazardous to eat some species of the larger fungi, i.e. mushrooms and "toadstools", but until comparatively recently the occurrence of common moulds on foods has generally been considered an aesthetic problem, not a health hazard. The realization that metabolites of some common foodborne fungi were responsible for animal disease and death came only in the 1960s, despite a few excellent studies in the first half of the 20th century. It is now well established that mycotoxins have been responsible for major epidemics in humans and animals during recent historical times. The most important epidemics have been ergotism, which has killed hundreds of thousands of people in Europe during the last millennium (Smith and Moss, 1985); alimentary toxic aleukia (ATA), which was responsible for the deaths of at least 100 000 people in the USSR between 1942 and 1948 (Joffe, 1978); stachybotryotoxicosis, which killed tens of thousands of horses in the USSR in the 1930s (Moreau, 1979); and aflatoxicosis, which killed 100 000 young turkeys in the United Kingdom in 1960 and has caused disease and death in many other animals, including humans (Rodricks *et al*., 1977; Lubulwa and Davis, 1994).

By general consent, the name "mycotoxin" is usually restricted to toxic compounds produced by microfungi and excludes toxins formed by the Basidiomycetes, the mushrooms or macrofungi consumed as foods in many parts of the world.

Many thousands of metabolites have been described from microfungi; even the large number dealt with in extensive reviews (Cole and Schweigert, 2003a, 2003b; Cole *et al*., 2003) are only a fraction of those known. It seems likely that many of these metabolites are produced not randomly but in attempts to alter the ecology surrounding the fungus, by inhibiting growth of competitor microorganisms, insects, etc. Only a limited number of compounds, a few hundred, are known to be toxic to humans or domestic animals.

2. Mycotoxigenic fungi

2.1 Which genera are important?

If we look at the worldwide occurrence of fungi in foods, and at which might be capable of mycotoxin production, three genera stand out: *Aspergillus*, *Penicillium*, and *Fusarium* (Pitt and Hocking, 2009). *Fusarium* species are destructive pathogens on cereal crops and other commodities, and produce mycotoxins before, or immediately after, harvest. Certain species of *Aspergillus* and *Penicillium* are also plant pathogens or commensals, but these genera are more commonly associated with commodities and foods during drying and storage. *Aspergillus flavus* is an exception: it can be a pathogen, a commensal (growing in a plant without affecting it), or a storage fungus, and it produces mycotoxins under all three conditions.

While not of worldwide significance, a fourth genus is of sufficient importance to be included here. *Claviceps* is pathogenic on a wide variety of cereals and other crops, producing resting structures called ergots, which often contain toxins.

2.2 Which mycotoxins are important?

As noted above, several hundred compounds are known to be toxic to humans or animals. However, many of these cause little concern because they are produced by fungi that are rarely encountered in foods or feeds. Many species of *Penicillium*, for example, are found almost exclusively in soils and rarely, if ever, in foods or feeds. Therefore, many highly toxic compounds produced by *Penicillium* species have not been found in foods or feeds in appreciable quantities. These include verruculogen, produced by *P. simplicissimum* and related species; janthitrems, produced by *P. janthinellum*; rugulosins, produced by *P. rugulosum* and *P. variabile*; and many others.

In a second category are compounds that are demonstrably toxic under some test conditions, e.g. by injection, but are not toxic when taken by a natural route. Compounds of this type may be inactivated by stomach acids or are so insoluble as to be excreted without harm. Sterigmatocystin, produced by the quite common storage fungus *Aspergillus versicolor*, is so insoluble in water, or acid, that its true toxicity to mammals has been difficult to measure, and it has not been known to cause illness.

In a third category, of potentially greater human health significance, are compounds that are produced by fungi known to occur in foods but that

under normal conditions are present in such low concentrations that they present no real hazard, i.e. their effects, if any, are not measurable. In most cases the reason is that although the fungi are readily isolated from some types of foods, they do not normally grow to the extent required to produce hazardous levels of toxin. Many examples exist: cyclopiazonic acid, from *Aspergillus flavus*; citrinin, from *Penicillium citrinin* and several other species; citreoviridin, produced by *P. citreonigrum* and *Eupenicillium ochrosalmoneum*; roquefortine, produced by *P. roqueforti* and related species; penitrem A, from *P. crustosum*; many of the trichothecenes produced by various *Fusarium* species; and tenuazonic acid, from *Alternaria* species. Under favourable growth conditions, however, the fungi in this category are capable of extensive growth and significant toxin production, so these and some other toxins should be kept in mind when fungal spoilage of foods and feeds occurs.

Some toxins are produced by rare species. For example, the species that produces rubratoxins is known from only a few isolates and does not even have a recognized name. Rubratoxin A is known to have caused one disease outbreak, in two people who consumed mouldy home-made rhubarb wine (Richer *et al*., 1997). However, rubratoxin can be ignored when overviewing mycotoxin occurrence worldwide.

A few mycotoxins are considered to be significant in feeds but not foods. These are of known toxicity to birds, in particular, and are mainly water-soluble toxins. The reason appears simple: whereas mammals excrete water-soluble toxins, often with little ill effect, birds excrete only solid waste, so are unable to get rid of these toxins so readily. In this category are cyclopiazonic acid, citrinin, and tenuazonic acid.

Some other mycotoxins are important in limited areas of the world. Sporidesmin is a mycotoxin that causes facial eczema in sheep. It is produced in pasture by the fungus *Pithomyces chartarum* in some areas of New Zealand and Australia, and can cause large economic losses in local areas. The fungus *Phomopsis leptostromiformis* produces the mycotoxin phomopsin in lupin plants and seeds in Western Australia, and phomopsin is of great importance to the cattle raising and lupin seed export industry in that state. However, its global impact is minimal.

Patulin is sometimes included in lists of important mycotoxins, and concentrations in foods are subject to regulatory control in some countries. Patulin is produced by the growth of *Penicillium expansum* in apples and pears. The production of significant levels of patulin is accompanied by visible rotting of the fruit, so patulin is primarily of concern in juices. Nearly all the toxin can be removed if rotting fruit are rejected by visual inspection or rotten parts are removed by hand trimming or by washing them out with high-pressure water jets. Hence, patulin in apple juices and other products is controllable by simple food technological procedures, and its occurrence does not warrant consideration here.

The mycotoxins treated in detail in this book are based on those considered by Miller (1995) to be the most important on a worldwide basis: aflatoxins, ochratoxin A, fumonisins, specific trichothecenes (deoxynivalenol and nivalenol), and zearalenone. These toxins are produced in foods and feeds by species of *Aspergillus*, *Penicillium*, and *Fusarium*. A limited taxonomic treatment of these fungi and the species producing important mycotoxins is given in this chapter. Also included is *Claviceps purpurea*, the species that produces ergot and ergot toxins in small grains.

3. Taxonomic overview of the fungal genera producing important mycotoxins

The genera *Aspergillus*, *Fusarium*, and *Penicillium* all reproduce by asexually produced spores, known as conidia, which are formed from specialized cells called phialides, where mitosis takes place and from which conidia are generated rapidly and in great numbers. Some species in each genus also produce a sexual stage defined by the production of asci, specialized cells that result from meiosis, usually in well-defined macroscopic bodies (up to 1 mm in diameter) called cleistothecia (Kirk *et al*., 2001). A few species produce hard resting cells called sclerotia, essentially immature cleistothecia.

In *Aspergillus* and *Penicillium*, phialides are borne in clusters, while conidia are single-celled, more or less spherical, and very small, usually not exceeding 5 µm in diameter. The two genera are closely related and are distinguished by the way in which phialides are grouped. In *Aspergillus*, phialides are always borne in tight clusters around the swollen apices (vesicles) of long stalks (stipes), with or without an intermediate row of supporting cells called metulae (Raper and Fennell, 1965; Pitt and Hocking, 2009; Samson *et al*., 2010); see Figs 1.1–1.5. In *Penicillium*, phialides are usually borne in finger-like clusters on more diminutive stipes, again with or without one or two intermediate rows of supporting cells (metulae and rami) (Pitt, 1979; Pitt and Hocking, 2009); see Fig. 1.6. Colonies of *Penicillium* species grown on identification media in Petri dishes are usually green, the colour of *Penicillium* conidia, and often have other pigments from the mycelium or excreted from the colonies. In *Aspergillus*, colony colours are those of the conidia, which may be black, yellow, brown, white, or green.

Colonies of most *Aspergillus* species show no other colours. *Fusarium* colonies generally consist of loose, fluffy mycelium, coloured white, pink, or purple, and often show similar colours in the colony reverse. In *Fusarium*, phialides are not clustered, and conidia may be of two types: those characteristic of the genus are large and crescent-shaped (although sometimes formed only under natural conditions or on special media), whereas the second type, produced by only some species, are small and usually cylindrical (Marasas *et al*., 1984; Pitt and Hocking, 2009; Samson *et al.* 2010); see Figs 1.7–1.9.

The taxonomy of all three genera is complex. Overall taxonomies for mycotoxigenic fungi occurring in foods are given in *Fungi and Food Spoilage* (Pitt and Hocking, 2009) and *Food and Indoor Fungi* (Samson *et al*., 2010). The most useful introductions to the major species in each genus are found in laboratory guides: *A Laboratory Guide to Common* Penicillium *Species* (Pitt, 2000), *Identification of Common* Aspergillus *Species* (Klich, 2002), and *The* Fusarium *Laboratory Manual* (Leslie and Summerell, 2006).

Claviceps differs from the three genera mentioned above because most species cannot be cultivated in the laboratory. Species of *Claviceps* grow on a wide variety of grasses, where they infect only the ovaries, forming hard bodies called sclerotia that replace normal seed heads. Conidia are released in droplets called honeydew, attractive to insects, which then disseminate the fungus throughout the crop.

The taxonomy (classification) of these genera is described in more detail below.

4. Genus *Aspergillus*

Aspergillus is a large genus, with 100 or more recognized species, most of which grow and sporulate well on common synthetic or semisynthetic media. The most widely used taxonomy is by Raper and Fennell (1965), although some of their concepts are out of date. For more modern taxonomic concepts, see Samson and Pitt (1990) or Klich (2002). A minority of *Aspergillus* species make a sexual stage (known as a teleomorph) in which spores (ascospores) are borne in asci, in turn borne in cleistothecia. Species with teleomorphs are correctly classified in teleomorph genera, of which *Eurotium*, *Neosartorya*, and *Emericella* are the best known. Few of the important mycotoxigenic species produce teleomorphs.

The most significant mycotoxigenic species in *Aspergillus* are *A. flavus* and *A. parasiticus*, which make aflatoxins, and the species that make ochratoxin A: *A. ochraceus* and related species, the black species *A. carbonarius*, and (uncommonly) *A. niger. A. flavus* and *A. parasiticus* are very closely related and are treated together.

4.1 Fungi producing aflatoxins: A. flavus *and* A. parasiticus

Taxonomy. Aflatoxins are now known to be produced by at least 10 *Aspergillus* species. However, most are rare or are rarely found in foods: the principal fungi producing aflatoxins remain *A. flavus* and *A. parasiticus*. Of some importance is a new species found in groundnuts in the southern hemisphere, called *A. minisclerotigenes*. This species looks like a variant of *A. flavus* that produces unusually small sclerotia, but like *A. parasiticus* it produces both B and G aflatoxins. The other species of some importance is *A. nomius*, which also makes B and G aflatoxins and looks like *A. flavus* but produces bullet-shaped sclerotia. *A. nomius* is associated with insects, and not usually with foods, but recently has been shown to be a common cause of aflatoxin production in Brazil nuts (Olsen *et al*., 2008).

Enumeration. Satisfactory enumeration of *A. flavus* and *A. parasiticus* can be achieved on any antibacterial enumeration medium that contains appropriate inhibitors to reduce colony spreading. Dichloran rose bengal chloramphenicol agar (DRBC) or dichloran 18% glycerol agar (DG18) are recommended (Pitt and Hocking, 1997, 2009; Samson *et al*., 2010). Relatively rapidly growing, moderately deep, yellow green colonies exhibiting "mop-like" fruiting structures under the stereomicroscope can be presumptively counted as *A. flavus* plus *A. parasiticus*. Microscopic examination of colonies can provide supporting evidence, but representative colonies must be grown on standard identification media for confirmation.

A more effective medium for enumerating these species is Aspergillus flavus and parasiticus agar (AFPA; Pitt *et al*., 1983), a medium formulated specifically for this purpose. AFPA has two major advantages: enumeration can be carried out after incubation at 30 °C for 2 days, and both species are readily recognized, even by untrained eyes, by intense orange yellow colours in the reverses of the colonies. Under the incubation conditions specified for AFPA (30 °C for 42–48 hours), the presence of the bright orange yellow reverse is diagnostic for these species.

Descriptions. The descriptions of *Aspergillus* species given here are taken from *Fungi and Food Spoilage* (Pitt and Hocking, 2009). The fungi are grown as colonies on Czapek yeast extract agar (CYA) and malt extract agar (MEA) at 25 °C and CYA at 37 °C for 7 days. Fungi are inoculated onto these plates at three

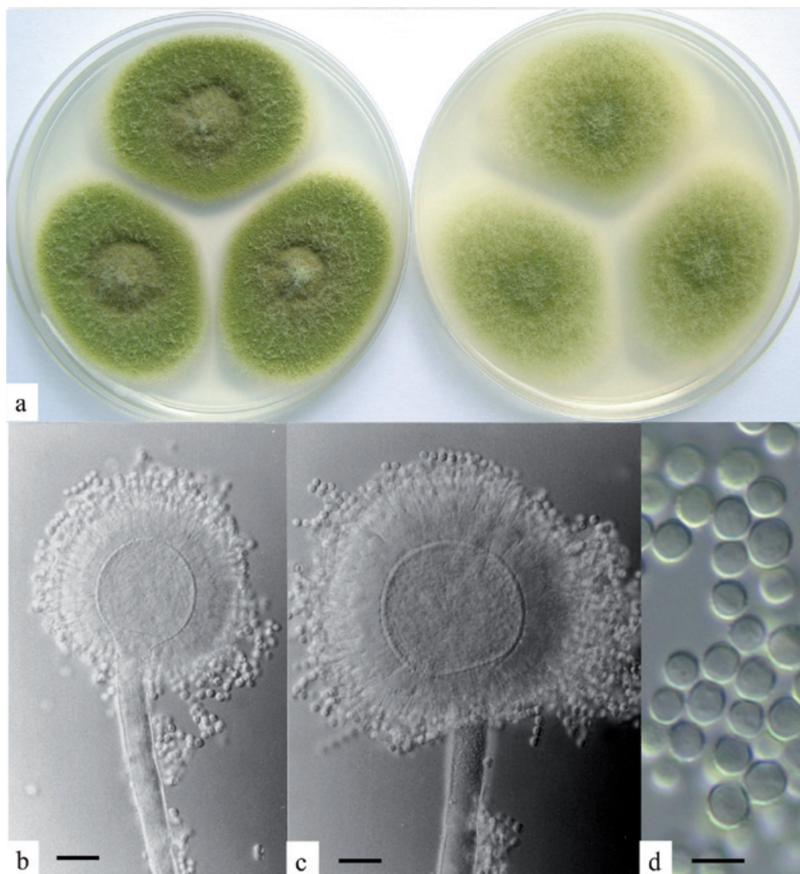
points, equidistant from each other and midway between the rim and centre of the Petri dish. Inoculation is facilitated by first dispersing a needle point of conidia in small vials containing 0.2 mL of 0.2% agar and 0.05% Tween 80 or similar detergent (Pitt and Hocking, 2009), as this reduces colonies from stray spores. Formulations for these and other media are given in the Annex (p. 29).

