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REVIEW ARTICLE

Current trends in aflatoxin research

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ABSTRACT Aflatoxins are decaketide-derived secondary metabolites which are produced by a complex biosynthetic pathway. Aflatoxins are among the economically most important mycotoxins. Aflatoxin B_1 exhibits hepatocarcinogenic and hepatotoxic properties, and is frequently referred to as the most potent naturally occurring carcinogen. Acute aflatoxicosis epidemics occurred in several parts of Asia and Africa leading to deaths of several hundred people. Recent data indicate that aflatoxins are produced by 20 species assigned to three sections of the genus *Aspergillus*: sections *Flavi*, *Nidulantes* and *Ochraceorosei*. The economically most important producer is *A. flavus* and its relatives. Compounds with related structures include sterigmatocystin, an intermediate of aflatoxin biosynthesis produced by several Aspergilli and species assigned to other genera, and dothistromin produced by a range of non-*Aspergillus* species. Aflatoxin producers and consequently aflatoxin contamination occur frequently in various food products mainly in tropical and subtropical areas of the world. However, climate change led to the occurrence of aflatoxin producing species, especially *A. flavus* in areas where they were not prevalent previously. Molecular genetic and genomic studies led to the clarification of aflatoxin and sterigmatocystin biosynthetic pathways in a range of producing organisms, and provided insight into the metabolism and effect of aflatoxins. In this review, we wish to give an overview on recent