Aspergillus flavus Link. See Fig. 1.1. Colonies on CYA 60–70 mm in diameter; conidial heads usually borne uniformly over the whole colony but sparse or absent in areas of floccose (cotton wool) growth or sclerotial production, characteristically coloured greyish green but sometimes pure yellow, becoming greenish in age; sclerotia produced by about 50% of isolates, at first white, becoming deep reddish brown, density varying from inconspicuous to dominating colony appearance and almost entirely suppressing conidial production. Colonies on MEA 50–70 mm in diameter, similar to those on CYA, although usually less dense. At 37 °C, colonies usually 55–65 mm in diameter, similar to those on CYA at 25 °C.

Sclerotia spherical, usually 400–800 μm in diameter. Teleomorph developing from sclerotia, but only after selected isolates are mated. Structures bearing conidia 400 μm to 1 mm or more long; vesicles (terminal swellings) spherical, 20–45 μm in diameter, fertile over three quarters of the surface, typically bearing both phialides and metulae (cells supporting phialides), but in some isolates a proportion of, or even most, heads bear phialides alone; conidia spherical or nearly, usually 3.5–5.0 μm in diameter, with relatively thin walls, finely roughened or, rarely, smooth.

The teleomorph is *Petromyces flavus* (Horn *et al*., 2011), but in culture is seen only after suitable strains are mated (Horn *et al*., 2009b).

Fig. 1.1. *Aspergillus flavus* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c) heads, bars = 20 μm; (d) conidia, bar = 5 μm. Source: Pitt and Hocking (2009), Fig. 8.13, p. 305; reproduced with kind permission from Springer Science+Business Media B.V.



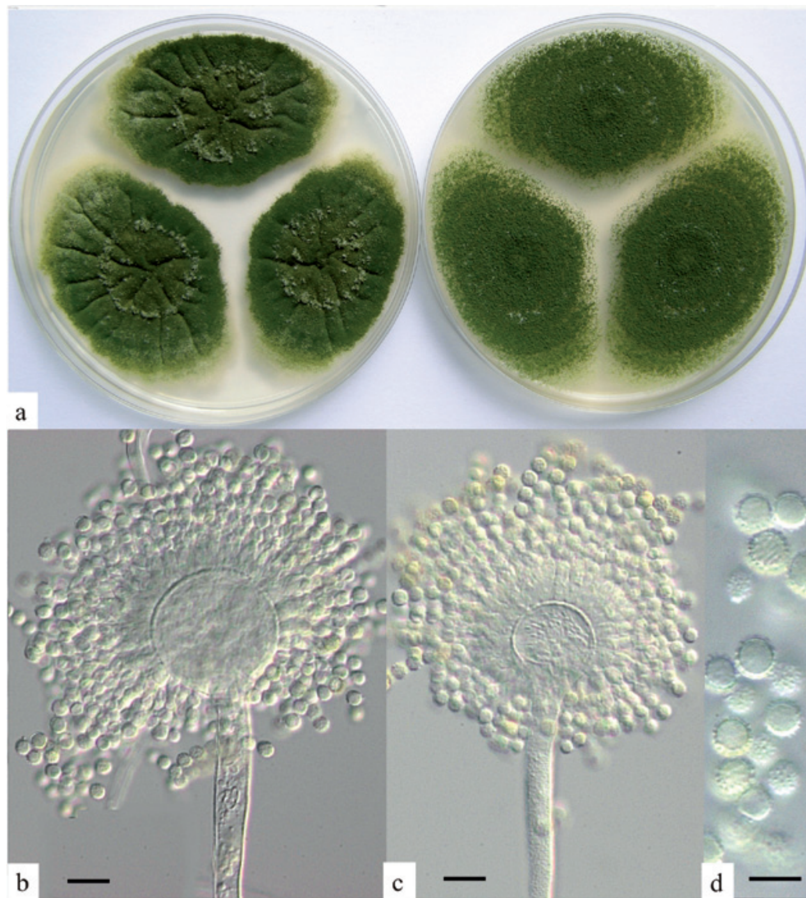
Aspergillus parasiticus Speare. See Fig. 1.2. Colonies on CYA 50–70 mm in diameter, conidial heads in a uniform, dense layer, coloured dark yellowish green; sclerotia occasionally produced. Colonies on MEA 50–65 mm in diameter, generally similar to those on CYA. At 37 °C, colonies covering the available area, similar to those on CYA at 25 °C.

Sclerotia occasionally produced, white at first, becoming black, spherical, 400–800 μm in diameter. Teleomorph developing from sclerotia, but only after selected isolates are mated. Structures bearing conidia 250–500 μm long; vesicles spherical, 20–35 μm in diameter, fertile over three quarters of the surface, mostly bearing phialides only, but in some isolates up to 20% of heads bearing metulae as well; conidia spherical, mostly 4.0–6.0 μm in diameter, with distinctly roughened walls.

The teleomorph is *Petromyces flavus* (Horn *et al*., 2011), but in culture is seen only after suitable strains are mated (Horn *et al*., 2009a).

Distinctive features. *Aspergillus flavus* and *A. parasiticus* together are distinguished by their rapid growth at both 25 °C and 37 °C and their bright yellow green (or, less commonly, yellow) conidial colour. The definitive difference between the two species is that *A. flavus* produces conidia that are rather variable in shape and size,

Fig. 1.2. *Aspergillus parasiticus* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c) heads, bars = 10 μm; (d) conidia, bar = 5 μm. Source: Pitt and Hocking (2009), Fig. 8.18, p. 321; reproduced with kind permission from Springer Science+Business Media B.V.



have relatively thin walls, and range from smooth to moderately rough, the majority being finely rough. In contrast, conidia of *A. parasiticus* are spherical and have relatively thick, rough walls. In addition, vesicles of *A. flavus* are larger, up to 50 μm in diameter, and usually bear metulae, whereas vesicles of *A. parasiticus* rarely exceed 30 μm in diameter and metulae are uncommon (Klich and Pitt, 1988). The teleomorphs are not seen in pure culture of single isolates.

Differentiating toxigenic from non-toxigenic isolates of *A. flavus* or *A. parasiticus* can also be of value. A variety of media have been proposed to achieve this, one of which is coconut cream agar (Dyer and McCammon, 1994). Recently, the use of a cyclodextrin, incorporated into any standard medium, has been proposed (Jaimez Ordaz *et al*., 2003). Visualization of aflatoxin production is by examination of the reverse of colonies on Petri dishes under ultraviolet (UV) light.

Factors influencing growth. Growth temperatures for *A. flavus* most often reported are a minimum of 10–12 °C, a maximum of 43–48 °C, and an optimum of about 33 °C (Pitt and Hocking, 2009). The minimum water activity (a_w) permitting growth is 0.82 at 25 °C, 0.81 at 30 °C, and 0.80 at 37 °C (Pitt and Miscamble, 1995). A predictive model for *A. flavus* growth in relation to a_w and temperature was derived from those data (Gibson *et al*., 1994). Growth of *A. flavus* occurred over the pH range 2.1–11.2 (the entire range examined) at 25, 30, and 37 °C, with optimal growth over a broad pH range of 3.4–10 (Wheeler *et al*., 1991).

The heat resistance of *A. flavus* has been studied under various conditions by several authors. The most reliable figures indicate a D_{45} value (the time required at 45 °C to kill 90% of the population) of more than 160 hours, a D_{50} of 16 hours, a D_{52} of 40–45 minutes, and a D_{60} of 1 minute, at neutral pH and high a_w, with z values (the increase in temperature required to reduce the D value by 90%) of 3.3–4.1 C° (ICMSF, 1996).

The addition of phosphine, used to control insects, to grain at 0.80 or 0.86 a_w reduced growth of *A. flavus* while having little effect on the survival of conidia (Hocking and Banks, 1991, 1993).

Available data indicate that the influence of physical factors on the growth of *A. parasiticus* is very similar to that on *A. flavus* (Pitt and Hocking, 2009). However, *A. parasiticus* grows at somewhat lower temperatures, up to 42 °C. The effect of a_w is similar to that found for *A. flavus* (Pitt and Miscamble, 1995).

4.1.1 Commodities and foods at risk from aflatoxin contamination

Aspergillus flavus and to a lesser extent *A. parasiticus* have been isolated from a very wide range of food commodities (Pitt and Hocking, 2009). Indeed *A. flavus* may be regarded as truly ubiquitous in foods produced in tropical and subtropical countries. Although evidence of *A. flavus* at low levels in foods cannot be taken as an indicator of the presence of aflatoxins, high levels of infection, i.e. plate counts of greater than 10^5/g, or infection levels of more than

50% of grains when direct plated, provide reasonable presumptive evidence that aflatoxin may be present. However, quantification of the association between levels of *A. flavus* infection and aflatoxin production is not possible, so these figures can only serve as guidelines.

Although it is possible to induce aflatoxin production in a very wide range of foods or raw materials under experimental conditions, research and experience have shown that only certain commodity types are likely to contain aflatoxin in the absence of obvious signs of fungal growth or other deterioration in appearance.

Based on the results of many surveys, commodities most at risk in international trade are groundnuts, maize, and cottonseed. Lesser, but still substantial, risk is associated with tree nuts of all types, especially Brazil nuts, pistachio nuts, and semi-processed coconuts (i.e. copra). Walnuts, hazelnuts, and cashews are only occasionally affected. Spices from tropical countries are also a frequent source of aflatoxin, but these spices are usually present at only low levels in foods. Oilseeds of all kinds are affected from time to time (Pohland and Wood, 1987), and figs may carry a substantial risk (Le Bars, 1990).

Aflatoxins have been reported from smoked and dried or cured fish in Sierra Leone (Jonsyn and Lahai, 1992) but are not considered to be a problem in salted dried fish produced under South-East Asian conditions (Pitt and Hocking, 1996). Other meat products, including prepared hams (Rojas *et al*., 1991), are not considered to be at risk.

Cereals, legumes, and pulses may also be infected with *A. flavus* (Pitt and Hocking, 2009), but unacceptable levels of aflatoxin occur only under poor storage conditions and are rarely of concern.

Aspergillus flavus is capable of causing spoilage of some kinds of fresh fruit and vegetables, including citrus, tomatoes, peppers, litchis, pineapples, and pomegranates (Snowdon, 1990, 1991), but aflatoxin production is unlikely.

4.1.2 Formation of aflatoxins in susceptible crops

Groundnuts. Groundnuts are susceptible to infection by both *A. flavus* and *A. parasiticus* (Hesseltine *et al*., 1973; Diener *et al*., 1987; Pitt and Hocking, 2009). The primary source of these fungi is soil, where high numbers may build up because some groundnuts are not harvested but remain in the ground and act as a nutrient source (Griffin and Garren, 1976a). Uncultivated soils contain very low numbers of *A. flavus*, but soils in groundnut fields usually contain 100–1000 propagules/g (Pitt, 1989). Under drought stress conditions, this number may rise to 10^4–10^5/g (Horn *et al*., 1995). Large numbers of *A. flavus* spores are also airborne over susceptible crops (Holtmeyer and Wallin, 1981).

Direct entry to developing groundnuts through the shell by *A. flavus* in the soil appears to be the main method for nut infection (Diener *et al*., 1987). Infection can also occur through the pegs and flowers (Wells and Kreutzer, 1972; Griffin and Garren, 1976b; Pitt, 1989). *A. flavus* sometimes grows within groundnut plants themselves: growth in plant tissue is not pathogenic but commensal. The seedpod (Lindsey, 1970) or the plant (Pitt, 1989; Pitt *et al*., 1991) show no visible sign of colonization by the fungus.

A variety of factors influence invasion of developing groundnuts by *A. flavus*. Infection before harvest occurs only if substantial numbers of fungal propagules (perhaps 10^3/g) exist in the soil. Other important factors are drought stress (Sanders *et al*., 1981) and soil temperatures around 30 °C (Blankenship *et al*., 1984; Sanders *et al*., 1984; Cole *et al*., 1985; Cole, 1989; Dorner *et al*., 1989) during the last 30–50 days before harvest (Sanders *et al*., 1985).

Maize. Maize is usually infected only by *A. flavus*, not by *A. parasiticus* (Lillehoj *et al*., 1980; Angle *et al*., 1982; Horn *et al*., 1995). It appears probable that the most important route for entry of *A. flavus* to maize is through insect damage (Lillehoj *et al*., 1982; Bilgrami *et al*., 1992). Invasion down the silks is also possible (Marsh and Payne, 1984; Diener *et al*., 1987). High temperature stress increases infection (Jones *et al*., 1980), the critical time for infection being between 16 and 24 days after inoculation at silking (Jones *et al*., 1980; Payne, 1983).

Cottonseed. *A. flavus* is also a commensal in the cotton plant (Klich *et al*., 1984). Infection occurs through the nectaries, natural openings in the cotton stem that are important in pollination (Klich and Chmielewski, 1985), or through cotyledonary leaf scars (Klich *et al*., 1984). Upward movement occurs in the stem towards the boll, but not downwards from boll to stem (Klich *et al*., 1986). Insect damage is also a potential cause of infection (Lee *et al*., 1987), but insects are often well controlled in cotton crops. As in groundnuts and maize, temperature appears to be a major environmental factor in pre-harvest infection of cottonseed (Marsh *et al*., 1973; Simpson and Batra, 1984). High minimum temperatures, above 24 °C, appear to lead to high aflatoxin formation (Diener *et al*., 1987).

4.1.3 Formation of aflatoxins in other crops

With other crops, *Aspergillus flavus* is not associated with the plant, so entry to nuts or seeds or other food parts is opportunistic and usually occurs

only after the crop matures. Entry of *A. flavus* into pistachio nuts depends on the time of splitting of hulls. Nuts in which hull splitting occurs early are much more susceptible to *A. flavus* invasion on the tree (Doster and Michailides, 1994). It is known that some cultivars are more prone to early splitting than others, and this is especially important where nuts are harvested from the ground, after contact with the soil.

Brazil nuts are harvested from the ground, beneath trees growing naturally in Amazonian forests. Harvesting is intermittent, up to a month apart, providing time for the ever present *A. flavus* and other related species to infect the nuts (Johnsson *et al*., 2008). *A. nomius* appears to be an important source of aflatoxins in Brazil nuts (Olsen *et al*., 2008).

In other tree nuts, formation of aflatoxins occurs sporadically, usually as the result of insect damage or poor storage practices.

Figs are sometimes infected by *A. flavus*. The unique structure of the fruit evolved to enable fertilization by insects, and insects may carry *A. flavus* spores into the seed cavity. Also, figs are harvested from the ground in some countries. Immature figs are not colonized by *A. flavus*, but once they are ripe infection occurs readily and fungal growth continues during drying (Buchanan *et al*., 1975; Le Bars, 1990). The proportion of the crop infected is low, 1% or less (Steiner *et al*., 1988). The problem has been serious for some exporting countries (Sharman *et al*., 1991) but is now well controlled by examination of individual figs under UV light.

4.1.4 Formation of aflatoxins in storage

Crops susceptible to aflatoxin formation are mostly nuts and oilseeds, where soluble solids (sugars) in the dried commodity are low, and oil content high. Sorption isotherms of these commodities are similar (Iglesias and Chirife, 1982). *A. flavus* and *A. parasiticus* cannot grow below about 0.80 a_w, equivalent to about 10% moisture content in these commodities. However, storage above 8% moisture content (about 0.7 a_w) can lead to fungal spoilage. Fungal growth may result in a moisture increase, creating conditions under which *A. flavus* can grow, so 8% moisture must be considered the safe moisture content for these commodities.

Such a low moisture content can be difficult to maintain in practice. Shipment of nuts in containers across the tropics is a particular hazard as unsuitable stowage, on decks or near engines, can lead to moisture migration sufficient to cause sporadic spoilage or even total loss. Cases of rampant growth of *A. flavus* accompanied by high aflatoxin production have been observed under these conditions.

Storage of nuts in tropical countries is sometimes inadequate, again leading to spoilage or aflatoxin production.

4.2 Ochratoxin A

4.2.1 Formation of ochratoxin A

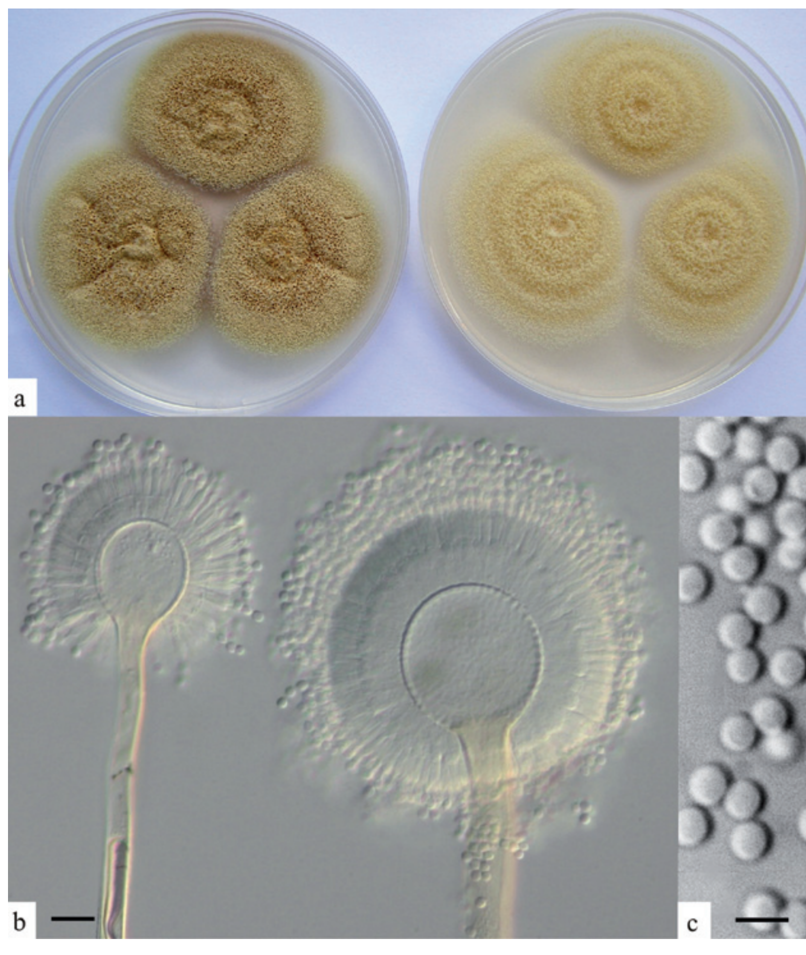
Ochratoxin A (OTA) was originally described as a metabolite of *Aspergillus ochraceus* from laboratory experiments (van der Merwe *et al*., 1965), and subsequently from several related *Aspergillus* species. However, the first report of natural occurrence, and the potential importance, of OTA was from a *Penicillium* species (Scott *et al*., 1970; Krogh *et al*., 1973), reported as *P. viridicatum*, but later corrected to *P. verrucosum* (Pitt, 1987). Larsen *et al*. (2001) reported that *P. nordicum*, closely related to *P. verrucosum*, is also a producer of OTA. Many reports refer to OTA production by other, often unspecified, *Penicillium* species, but these reports are known to be erroneous (Frisvad 1989; Frisvad and Filtenborg, 1989; Pitt and Hocking, 2009).

Recently, *Aspergillus carbonarius* has been identified as a third major source of OTA, together with a low percentage of isolates of the closely related species *A. niger* (Abarca *et al*., 1994; Téren *et al*., 1996). It is now clear that OTA is produced by two closely related *Penicillium* species, *P. verrucosum* and *P. nordicum*, and by a rather remarkable range of *Aspergillus* species. The following sections deal with these species in more detail.

4.2.2 Aspergillus ochraceus *and related species*

Taxonomy. Recent work has shown that *Aspergillus ochraceus* is not a common producer of OTA. Most OTA in foods originally attributed to *A. ochraceus* is now known to be due to *A. westerdijkiae* and *A. steynii*, newly described species very similar morphologically to *A. ochraceus* (Frisvad *et al*., 2004). Apart from these species, this group of OTA producers includes two ascosporic fungi, *Neopetromyces muricatus* (asexual state *A. muricatus*) and *Petromyces alliaceus* (asexual state *A. alliaceus*), and two that do not produce a teleomorph, *A. sclerotiorum* and *A. sulphureus*. *N. muricatus* is the correct name for isolates that produce OTA, previously identified as *A. melleus*. Both *N. muricatus* and *P. alliaceus* are uncommon species. *A. sclerotiorum* isolates produce OTA only rarely, and although isolates of *A. sulphureus* are usually OTA producers, this is a rare species. Apart from *A. westerdijkiae* and *A. steynii*, all of these species are very uncommon in foods and are not known to cause food spoilage.

Fig. 1.3. *Aspergillus ochraceus* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b) heads, bar = 20 µm; (c) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.17, p. 318; reproduced with kind permission from Springer Science+Business Media B.V.



Enumeration. *Aspergillus ochraceus*, *A. westerdijkiae*, and *A. steynii* grow slowly on media of high a_w as they are all xerophilic. Enumeration on a medium of reduced a_w, such as DG18, is recommended (Pitt and Hocking, 2009).

Colonies of *A. ochraceus* and closely related species can be presumptively recognized by relatively deep colonies, uniformly coloured pale brown to yellow brown, that under the low-power stereomicroscope exhibit long fruiting stalks bearing radiate *Aspergillus* heads, with spore chains splitting into two or three dense columns in age. Confirmation of identity by growth in pure culture is necessary.

Satisfactory enumeration should usually be possible also on DRBC, a selective medium of higher a_w (King *et al*., 1979). Dilute media, such as potato dextrose agar (PDA) or bacteriological enumeration media, and incubation temperatures above 25 °C, are unsatisfactory.

Aspergillus ochraceus Wilhelm. See Fig. 1.3. Colonies on CYA 40–55 mm in diameter; conidial heads closely packed, coloured light to golden yellow; sclerotia sometimes produced, white when young, later pink to purple. Colonies on MEA 40–55 mm in diameter, plane, similar to those on CYA but quite sparsely sporing. At 37 °C, colonies of 25–30 mm in diameter produced.

Structures bearing conidia 1.0–1.5 mm long, with yellowish to pale brown walls, finely to conspicuously roughened; vesicles spherical, 25–50 µm in diameter, bearing tightly packed metulae and phialides over the entire surface; conidia spherical or near, 2.5–3.5 µm in diameter, with smooth to finely roughened walls.

Distinctive features. *Aspergillus ochraceus* and closely related species producing ochratoxin all grow moderately slowly on standard identification media such as CYA and MEA (Pitt and Hocking, 2009). Colonies are coloured pale brown to yellow brown from the conidia. *A. ochraceus* grows strongly at 37 °C, whereas the closely related species *A. westerdijkiae* and *A. steynii* do not grow at that temperature. Apart from that distinction, *A. westerdijkiae* produces spherical, finely roughened conidia, whereas those of *A. steynii* are smooth-walled and ellipsoidal, not spherical.

Factors influencing growth. *Aspergillus ochraceus* and the closely related species described here are mesophilic xerophiles. Growth occurs between 8 °C and 37 °C, with the optimum at 24–31 °C (Pitt and Hocking, 2009). Optimal conditions for growth are 0.95–0.99 a_w, while the lower limit for growth is 0.79 a_w on media containing sugars and down to 0.81 a_w on media based on NaCl. *A. ochraceus* grows slowly at pH 2.2 and well between pH 3 and 10 (Pitt and Hocking, 1977).

Commodities and foods at risk. *Aspergillus ochraceus* has been reported from a wide range of food products, more commonly in dried and stored foods than elsewhere. However, it is likely that many of these reports relate to the recently described *A. westerdijkiae* or *A. steynii*. Stored foods from which these species have been isolated include

smoked or salted dried fish and meat, beans, chickpeas, and nuts, especially pecans and pistachios. These species have been reported (usually as *A. ochraceus*) from cereals and cereal products but, rather infrequently, also from cheese, spices, black olives, and cassava. However, these species rarely cause spoilage, and are often found in foods at only low levels, so their presence is not a good indicator of significant mycotoxin production (Pitt and Hocking, 2009).

Several studies have detected *A. ochraceus* in green coffee beans (Levi *et al*., 1974; Cantafora *et al*., 1983; Tsubouchi *et al*., 1984; Micco *et al*., 1989; Studer-Rohr *et al*., 1994). Coffee cherries are usually picked by hand, or sometimes mechanically on large farms, and are usually dried in the sun. The beans may be dried directly and separated from the hull afterwards, or mechanically dehulled and dried, or dehulled by fermentation before drying. Coffee beans are stored after drying (as "green" coffee), then graded and shipped to manufacturers.

Picking cherries and spreading them on drying yards frequently causes damage, allowing ingress of fungi. If cherries are picked from the ground, contamination is likely to be high. Drying is often a slow process, in particular because of the environment in which coffee is grown. Coffee trees will not flower above 19 °C but require high temperatures to mature, so coffee is commonly grown on upland areas in the tropics. In consequence, drying is often conducted under less than ideal conditions, with morning mists or rain common in some growing areas (Teixera *et al*., 2001). Fungal growth frequently occurs.

Although the possibility of significant levels of OTA being present in coffee beans has been known for some time, the fungal cause remained elusive. Only recently has it been established that *Aspergillus ochraceus*, and no doubt its close relatives *A. westerdijkiae* and *A. steynii*, are major sources of OTA in coffee (Taniwaki *et al*., 1999, 2003; Pitt *et al*., 2001; Batista *et al*., 2003). Other known OTA producers, *A. niger* and *A. carbonarius*, have also been isolated from coffee (Frank, 2001; Pitt *et al*., 2001). As detailed mycological studies have not yet been conducted in some major coffee growing areas, the relative importance of *A. ochraceus* and *A. carbonarius* as the main source of OTA in coffee is difficult to assess.

Available evidence indicates that the sources of these fungi are environmental and that entry to cherries is gained during picking and drying (Taniwaki *et al*., 1999). OTA is produced during drying (Taniwaki *et al*., 1999; Bucheli *et al*., 2000; Teixera *et al*., 2001). Coffee picked and dried under good agricultural practice appears to contain OTA only rarely (Taniwaki *et al*., 1999, 2003).

4.2.3 Aspergillus carbonarius *and related species*

Taxonomy. *Aspergillus carbonarius* was recognized as a source of OTA relatively recently (Horie, 1995; Téren *et al*., 1996; Wicklow *et al*., 1996). It is now known that most, if not all, isolates of *A. carbonarius* produce OTA when grown in pure culture (Heenan *et al*., 1998; Taniwaki *et al*., 1999), although the extent of production is variable. The closely related species *A. niger* has also been reported reliably as a producer (Ueno *et al*., 1991; Abarca *et al*., 1994; Heenan *et al*., 1998; Taniwaki *et al*., 1999). However, all reports agree that OTA production by *A. niger* is very uncommon; OTA is formed under pure culture conditions by only 1–2% of isolates.

Enumeration. Satisfactory enumeration of *A. niger* and *A. carbonarius* and the closely related (but always non-toxigenic) species *A. japonicus* can be achieved on any antibacterial enumeration medium that contains appropriate inhibitors to reduce colony spreading. DRBC or DG18 is recommended (Pitt and Hocking, 1997; Samson *et al*., 2010). Rapidly growing, very dark brown to black colonies exhibiting "mop-like" fruiting structures under the stereomicroscope can be presumptively counted as *A. niger* plus *A. carbonarius*. Microscopic examination of colonies can provide supporting evidence, but representative colonies must be grown on standard identification media for confirmation.

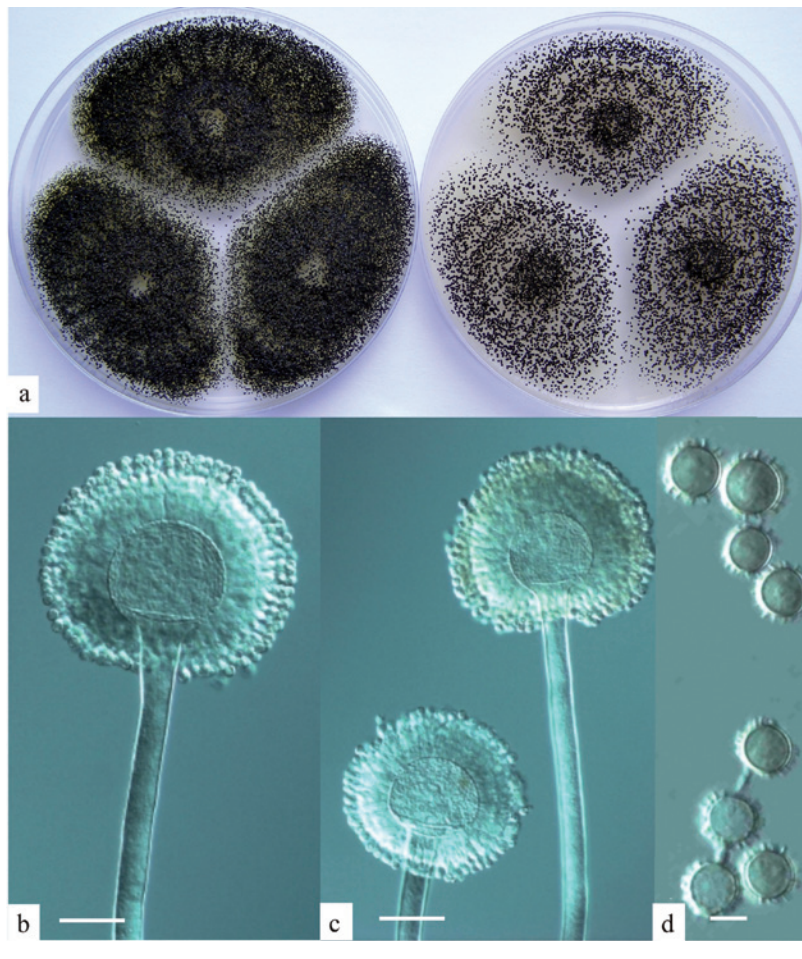
Aspergillus carbonarius (Bainier) Thom. See Fig. 1.4. Colonies on CYA 60 mm or more in diameter, usually covering the whole Petri dish; conidia black or nearly black. Colonies on MEA 50–60 mm in diameter, usually smaller than those on CYA, otherwise similar. At 37 °C, colonies 10–20 mm in diameter.

Structures bearing conidia 1.0–3.0 mm long, with heavy, hyaline, smooth walls; vesicles spherical, usually 60–85 µm in diameter, bearing closely packed metulae and phialides over the whole surface; conidia spherical, 7–10 µm in diameter, black, with walls extremely roughened.

Aspergillus niger Tiegh. See Fig. 1.5. Colonies on CYA 60 mm or more in diameter, usually covering the whole Petri dish; conidia black. Colonies on MEA varying from 30 mm to 60 mm in diameter, usually smaller than those on CYA and often quite sparse. At 37 °C, colonies 60 mm or more in diameter, covering the available space.

Structures bearing conidia 1.0–3.0 mm long, with heavy, hyaline, smooth walls; vesicles spherical, usually 50–75 µm in diameter, bearing closely packed metulae and phialides over the whole surface; conidia spherical, 4–5 µm in diameter, brown, with walls conspicuously roughened or sometimes striped.

Fig. 1.4. *Aspergillus carbonarius* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c) heads, bars = 40 μm; (d) conidia, bar = 5 μm. Source: Pitt and Hocking (2009), Fig. 8.10, p. 300; reproduced with kind permission from Springer Science+Business Media B.V.



Distinctive features. Differentiation of *Aspergillus niger*, *A. carbonarius*, and *A. japonicus* from nearly all other species is not difficult. These species grow rapidly and produce very dark brown to black conidia. *A. niger* and *A. carbonarius* produce metulae, whereas *A. japonicus* does not. *A. niger* produces conidia that are 4–5 μm in diameter, whereas those of *A. carbonarius* are larger, on average 7 μm or more in diameter. If these species are grown on CYA for 7 days at 37 °C, separation on colony diameters can be very useful: *A. niger* grows very quickly (60 mm or more), whereas *A. carbonarius* and *A. japonicus* grow much more slowly (less than 20 mm) (Mitchell *et al*., 2003).

It is important to remember that very few isolates of *A. niger* produce OTA. *A. niger* is an exceptionally common species, and recovery of this species from foods should not be regarded as evidence that OTA is likely to be present.

Factors influencing growth. *Aspergillus carbonarius* can grow down to 10 °C, with an optimum near 30 °C and a maximum near 41 °C. The optimal a_w for growth is 0.96–0.98, with a minimum near 0.85 at 25 °C. *A. niger* grows up to 45 °C, with an optimum of 35–37 °C, and has been reported to germinate down to 0.77 a_w (Pitt and Hocking, 2009).

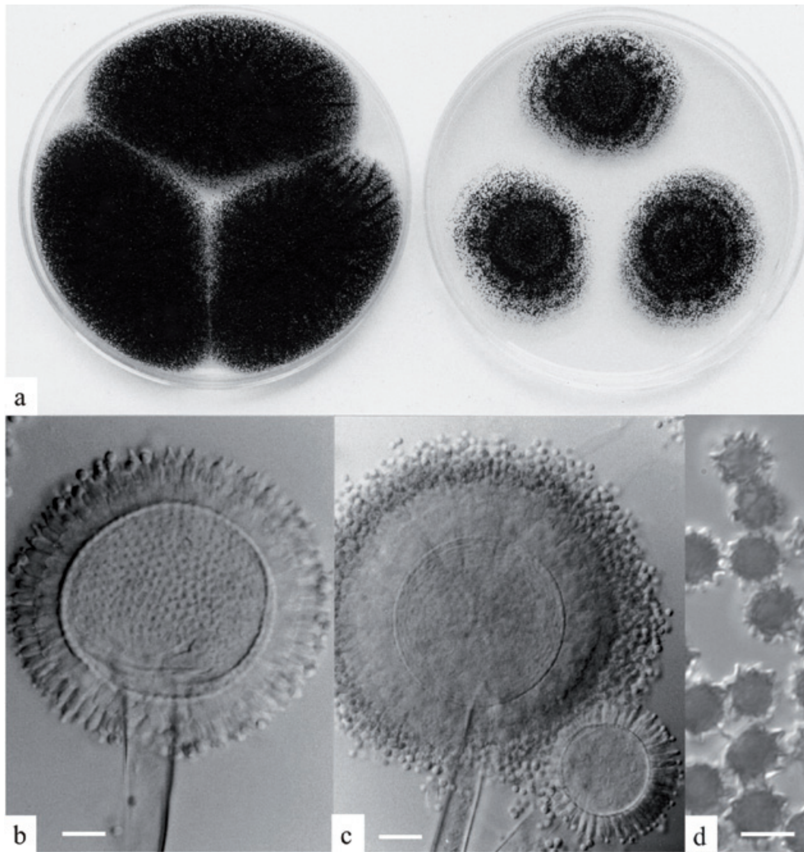
Commodities and foods at risk. Some black *Aspergillus* species, i.e. *A. niger*, *A. carbonarius*, and *A. japonicus*, are common inhabitants of vineyards, as these fungi grow rapidly at relatively high temperatures (above 30 °C) and their pigmentation renders them highly resistant to the deleterious effects of sunlight and UV light. These species appear to have no pathogenicity towards grapes and to be unable to penetrate an intact grape skin. Entry to maturing grapes results from attack by other pathogenic fungi (e.g. *Rhizopus stolonifer*, *Botrytis cinerea*, or powdery mildews), from mechanical damage due to cultivating or harvesting equipment, or, in some cultivars, from the splitting of berry skins that results from rain near harvest time. Once entry to a berry is gained, these fungi thrive in the acid, high-sugar environment.

Where grapes are dried, the black Aspergilli enjoy a considerable ecological advantage, and will continue to grow and produce OTA until the grapes dry below 0.8 a_w. As grapes are normally dried in the sun, this usually takes several days, allowing ample time for OTA production to occur (Hocking *et al*., 2003).

None of the three species of black Aspergilli appears to enjoy a particular ecological advantage, at least in Australia, as all three species are commonly recorded from maturing grapes, with proportions of each varying with seasonal factors (Leong *et al*., 2004). For OTA formation, *Aspergillus carbonarius* is the significant species: only a small proportion of *A. niger* isolates are capable of producing OTA, and *A. japonicus* isolates do not produce this toxin.

In grapes intended for wine-making, the time interval is usually short between infection and crushing, when fermentation stops fungal growth

Fig. 1.5. *Aspergillus niger* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b) head, bar = 15 µm; (c) heads, bar = 10 µm; (d) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.15, p. 314; reproduced with kind permission from Springer Science+Business Media B.V.



and toxin production ceases. So control of OTA in wines relies on good vineyard management, i.e. control of bunch rots and skin splitting, and short time intervals between harvest and crushing.

Occasional contamination of figs with OTA has been reported (Özay and Alperden, 1991). In a study of Turkish figs sampled during different stages of processing, only 3 of 100 samples contained OTA, and in each case levels were between 5 µg/kg and 10 µg/kg.

Aspergillus niger and *A. carbonarius* also occur in a wide variety of other fruits (Snowdon, 1990, 1991). Generally, other fruits are handled in ways that minimize fungal infection, or damaged fruit is discarded, not eaten, so OTA formation is not a hazard.

5. Genus *Penicillium*

Taxonomy. *Penicillium* is a large genus, with more than 200 recognized species, of which 50 or more are of common occurrence (Pitt, 2000). All common species grow and sporulate well on synthetic or semisynthetic media, and usually can be readily recognized at genus level. Most *Penicillium* species grow slowly, and have green conidia.

Classification within *Penicillium* is based primarily on microscopic morphology: the genus is divided into subgenera based on the number and arrangement of phialides (elements producing conidia) and metulae and rami (elements supporting phialides) on the main stalk cells. The classification of Pitt (1979, 2000) includes four subgenera: *Aspergilloides*, in which phialides are borne directly on the stalk cells without intervening supporting elements; *Furcatum* and *Biverticillium*, in which phialides are supported by metulae; and *Penicillium*, in which both metulae and rami are usually present. The majority of important toxigenic and food spoilage species are found in subgenus *Penicillium*.

Enumeration. Enumeration procedures suitable for all common *Penicillium* species are similar. Any effective antibacterial enumeration medium can be expected to give satisfactory results. However, some *Penicillium* species grow rather weakly on dilute media, such as PDA or dichloran chloramphenicol peptone agar (DCPA), so DRBC is recommended. Penicillia can also be effectively enumerated on DG18 (Pitt and Hocking, 1997; Samson *et al.*, 2010).

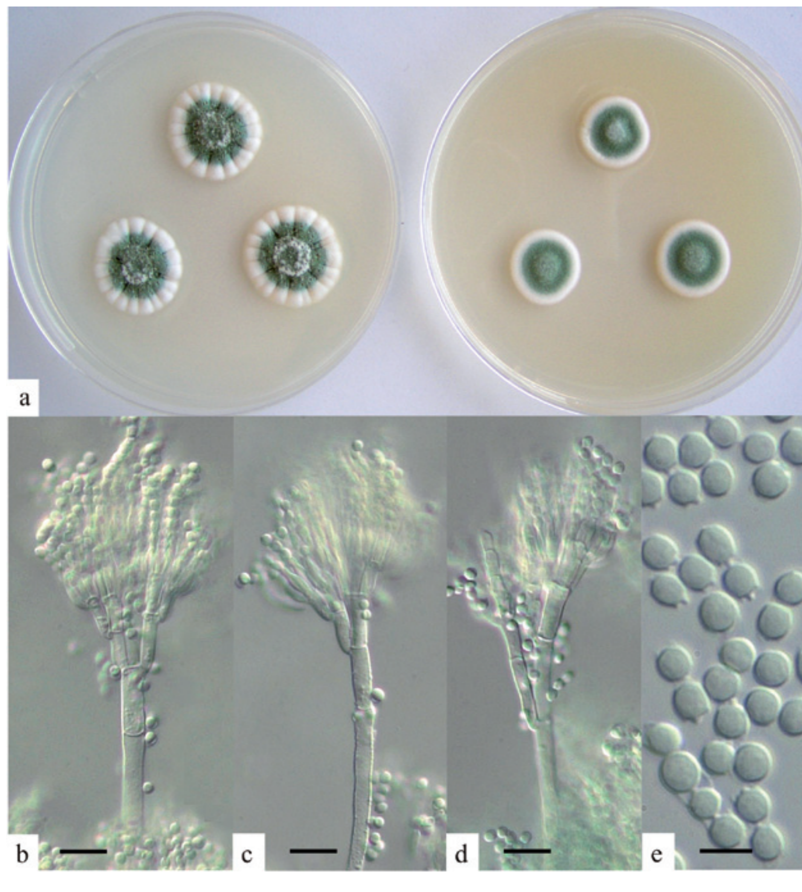
Identification. For a comprehensive taxonomy of *Penicillium*, see Pitt (1979). Keys and descriptions to common species are provided by Pitt (2000) and to foodborne species by Pitt and Hocking (2009) and Samson *et al.* (2010).

Identification of *Penicillium* isolates to species level is not easy, preferably being carried out under carefully standardized conditions of media, incubation time, and temperature. As well as microscopic morphology, gross physiological features, including colony diameters, colours of conidia, and colony pigments, are used to distinguish species.

5.1 Ochratoxin A production by Penicillium verrucosum

Soon after the discovery of OTA from *Aspergillus ochraceus*, the formation of OTA by a *Penicillium* species, *P. viridicatum*, was reported (van Walbeek *et al.*, 1969) and natural occurrence confirmed (Krogh *et al.*,

Fig. 1.6. *Penicillium verrucosum* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c, d) penicilli, bars = 10 µm; (e) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 7.48, p. 260; reproduced with kind permission from Springer Science+Business Media B.V.



1973). The view that *P. viridicatum* was a major source of OTA contamination in foods and feeds in some parts of the world was accepted for more than a decade. The species involved was later correctly identified as *P. verrucosum* (Pitt, 1987), and this was confirmed (Frisvad, 1989; Frisvad and Filtenborg, 1989). *P. viridicatum* does produce mycotoxins, but these have only rarely been implicated in animal health.

Penicillium verrucosum, and the closely related *P. nordicum*, are the only *Penicillium* species that produce OTA. *P. verrucosum* commonly occurs in cereals in temperate climates, whereas *P. nordicum* has been isolated, uncommonly, from processed meats.

Enumeration. The media specified above for general enumeration of *Penicillium* species are effective for *P. verrucosum*. On dichloran rose bengal yeast extract sucrose agar (DRYS), a selective medium for the enumeration of *P. verrucosum* and *P. viridicatum*, *P. verrucosum* produces a violet brown reverse colouration (Frisvad, 1983). Isolation and identification of *P. verrucosum* in pure culture is essential for confirmation.

Penicillium verrucosum Dierckx. See Fig. 1.6. Colonies on CYA 15–25 mm in diameter; mycelium white; conidial formation light to moderate, grey green to dull green; reverse yellow brown to deep brown. Colonies on MEA 12–15(–20) mm in diameter; mycelium white; conidial production moderate, coloured as on CYA; reverse dull brown or olive. No growth at 37 °C. Structures bearing conidia 200–500 µm long, with walls finely to conspicuously roughened; fruiting structures variable, with two or three supporting cells beneath phialides; conidia usually spherical, 2.5–3.0 µm in diameter, with smooth walls.

Distinctive features. *Penicillium verrucosum* is characterized by slow growth on CYA and especially on MEA, by conidia coloured relatively bright green, by the absence of other conspicuous pigmentation, and by rough walls on the stalk cells (Pitt, 2000). It is similar in general appearance to *P. viridicatum* and *P. solitum*. *P. verrucosum* and *P. viridicatum* produce a distinctive violet brown reverse on DRYS (Frisvad, 1983). It should be noted that recognition of this species requires specialist knowledge, or detailed comparison with known cultures (of this and other related species).

Only one other species of *Penicillium* is known to produce ochratoxin A: *P. nordicum*. This species was segregated from *P. verrucosum* by small physiological differences (it produces a yellow reverse on DRYS; Larsen *et al.*, 2001), but it is ecologically distinct, occurring mainly on meat and cheese. Its significance in terms of human health is unknown.

Factors influencing growth. *P. verrucosum* grows from 0 °C to 31 °C, with the optimum at 20 °C. The minimum a_w for growth is about 0.80 (Pitt and Hocking, 2009). Growth occurs over the pH range 2.1–10.0 at least (Wheeler *et al.*, 1991). The ability of *P. verrucosum* to produce significant levels of OTA at 4 °C and a_w as low as 0.86 is noteworthy (Northolt *et al.*, 1979). The physiology of *P. nordicum* is likely to be very similar.

Commodities and foods at risk. The major food habitat for *P. verrucosum* is cereal crops grown in cool temperate climates, ranging across northern and central Europe and Canada. The occurrence of this species in European cereals has two consequences: OTA is present in many kinds of European cereal products, especially bread and flour-based foods, and in animals that eat cereals as a major dietary component. OTA was detected in Danish pig meats nearly 40 years ago (Krogh *et al*., 1973), and its implications for human and animal health were recognized at the same time. As bread and other cereal products and pig meats are major components of the European diet, the further consequence is that most Europeans who have been tested have shown appreciable concentrations of OTA in their blood (WHO, 2007).

Recent information indicates that *P. verrucosum* is not a commensal on cereal crops, i.e. it does not grow in grain crops before harvest, but its presence is due to post-harvest contamination. The primary sources of infection of the grain appear to be from harvesting, processing, and storage equipment (Magan and Olsen, 2004; Olsen *et al*., 2004).

A maximum growth temperature near 30 °C restricts *Penicillium verrucosum* geographically. It appears to be uncommon, indeed almost unknown, in warm temperate or tropical climates, or in other kinds of foods. *P. verrucosum* is never a source of OTA in foods from warmer climates, such as coffee, wines, or other grape products.

Penicillium nordicum has been isolated quite commonly from meats, especially refrigerated products.

6. Genus *Fusarium*

Fusarium is one of the most important genera of plant pathogenic fungi, with a record of devastating infections in many kinds of economically important plants. *Fusarium* species are responsible for wilts, blights, root rots, and cankers in legumes, coffee, wheat, maize, carnations, pine trees, and grasses. The importance of *Fusarium* species in the current context is that infection may sometimes occur in developing seeds, especially in cereals, and also in maturing fruits and vegetables. An immediate potential for toxin production in foods is apparent.

The very important role of *Fusarium* species as mycotoxin producers appears to have remained largely unsuspected until the 1970s. Research has now strongly associated alimentary toxic aleukia (ATA) with *Fusarium* species. An epidemic of this human mycotoxicosis in the USSR killed at least 100 000 people between 1942 and 1948 (Joffe, 1978). ATA outbreaks are also known to have occurred in the Russian Federation in 1932 and 1913, and there is little doubt that outbreaks occurred in earlier years as well (Joffe, 1978). Matossian (1981, 1989) has argued persuasively that ATA outbreaks occurred in other countries, including England, in the 16th to 18th centuries at least.

Research since 1970 has shown that *Fusarium* species are capable of producing a bewildering array of mycotoxins. Foremost among these are the trichothecenes, of which at least 50 are known; the majority are produced by *Fusarium*. The most notorious trichothecene is T-2 toxin, which was linked to ATA. Of no less importance in modern times are the fumonisins, which are especially toxic to horses, and are suspected to be responsible for chronic human diseases also.

Taxonomy. The signature micromorphological characteristics of *Fusarium* species are uncoloured, multiseptate, large (25 µm to 50 µm or more) curved conidia called macroconidia, which are produced from phialides. Most species produce macroconidia in cushion-like structures called sporodochia. In some species, macroconidia are sparsely formed in Petri dish culture and recognition of these species as belonging to *Fusarium* requires experience.

In addition to macroconidia, some *Fusarium* species can make one or two kinds of smaller, one- or two-celled conidia called microconidia. Microconidia are usually produced in the aerial mycelium in culture. Most often, microconidia are produced in slimy heads, but some species produce them in chains or singly. Microconidia are also produced from phialides, which may have a single spore-bearing opening (monophialides) or multiple openings (polyphialides).

The taxonomy of *Fusarium* has been difficult, with several competing taxonomic schemes, recognizing from 9 to 60 species in the genus. The taxonomy of Nelson *et al.* (1983), which accepted 30 species, has met with widespread approval, and is still widely used in conjunction with the laboratory manual of Leslie and Summerell (2006). Recent molecular studies have suggested that Nelson *et al.* (1983) greatly underestimated species numbers in *Fusarium*. For example, O'Donnell *et al.* (1998) recognized 36 phylogenetic species (species recognizably different by molecular techniques) in a grouping corresponding to *Fusarium* section Liseola in which four species had been recognized by Nelson *et al.* (1983). Nirenberg and O'Donnell (1998) described 10 new species in this section. However, for practical identification of the species important for mycotoxin production, the manual of Leslie and Summerell (2006) is recommended.

Table 1.1. Media of value for isolation and enumeration of *Fusarium* species

Medium	Advantages	Disadvantages	Reference
Pentachloronitrobenzene (PCNB) agar	Often used	Carcinogenic; no spore production by *Fusarium* species	Snyder and Hansen (1940)
Dichloran chloramphenicol peptone agar (DCPA)	Sporulation allows recognition of *Fusarium* species	Little pigmentation for differentiating *Fusarium* species	Andrews and Pitt (1986)
Czapek–Dox iprodione dichloran agar (CZID)	Pigmentation helpful in distinguishing *Fusarium* species	Sterile colonies do not permit ready identification of *Fusarium* species	Abildgren *et al.* (1987)
Dichloran 18% glycerol agar (DG18)	Sporulation allows recognition of *Fusarium* species	Low-a_w medium, not ideal for *Fusarium* growth	Hocking and Pitt (1980)

A direct consequence of confusion in taxonomy has been confusion over species–mycotoxin associations. *Fusarium* isolates producing a particular toxin have often been given different names. However, Desjardins (2006) has provided a comprehensive clarification of the important mycotoxigenic species and the mycotoxins they each produce. The species judged to be most important from the viewpoint of human health are discussed here.

Enumeration and isolation. Growth of *Fusarium* species is favoured by dilute media of high a_w. Enumeration of Fusaria can be effectively carried out on media such as PDA provided chloramphenicol or other broad-spectrum antibiotics are added to suppress bacteria. However, acidified PDA, a frequently used antibacterial medium, is not recommended because it may inhibit sensitive cells. DCPA (Andrews and Pitt, 1986) and Czapek–Dox iprodione dichloran agar (CZID) (Thrane, 1996) are effective enumeration and isolation media for most foodborne *Fusarium* species. In addition, half-strength PDA is used by many laboratories isolating directly from plant tissue, where the number of unwanted fungi is much lower than in soil or plant debris. It should be pointed out that, although pentachloronitrobenzene (PCNB) agar is still widely used, PCNB is a known carcinogen. DCPA is to be preferred as it contains pentachloronitroaniline (dichloran), a molecule with similar properties to PCNB but that is not carcinogenic.

Recognition of *Fusarium* colonies on these media requires careful observation and experience. Presumptive identification to genus level can usually be made from colony appearance: low to floccose colonies, coloured white, pink, or purple, with pale to red or purple reverses, are indicative of *Fusarium*. Confirmation requires microscopic examination, in which the crescent-shaped macroconidia characteristic of the genus should be observed. However, these are not always produced on enumeration media, especially PDA. Differentiation of some species on enumeration media is possible, but also requires experience.

Identification. All contemporary identification schemes based primarily on morphology use two media: a weak medium for stimulation of sporulation and a richer medium for measuring growth rates and for stimulation of diagnostic pigment production. The most commonly used rich medium is PDA. Potato sucrose agar (PSA) was used in the manual of Booth (1971) and is still used in some laboratories instead of, or in addition to, PDA. Nelson *et al.* (1983) among others advocated the use of PDA made from old potatoes, rather than commercial formulations, but many laboratories use commercial PDA with satisfactory results. Oatmeal agar is used in some laboratories. The most commonly used weak media are carnation leaf agar (CLA) and Synthetischer Nährstoffarmer Agar (often now called synthetic nutrient agar [SNA]). SNA has the advantage of being a defined medium, but CLA supports superior sporodochial production in some species. However, use of CLA requires access to a source of gamma-irradiated carnation leaves. Banana leaf agar (autoclave-sterilized banana leaves on half-strength cornmeal agar) is also a good medium for stimulation of sporulation, but is not yet widely used. To maintain uniformity for descriptions of all foodborne fungi, Pitt and Hocking (2009) provided *Fusarium* descriptions on PDA, together with media used in *Aspergillus* and *Penicillium* identification. Some readily prepared media of value in *Fusarium* identification are given in Table 1.1.

Opinions differ regarding the necessity to make a single conidium isolate of *Fusarium* species before identification. Some laboratories make a new single conidium isolate at every transfer of the culture. This ensures a highly reproducible growth rate. Other laboratories prefer not to make single spore cultures because this may decrease the vigour of the culture. Because *Fusarium* species often grow in mixed colonies when isolated from soil or plant material, at least one generation of single conidium isolates is advisable for cultures intended for experimental use, especially genetic studies.

Traditionally, *Fusarium* cultures have been cultivated for 7–10 days under mixed fluorescent/near UV light at room temperature, near 25 °C. Recently, particularly for the group of species producing fumonisin, the importance of also growing the

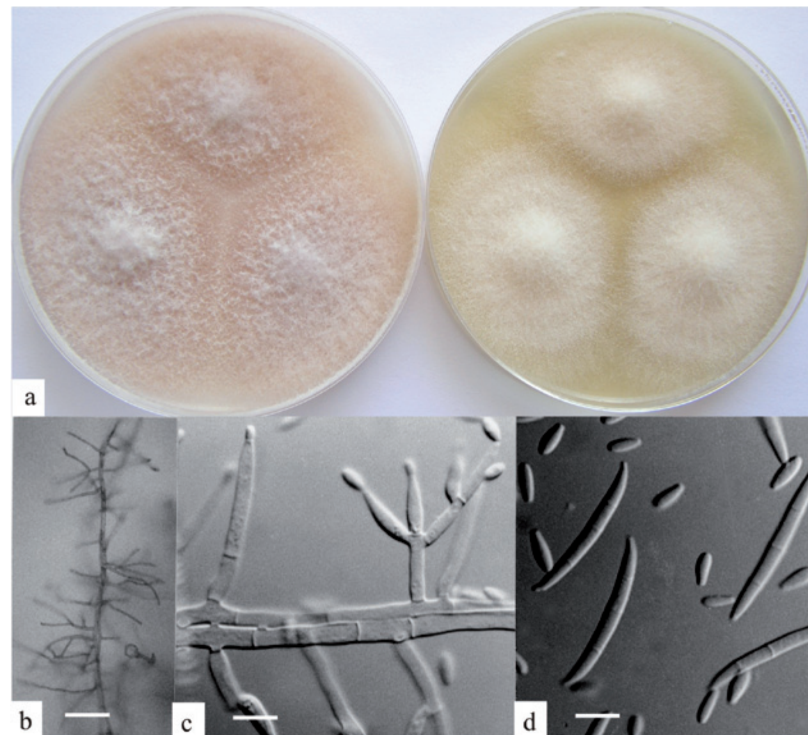
same cultures in darkness to allow the development of some diagnostic features has been emphasized.

The only definitive taxonomy for *Fusarium* is that of Nelson *et al*. (1983). The most useful and up-to-date guide to important species is that provided by Leslie and Summerell (2006). Pitt and Hocking (2009) and Samson *et al*. (2010) provide keys and descriptions for common foodborne species. The descriptions of species given here have been provided by Dr K.A. Seifert (Agriculture and Agri-Food Canada, Ottawa) and are based on the protocols and media described above.

6.1 Fumonisin production by F. verticillioides *and* F. proliferatum

Fusarium verticillioides (Sacc.) Nirenberg. See Fig. 1.7. Colonies on rich media (PDA or PSA) at 25 °C grow moderately rapidly, 3.5–5.5 cm in diameter in 4 days. Abundant aerial mycelium is produced, and the reverse usually has rays or large patches of violet or purple. On weak media (CLA or SNA) at 25 °C, sporodochia are sparsely produced or not present in most isolates. When present they are inconspicuous and almost colourless, on the agar surface beneath the often dense aerial mycelium. Macroconidia are usually 3–5 septate, mostly 30–45 µm long, straight or variably curved, with more or less parallel walls and with the widest point near the middle. Microconidia are abundantly produced in the aerial mycelium on highly branched conidiophores. The conidiogenous cells are monophialides that often collapse before the cultures are about 10 days old. Microconidia are produced in long, dry chains visible with the stereomicroscope; these chains, which are often coiled, give the colonies a distinctive texture similar to curly hair. Individual conidia are ellipsoidal, 0–1 septate, 4–19 × 1.5–4.5 µm.

Fig. 1.7. *Fusarium verticillioides* (a) colonies on PDA (left) and DCPA (right), 7 days, 25 °C; (b) phialides bearing chains of microconidia, bar = 50 µm; (c) phialides, bar = 10 µm; (d) macroconidia and microconidia, bar = 10 µm. Source: Pitt and Hocking (2009), Fig. 5.36, p. 120; reproduced with kind permission from Springer Science+Business Media B.V



The teleomorph of *F. verticillioides* is *Gibberella moniliformis*.

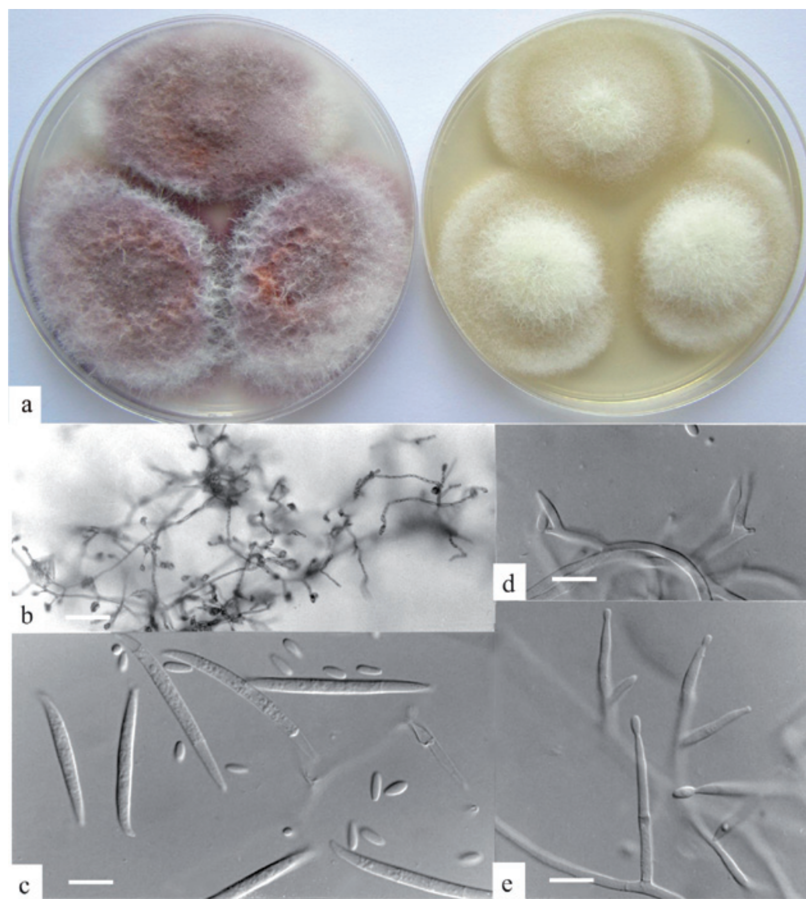
Distinctive features. *Fusarium verticillioides* is recognized by the combination of purplish colours on PDA, and the production of long, curly chains of microconidia from monophialides in the aerial mycelium. Although macroconidia are found in some cultures, many strains do not produce them. Therefore, an experienced eye can be necessary to recognize those strains as belonging to *Fusarium*. The teleomorph, *Gibberella moniliformis*, is produced in culture only when strains of opposite mating type are crossed under appropriate conditions.

Until recently, *Fusarium verticillioides* was known as *F. moniliforme*, a name that is no longer used on the recommendation of an expert committee (Seifert *et al*., 2003). The name *F. moniliforme* is predated by *F. verticillioides* and also cannot reliably be linked to modern species concepts.

Fusarium thapsinum (teleomorph: *Gibberella thapsina*) is a closely related species, found primarily on sorghum. It has similar micromorphology, but PDA colonies lack purplish pigmentation and tend to be yellow to yellow brown. Both *F. verticillioides* and *F. thapsinum* produce longer microconidial chains and lack the polyphialides that characterize *F. proliferatum*.

Factors influencing growth. The maximum temperature for growth of *Fusarium verticillioides* has been reported as 32–37 °C, the minimum as 2.5–5 °C, and the optimum near 25 °C. The minimum a_w for growth is about 0.87 (Pitt and Hocking, 2009). Values of these parameters for *F. proliferatum* are essentially identical.

Fig. 1.8. *Fusarium proliferatum* (a) colonies on PDA (left) and DCPA (right), 7 days, 25 °C; (b) phialides bearing microconidia in chains and false heads in situ, bar = 50 µm; (c) macroconidia and microconidia, bar = 10 µm; (d) polyphialides, bar = 10 µm; (e) monophialides, bar = 10 µm. Source: Pitt and Hocking (2009), Fig. 5.31, p. 111; reproduced with kind permission from Springer Science+Business Media B.V.



Fusarium proliferatum (Matsush.) Nirenberg. See Fig. 1.8. Colonies on rich media (PDA or PSA) at 25 °C grow moderately rapidly, 3.5–5.5 cm in diameter in 4 days. There is abundant white to pink or slightly orange aerial mycelium, and the reverse usually has rays or large patches of violet or purple. On weak media (CLA or SNA) at 25 °C, sporodochia are usually sparsely produced and often are not present. When present they are inconspicuous and almost colourless, on the agar surface beneath the often dense aerial mycelium. Macroconidia are usually 3–5 septate, 30–45 µm long, straight or variably curved, with more or less parallel walls and with the widest point near the middle. Microconidia are abundantly produced in the aerial mycelium on divergently branched conidiophores. The cells producing conidia are predominantly monophialides, but up to 20% may be polyphialides. Microconidia are produced in short, dry chains visible with the stereomicroscope, ovoid to ellipsoidal, 0–1 septate, 7–12.5 × 2–3 µm.

The teleomorph of *F. proliferatum* is *Gibberella intermedia*.

Distinctive features. The production of purplish pigments on PDA, and the occurrence of short, dry chains of microconidia in the aerial mycelium and the sometimes sparse occurrence of polyphialides characterize *F. proliferatum*. Although macroconidia are found in some cultures, many strains do not produce them. Therefore, an experienced eye can be necessary to recognize those strains as belonging to *Fusarium*. The teleomorph, *Gibberella intermedia*, is produced in culture only when strains of opposite mating type are crossed under appropriate conditions.

Fusarium proliferatum is distinguished from *F. verticillioides* by the shorter chains of microconidia and the occurrence of polyphialides in the aerial mycelium. Another frequently isolated species that occupies the same ecological niche is *F. subglutinans*, which does not produce fumonisins. It produces slimy heads rather than chains of microconidia from a mixture of monophialides and polyphialides; strains of this species are more likely to produce macroconidia than either *F. verticillioides* or *F. subglutinans*. *F. nygamai* is a similar species that also produces fumonisin in some strains; it can be distinguished from *F. proliferatum* by the production of chlamydospores.

Other species making fumonisins. Several other *Fusarium* species are known to produce fumonisins, including *F. anthophilum*, *F. beomiforme*, *F. dlamini*, *F. globosum*, *F. napiforme*, *F. nygamai*, *F. oxysporum*, *F. polyphialidicum*, and *F. subglutinans* (IARC, 2002). None is of major importance in fumonisin production in foods.

Commodities and foods at risk. *Fusarium verticillioides* and *F. proliferatum*, the major sources of fumonisins, are the most common fungi associated with maize. *F. verticillioides* has been known for many years to occur systemically in leaves, stems, roots, and kernels of maize (Foley, 1962). These fungi can be recovered from virtually all maize kernels worldwide, including those that are healthy (e.g. Hesseltine *et al.*, 1981; Pitt *et al.*, 1993; Miller,

1994; Miller *et al*., 1995; Ramirez *et al*., 1996; Logrieco *et al*., 2002). *F. verticillioides* has been reported to suppress the growth of other ear fungi (Reid *et al*., 1999), and kernels heat-treated to destroy the fungus germinate but do not thrive (Foley, 1962). Strains of *F. verticillioides* isolated from maize have the potential to produce fumonisins, even for maize from regions where fumonisin accumulations in maize are historically uncommon. These include Africa, Asia, Europe, North America (Canada, the USA, and Mexico), and South America (WHO, 2000).

Fusarium kernel rot in maize due to the growth of *F. verticillioides* and related species causes the formation of fumonisins, whereas Gibberella ear rot or pink ear rot, mainly caused by *F. graminearum*, is associated with deoxynivalenol and zearalenone production (see Section 6.3). These fungi grow under different environmental conditions but with overlap. *F. proliferatum*, which causes kernel rot, concurrently produces fumonisin and moniliformin. Slightly different environmental conditions appear to favour one or the other of the principal species that produce fumonisins. For these reasons, it is sensible to consider the two diseases Fusarium kernel rot and pink ear rot together. In North America and Europe, fumonisins can occur in maize crops in some seasons, but in others, deoxynivalenol can occur, sometimes accompanied by zearalenone. In other regions, such as Africa or South-East Asia, all three toxins can be seen together (Yamashita *et al*., 1995; Doko *et al*., 1996; Ali *et al*., 1998). These differences in mycotoxin occurrence have important toxicological implications.

Fusarium subglutinans is also common in maize kernels in North America (Miller, 1994; Munkvold, 2003), but this species apparently causes a higher level of ear rot in some European locations and results in the accumulation of moniliformin (Logrieco *et al*., 2002). From limited data, moniliformin is not commonly found in United States (Gutema *et al*., 2000) or Canadian maize (Farber *et al*., 1988).

After genotype susceptibility, temperature is the primary determining factor for maize diseases caused by *Fusarium* species (Miller, 1994; Munkvold, 2003). *F. graminearum* has a very narrow temperature window for growth in maize. The optimal temperature is between 26 °C and 28 °C. Its growth rate at 24 °C is one quarter that at 26–28 °C, while at 30 °C it is about one half. In contrast, *F. verticillioides* grows well above 26 °C (Miller, 2001; Munkvold, 2003).

In culture, fumonisin B_1 is produced under conditions known to favour the production of polyketides and sesquiterpenes. The toxin is optimally produced in media that have moderate a_w and are nitrogen-limited. Fumonisin is produced under relatively high oxygen tensions but apparently has an unusual requirement for low pH (about 2) for optimal production (Miller *et al*., 1995); such conditions arise only when the plant is dead or dying.

Fusarium kernel rot is associated with warm, dry years and insect damage and is caused by *F. subglutinans* (teleomorph: *Gibberella subglutinans*), *F. verticillioides* (teleomorph: *Gibberella moniliformis*), and *F. proliferatum* (Logrieco *et al*., 2002; Munkvold, 2003). In warmer parts of the USA and in the lowland tropics, *F. verticillioides* is one of the most important ear diseases (De Leon and Pandey, 1989).

Studies of the occurrence of fumonisin from natural occurrence and experimental infections clearly demonstrate the importance of drought stress and insect damage at the same time as temperatures are favourable. The fumonisin B_1 concentrations found in maize from the two Ontario, Canada, counties with the highest and lowest average concentration after the 1993 harvest were 1.4 and 0.4 times the state average, respectively. The average temperatures in the counties were similar, at 104% and 107% of the 30-year average, respectively. However, rainfall in the county with the highest fumonisin B_1 level was only 49% of normal, whereas in the county with the lowest level, it was 95% of normal (Miller *et al*., 1995). After experimental inoculation of 14 maize genotypes in Poland, the average temperature in the year with the highest fumonisin B_1 accumulation was 117% of the 30-year average; that in the year with the lowest fumonisin B_1 accumulation was 102% of normal. Rainfall in the year with the highest fumonisin B_1 accumulation was 6% of normal and in the year with the lowest, 65% of normal (Pascale *et al*., 1997).

A study of fumonisin occurrence in hybrids grown in the USA indicated that fumonisins are produced in higher concentrations in hybrids grown outside their area of adaptation. Fumonisin concentrations were inversely proportional to June rainfall (Shelby *et al*., 1994), again suggesting the important role of drought stress (Munkvold, 2003). Data from samples collected in Africa, Italy, and Croatia also indicate fumonisin accumulation in lines grown outside their area of adaptation, which includes tolerance to moisture stress (Doko *et al*., 1995; Visconti, 1996).

Hybrids with an increased likelihood of kernel splitting show higher levels of Fusarium kernel rot, and kernel splitting is generally worse under drought conditions. Drought stress also results in greater insect herbivory on maize; hence it is not possible to totally separate these variables. Further, in studies of experimental inoculation methods, the severity of Gibberella ear rot is related to wound size (Drepper and Renfro, 1990). It was observed early on that

a strong relationship exists between insect damage and Gibberella ear rot (Lew *et al.*, 1991). Transgenic Bt maize genotypes, which contain a gene from the soil bacterium *Bacillus thuringiensis* that results in the accumulation of proteins toxic to key insect pests of maize, had lowered levels of recovery of *F. verticillioides* and fumonisin (Munkvold *et al.*, 1997, 1999; Bakan *et al.*, 2002; Munkvold, 2003). Under conditions of high disease pressure, the Bt hybrids can make the difference between a crop being fit or unfit for human consumption (Hammond *et al.*, 2004; De La Campa *et al.*, 2004). In an examination of fumonisin concentration in relation to various climate variables under moderate insect pressure, most of the variation was explained by temperatures above 30 °C (about 40%), followed by insect pressure (about 20%) and hybrid (about 10%; De La Campa *et al.*, 2004).

Maize infected by other pathogens that damage ears (such as *F. graminearum*) may be predisposed to *F. verticillioides* damage and fumonisin accumulation. Ears inoculated with *F. graminearum*, *F. verticillioides*, and *F. subglutinans* by wounding produced visible symptoms on a 1–9 scale of 7.3, 4.4, and 4.7, respectively. Despite the fact that *F. graminearum* and *F. subglutinans* do not produce fumonisin, ears inoculated with these fungi contained 42 µg/g and 3 µg/g fumonisin B_1, respectively (Schaafsma *et al.*, 1993).

Breeding for resistance to Fusarium kernel rot has not been effective. Within areas of adaptation, there are apparent differences in symptom response (Miller, 2001; Munkvold, 2003). In a large trial at the International Maize and Wheat Improvement Center, in Mexico, slight improvements in symptom expression in some tropical maize genotypes were observed after many cycles of selection (De Leon and Pandey, 1989).

Factors that control insects, confer resistance to other ear diseases, and adaptations, including drought and temperature tolerance, are important in reducing the risk of fumonisin accumulations in maize.

Fumonisins are very uncommon in commodities or foods other than maize and maize products (IARC, 2002).

6.2 Fumonisin production by Aspergillus niger *and* Alternaria arborescens

It has been known for 20 years that one particular race of *Alternaria alternata*, described as *Alternaria alternata* f. sp. *lycopersici*, produces fumonisins (Chen *et al.*, 1992). This particular race is a host-specific pathogen that causes a stem canker disease on tomato plants. For that reason, fumonisin production by this species has been largely ignored in general discussions of fumonisins in foods and feeds. This taxon has been re-identified as *Alt. arborescens* (Frisvad *et al.*, 2007).

Recently, however, the picture has become more complicated. Studies on the genome sequence of *Aspergillus niger* showed, totally unexpectedly, the presence of the genes for fumonisin (Baker, 2006), and this was independently verified by Pel *et al.* (2007). It was soon confirmed that this gene cluster (consisting of at least 15 genes) was active and that at least some strains of *A. niger* can indeed produce fumonisins (Frisvad *et al.*, 2007).

The implications are vast. As described in Section 4.2.3, *A. niger* is a very common fungus, of which a few strains produce ochratoxin A. Foods in which OTA is found, produced by *A. niger* and the closely related species *A. carbonarius*, include grapes, dried vine fruits, wines, and coffee. *A. niger* is also common in some fresh fruits, particularly berries, and on onions (Pitt and Hocking, 2009). Therefore, fumonisins produced by *A. niger* can be expected to be widespread if more than a few isolates are producers. Recent information indicates that fumonisin production by *A. niger* is indeed common. In one study of *A. niger* strains from a sample of Californian raisins, 50 of 66 strains (77%) produced fumonisins (Mogensen *et al.*, 2010b). In a second study, where isolates were taken from 13 samples of dried vine fruits from several countries, 20 of 30 (67%) produced fumonisins (Varga *et al.*, 2010). Of an unstated number of isolates from Thai coffee, 67% were able to produce fumonisins (Noonim *et al.*, 2009). *A. niger* isolates do not usually produce fumonisin B_1; the major metabolite is fumonisin B_2, sometimes with lower amounts of fumonisin B_4.

So far, only a few studies have examined food products that are frequently infected by *A. niger* for the presence of fumonisins. Low levels (1–9.7 µg/kg) were found in 7 of 12 coffee samples (Noonim *et al.*, 2009). Levels of up to 7.8 mg/kg were found in inoculated dried fruits (Mogensen *et al.*, 2010b). More alarming, seven commercial dried fruit samples positive for *A. niger* were all positive for fumonisins B_1 to B_4: the average total fumonisin level was 7.2 mg/kg, with the range 4.6–35.5 mg/kg (Varga *et al.*, 2010). It is not surprising that wines can also contain fumonisins. Of 51 market samples of Italian wines, 9 (18%) contained fumonisin B_2, with levels ranging from 0.4 µg/L to 2.4 µg/L (Logrieco *et al.*, 2010). Seventy-seven wine samples from 13 countries were examined by Mogensen *et al.* (2010a), and 18 (23%) were found to contain fumonisins, with a range of 1 µg/L to 25 µg/L. These levels are low, but wine has a high consumption rate in some areas.

It has been found that fumonisin production in culture by *A. niger* is enhanced at a slightly reduced a_w, about 0.99, by the addition of 5% NaCl to CYA (Mogensen *et*

al., 2010c). This additive reduced fumonisin production by *Fusarium* species. Fumonisin was produced optimally by *A. niger* at 25–30 °C, whereas optimal temperatures for production were lower in *Fusarium* species (20–25 °C). Clearly, some evolution has occurred since the genes were transferred to *A. niger* (Mogensen *et al*., 2010c).

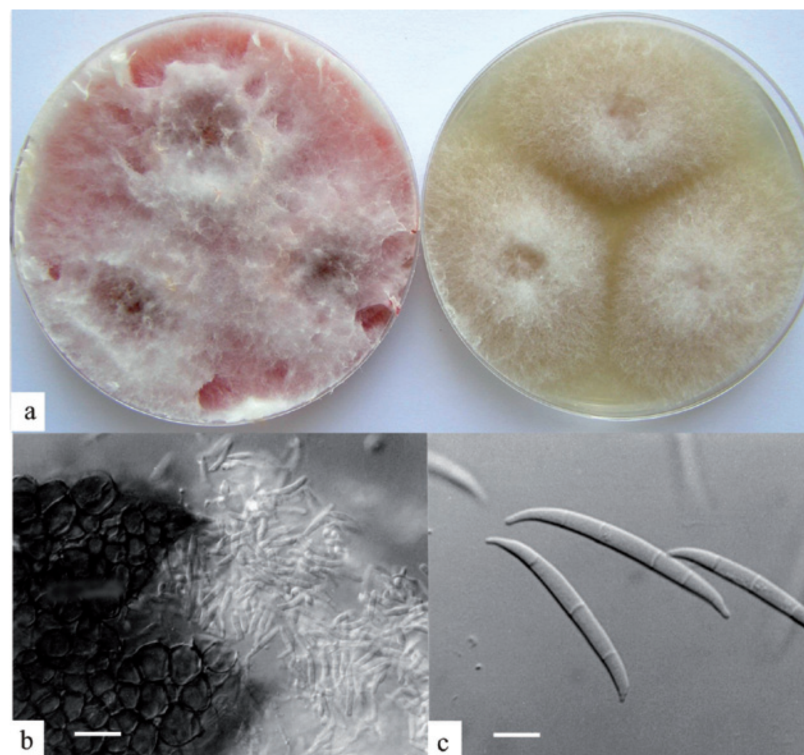
At this time, it is difficult to assess the relative importance of fumonisin production by *A. niger*, but the range of commodities in which fumonisins are found has been extended considerably. However, given the very high consumption of maize in some countries, production by *A. niger* will have lesser importance.

6.3 Deoxynivalenol, nivalenol, and zearalenone production by Fusarium graminearum *and related species*

The most important *Fusarium* species that produce the trichothecenes deoxynivalenol (DON) and (less commonly) nivalenol (NIV) in small grains are *F. graminearum*, *F. culmorum*, and, less frequently, *F. crookwellense*.

The name *Fusarium roseum* as used by Snyder and Hansen (1940) has caused a great deal of confusion in the literature as their very broad species concept included several well-known and important *Fusarium* species, including *F. graminearum*. The literature before the mid-1980s is therefore unreliable, both taxonomically and with respect to mycotoxin production. Since the publications by Nelson *et al*. (1983) and Marasas *et al*. (1984), *F. graminearum* and *F. culmorum* have become well established and species–mycotoxin relationships have become clear. *F. crookwellense* is a widespread species first described in 1982.

Fig. 1.9. *Fusarium graminearum* (a) colonies on PDA (left) and DCPA (right), 7 days, 25 °C; (b) *Gibberella zeae* perithecium and ascospores, bar = 25 μm; (c) macroconidia, bar = 10 μm. Source: Pitt and Hocking (2009), Fig. 5.27, p. 103; reproduced with kind permission from Springer Science+Business Media B.V.



Fusarium graminearum Schwabe.
See Fig. 1.9. Colonies on rich media (PDA or PSA) at 25 °C grow rapidly, 7.5–9 cm in diameter in 4 days. There is abundant white, reddish or yellowish brown aerial mycelium, and the reverse is usually distinctly red. About 5% of isolates have an orange brown reverse. On weak media (CLA or SNA) at 25 °C, almost colourless sporodochia are produced under a sparse layer of white aerial mycelium. Structures bearing conidia have 1–2 levels of branching, terminating with 1–4 phialides. Macroconidia are usually 4–6 septate and abundantly produced in fresh isolates, mostly 40–60 μm long, more or less straight with parallel walls. No microconidia are produced, although sometimes immature macroconidia are seen and could be confused with microconidia.

Sexual fruiting structures (perithecia) are often produced on CLA or SNA as the medium begins to desiccate, almost black, about 200 μm in diameter, with a warty wall, and exuding a light orange cloud of ascospores. Ascospores are generally fusiform to allantoid (slightly bean-shaped), light brown, and 20–30 μm long. Ascospores are forcibly discharged and can be found on the agar surface away from the perithecia, or on Petri dish lids. The teleomorph of *F. graminearum* is *Gibberella zeae* (Schw.) Petch.

Distinctive features. *Fusarium graminearum* produces straight macroconidia with parallel walls, which in combination with rapid growth and usually red pigments on PDA, are relatively distinctive. The frequent occurrence of perithecia of the teleomorph in culture is also a

reliable characteristic, occurring in about 90% of fresh isolates under appropriate lighting conditions. *F. pseudograminearum* (teleomorph: *Gibberella coronicola*) is an ecologically and phylogenetically distinct species that causes crown rot of wheat. The macroconidia are similar to those of *F. graminearum*, but are reportedly broadest above the middle; the growth rate on PDA is slower. Diagnostic PCR primers for *F. pseudograminearum* were designed by Aoki and O'Donnell (1999) based on β-tubulin sequences. This species also produces DON and zearalenone (ZEA).

Fusarium culmorum (W. G. Sm.) Sacc. Colonies on rich media (PDA or PSA) at 25 °C grow rapidly, 7.5–9 cm in diameter in 4 days. There is abundant white to slightly orange, brown or reddish aerial mycelium, and the reverse is usually distinctly red. About 5% of isolates have an orange brown or tan reverse. On weak media (CLA or SNA) at 25 °C, reddish brown or orange sporodochia are produced under a sparse layer of white aerial mycelium. Structures bearing conidia have up to 4 levels of branching, terminating with 1–4 phialides. Macroconidia are usually 3–6 septate and abundantly produced in fresh isolates, mostly 30–45 µm long, with the widest point above the middle and hence somewhat wedge-shaped, often appearing short and fat. No microconidia are produced.

Distinctive features. Broad, wedge-shaped macroconidia with short apical cells and basal cells are distinctive for *F. culmorum*. The related species *F. sambucinum* produces narrower macroconidia than *F. culmorum*. The wedge shape of the conidia distinguishes *F. culmorum* from *F. crookwellense*.

Fusarium crookwellense Burgess *et al.* Colonies on rich media (PDA or PSA) at 25 °C grow rapidly, 7.5–9 cm in diameter in 4 days. There is abundant aerial mycelium, white to pink, and the reverse is usually distinctly red. On weak media (CLA or SNA) at 25 °C, sporodochia are usually abundant and distinctly reddish brown. Structures bearing conidia, arising from the sporodochia, have 1–4 levels of branching, terminating with 1–6 phialides. Macroconidia are usually 5 septate, 35–60 × 4.5–6.5 µm, fairly uniform in shape, with a more or less straight ventral wall and curved dorsal wall, with the widest point near the middle, of medium size. Microconidia are not produced, although sometimes immature macroconidia give this impression.

Distinctive features. Distinctive macroconidia, with straight inner walls and curved outer walls, characterize *Fusarium crookwellense*.

Factors affecting growth. The optimal temperature for growth of *Fusarium graminearum* is 25 °C, and the maximum below 37 °C. The minimum a_w for growth is near 0.90 (Pitt and Hocking, 2009). *F. culmorum* is a psychrotroph, growing down to 0 °C but up to only 31 °C (Pitt and Hocking, 2009).

Trichothecenes in small grains: *Fusarium graminearum* and related species. Fusarium head blight is an important plant disease in temperate regions that affects small grains, mainly wheat, but also barley and triticale. Five or six *Fusarium* species are consistently isolated from small grains affected by this disease, and the most pathogenic species, *F. graminearum* and *F. culmorum*, are the most common. These two species are closely related and produce DON or NIV and ZEA, depending on the geographical origin of the isolate (Miller *et al.*, 1991; Waalwijk *et al.*, 2003). *F. graminearum* is common in wheat from North America, South America, and China (Miller, 1994). In cooler parts of Europe, *F. culmorum* has been dominant, but *F. graminearum* appears to be displacing it (Waalwijk *et al.*, 2003).

Concerning the other species involved in Fusarium head blight, *F. avenaceum* is also common in wheat from all regions studied. *F. crookwellense* is relatively common in Australia and South Africa, but is rare in wheat from Canada and the USA. *F. poae*, *F. langsethii*, *F. equiseti*, and *F. sporotrichioides* are also isolated from wheat kernels at low to moderate frequencies, more commonly under cooler conditions (Bottalico and Peronne, 2002). The distribution of head blight species is affected by pathogenicity, with a relative pathogenicity of *F. graminearum* > *F. culmorum* >> *F. crookwellense* > *F. avenaceum*. The regional and annual variation of the pathogenic species is most affected by temperature; species ranked from coldest to warmest areas are *F. culmorum* > *F. crookwellense* > *F. avenaceum* > *F. graminearum* (Miller, 1994; Bottalico and Peronne, 2002).

Isolates of *F. graminearum* and *F. culmorum* produce a fairly large number of other compounds as well as DON and ZEA. Isolates from North and South America produce 15-acetyl deoxynivalenol, the precursor to DON. If isolates from Asia and Europe produce DON or NIV, they also produce the respective 3-acetate, i.e. deoxynivalenol monoacetate or fusarenon-X. Strains of *F. crookwellense* produce NIV regardless of geographical origin (Miller *et al.*, 1991; Bottalico and Peronne, 2002, under the name *F. cerealis*). Some of the minor metabolites are found in small grains along with DON.

Although increased rainfall promotes Fusarium head blight, incidence is most affected by moisture at anthesis as long as the temperature remains in the favourable range for growth (Miller, 1994). Cultivar susceptibility and rainfall at anthesis explain most variability in infection,

but crop rotation also has a large effect. Growing wheat following maize increases disease under favourable weather conditions (Schaafsma *et al*., 2002). Reduced tillage is an equivocal source of variation in the amount of disease observed (Miller *et al*., 1998; Schaafsma *et al*., 2002).

Fusarium graminearum is a necrotrophic pathogen, i.e. it invades plants by killing host cells in advance. This was reported by the earliest investigators (see Schroeder and Christensen, 1963). Trichothecenes were recognized to be phytotoxic compounds at the time of their discovery (Brian *et al*., 1961). It was realized much later that large differences exist in the responses of wheat cultivars to Fusarium head blight. Coleoptile tissue of cultivars that were resistant to Fusarium head blight was 10 times as resistant to necrosis in the presence of DON than was that of disease-susceptible cultivars (Wang and Miller, 1988). This difference was shown to be due to the presence of a modified peptidyl transferase involved in protein synthesis (Miller and Ewen, 1997) and to unknown functional changes in the membranes of more resistant types (Snijders and Krechting, 1992; Cossette and Miller, 1995; Miller and Ewen, 1997). Earlier studies had shown that cultivars of wheat in the field appeared to be able to metabolize DON, and this was later shown to be the case in vitro in cultivars resistant to head blight (Miller and Arnison, 1986). Strains that produce high concentrations of DON in the field were more virulent (Snijders, 1994; Mesterhazy *et al*., 1999). This implied that one component of resistance to Fusarium head blight is related to reducing the phytotoxic impact of DON. In addition, DON has been found to appear in wheat kernels in advance of fungal mycelia (Snijders and Perkowski, 1990; Snijders and Krechting, 1992). The wheat cultivar Frontana is substantially more resistant to the membrane-damaging effects of DON than susceptible cultivars (Miller and Ewen, 1997).

7. Genus *Claviceps*

The following descriptions of *Claviceps* species are taken from Alderman *et al*. (1999).

The genus *Claviceps*, an Ascomycete with a conidial state, includes several species that are parasitic on grasses, including cultivated cereals throughout the temperate world. *Claviceps* species infect only the flowers of susceptible hosts. Infection involves replacement of the ovary by a specialized structure that develops into a sclerotium, a hard, compact mass of fungal tissue. The sclerotia are usually white, black, or tan and are 1–4 times as large as the seeds they replace. The sclerotia and diseases caused by *Claviceps* species go by the general name ergot. Ergots formed by the most important species, *C. purpurea*, are dark purple to black, and are most prevalent on rye, but also occur to some extent on barley, oats, and wheat, as well as wild and cultivated grasses.

During ergot development, conidia are produced by the fungus and are immersed in plant sap to produce a sugary liquid known as honeydew, which drips from the infected plant as large drops. These are attractive to insects, which act as vectors for dispersing the conidia and spreading infection throughout the crop.

Sclerotia are the resting stage of *Claviceps* species between seasons. Under favourable conditions sclerotia germinate, producing the ascomycete stage of the fungus. Ascospores are formed in closed bodies on stalks arising from the sclerotia, and in many species these provide the initial inoculum for infection of the next season's crop.

The sclerotia of many *Claviceps* species contain toxic alkaloids, poisonous to humans and animals, which have also found major use in pharmaceuticals.

Many *Claviceps* species are restricted to a few grass genera as hosts. However, the species most important from the mycotoxin viewpoint, *C. purpurea*, has a host range of more than 200 grass species. It is distributed worldwide in temperate climates and is responsible for the disease called ergotism in humans and domestic animals.

Identification. Species of *Claviceps* cannot be grown in culture, so must be identified by natural characteristics. *C. purpurea* is a pathogen on grasses, but not on sorghum or maize, whereas *C. africana*, also of importance in terms of mycotoxins, is a cause of a serious disease resulting in male-sterile sorghum seed.

Claviceps purpurea (Fr.: Fr.) Tul. The sclerotium of *Claviceps purpurea* comprises a compact mass of fungal tissue encased in a dark pigmented outer rind. This overwintering stage apparently requires 2 months of cold weather (0–10 °C) to induce germination. In warmer regions, sclerotia do not survive well.

In spring, the sclerotia germinate, producing stalked ascocarps, in which small, thread-like ascospores are produced. The ascospores are ejected forcibly from the ascocarp and are carried by air currents to grass flowers.

The period of susceptibility for most grasses is very brief, from flower opening to fertilization, as fertilized ovaries are resistant to infection. Environmental conditions that delay pollination, such as cool temperatures, increase the infective period. Male-sterile lines of grasses are especially susceptible to *Claviceps* infection because they are not pollinated.

Within a week after infection, conidia are produced in abundance, present in the sticky honeydew that drips from the flowers. The honeydew

acts as the main infective stage, being spread by insects, rain splash, or contact with uninfected flower heads.

Within about two weeks after infection, sclerotia begin to appear. Maturity of the sclerotia coincides with maturity of the infected grass seed heads.

8. Decision trees

The major commodities susceptible to mycotoxin formation are summarized in Figs 1.10 and 1.11, together with the major fungal species involved.

Fig. 1.10. Decision tree for directing risk management decisions or actions based on environmental considerations and probability of fungal contamination in warm climates. Expected toxic effects in susceptible animals are given for each group of mycotoxins.

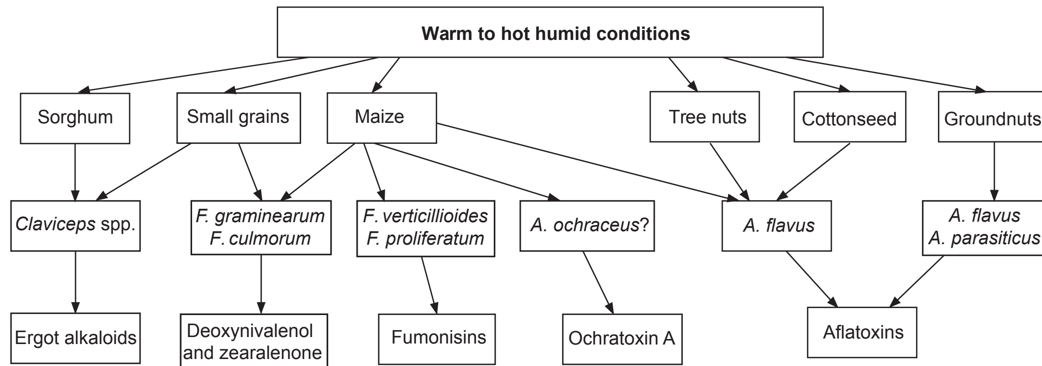
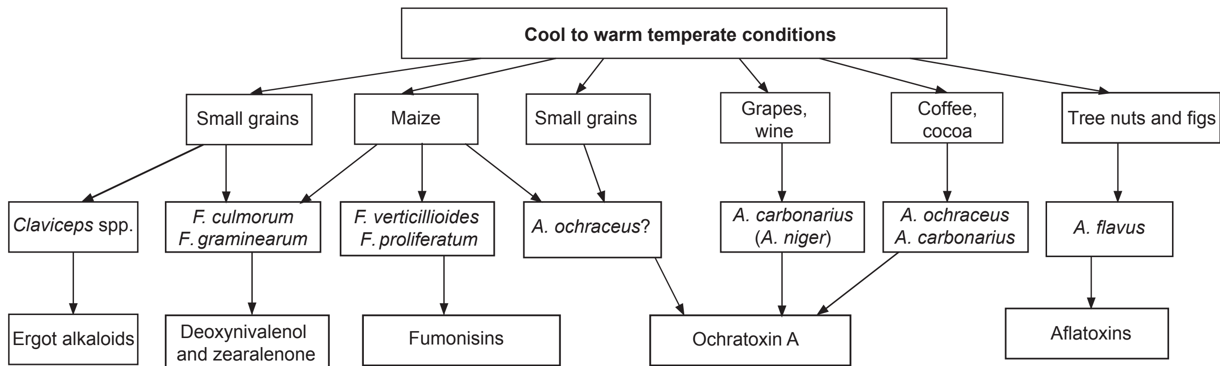


Fig. 1.11. Decision tree for directing risk management decisions or actions based on environmental considerations and probability of fungal contamination in cool climates. Expected toxic effects in susceptible animals are given for each group of mycotoxins.



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ANNEX. MEDIA

The formulations given below are from Pitt and Hocking (2009).

Aspergillus flavus and parasiticus agar (AFPA)

Peptone, bacteriological: 10 g
Yeast extract: 20 g
Ferric ammonium citrate: 0.5 g
Chloramphenicol: 100 mg
Agar: 15 g
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Water, distilled: 1 L

Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 6.0–6.5.

Czapek concentrate

$NaNO_3$: 30 g
KCl: 5 g
$MgSO_4.7H_2O$: 5 g
$FeSO_4.7H_2O$: 0.1 g
Water, distilled: 100 mL

Czapek concentrate will keep indefinitely without sterilization. The precipitate of $Fe(OH)_3$ that forms in time can be resuspended by shaking before use.

Czapek–Dox iprodione dichloran agar (CZID)

Sucrose: 30 g
Yeast extract: 5 g
Chloramphenicol: 100 mg
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Czapek concentrate: 10 mL
Trace metal solution: 1 mL
Agar: 15 g
Water, distilled: 1 L
Iprodione suspension: 1 mL

Sterilize by autoclaving at 121°C for 15 minutes. Add iprodione suspension (0.3 g Roval 50WP [Rhône-Poulenc Agrochimie, Lyon, France] in 50 mL sterile water, shaken before addition to medium) after autoclaving. This formulation is an adaptation of the original published formulation (Abildgren *et al.*, 1987), made from basic ingredients rather than using commercial Czapek–Dox broth. Chloramphenicol (100 mg/L) replaces the original combination of chlortetracycline (50 mg) and chloramphenicol (50 mg).

Czapek yeast extract agar (CYA)

K_2HPO_4: 1 g
Czapek concentrate: 10 mL
Trace metal solution: 1 mL
Yeast extract, powdered: 5 g
Sucrose: 30 g
Agar: 15 g
Water, distilled: 1 L

Refined table grade sucrose is satisfactory for use in CYA provided it is free of sulfur dioxide. Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 6.7.

Dichloran chloramphenicol peptone agar (DCPA)

Peptone: 15 g
KH_2PO_4: 1 g
$MgSO_4.7H_2O$: 0.5 g
Chloramphenicol: 0.1 g
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Agar: 15 g
Water, distilled: 1 L

Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 5.5–6.0.

Dichloran 18% glycerol agar (DG18)

Glucose: 10 g
Peptone: 5 g
KH_2PO_4: 1 g
$MgSO_4.7H_2O$: 0.5 g
Glycerol, A.R.: 220 g
Agar: 15 g
Dichloran (0.2% w/v in ethanol, 1.0 mL): 2 mg
Chloramphenicol: 100 mg
Water, distilled: 1 L

Add minor ingredients and agar to about 800 mL of distilled water. Steam to dissolve agar, then make up to 1 L with distilled water. Add glycerol; note that the final concentration is 18% w/w, not w/v. Sterilize by autoclaving at 121 °C for 15 minutes. Final a_w is 0.955; final pH is 5.5–5.8.

Dichloran rose bengal chloramphenicol agar (DRBC)

Glucose: 10 g
Peptone, bacteriological: 5 g
KH_2PO_4: 1 g
$MgSO_4.7H_2O$: 0.5 g
Agar: 15 g
Rose bengal (5% w/v in water, 0.5 mL): 25 mg
Dichloran (0.2% w/v in ethanol, 1.0 mL): 2 mg
Chloramphenicol: 100 mg
Water, distilled: 1 L

Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 5.5–5.8. Store prepared medium away from light; photoproducts of rose bengal are highly inhibitory to some fungi, especially yeasts. In the dark, the medium is stable for at least 1 month at 1–4 °C. The stock solutions of rose bengal and dichloran need no sterilization, and are also stable for very long periods.

Dichloran rose bengal yeast extract sucrose agar (DRYS)

Yeast extract: 20 g
Sucrose: 150 g
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Rose bengal (5% w/v in water, 0.5 mL): 25 mg
Chloramphenicol: 50 mg
Agar: 20 g
Water, distilled: to 1 L
Chlortetracycline (l% in water, filter-sterilized, 5.0 mL): 50 mg

Sterilize all ingredients except chlortetracycline by autoclaving at 121 °C for 15 minutes. Add

chlortetracycline after tempering to 50 °C. Chloramphenicol at twice the concentration specified (i.e. 100 mg/L) adequately controls bacteria in most situations, and this avoids the need for a second antibiotic that must be filter-sterilized.

25% Glycerol nitrate agar (G25N)

K_2HPO_4: 0.75 g
Czapek concentrate: 7.5 mL
Yeast extract: 3.7 g
Glycerol, analytical grade: 250 g
Agar: 12 g
Water, distilled: 750 mL

Glycerol for G25N should be of high quality, with a low (1%) water content. If a lower grade is used, allowance should be made for the additional water. Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 7.0.

Malt extract agar (MEA)

Malt extract, powdered: 20 g
Peptone: 1 g
Glucose: 20 g
Agar: 20 g
Water, distilled: 1 L

Commercial malt extract used for home brewing is satisfactory for use in MEA, as is bacteriological peptone. Sterilize by autoclaving at 121 °C for 15 minutes. Do not sterilize for longer as this medium will become soft on prolonged or repeated heating. Final pH is 5.6.

Potato dextrose agar (PDA)

Potatoes: 250 g
Glucose: 20 g
Agar: 15 g
Water, distilled: to 1 L

PDA prepared from raw ingredients is more satisfactory than commercially prepared media. Wash the potatoes, which should not be of a red skinned variety, and dice or slice, unpeeled, into 500 mL of water. Steam or boil for 30–45 minutes. At the same time, melt the agar in 500 mL of water. Strain the potato through several layers of cheesecloth into the flask containing the melted agar. Squeeze some potato pulp through also. Add the glucose, mix thoroughly, and make up to 1 L with water if necessary. Sterilize by autoclaving at 121 °C for 15 minutes.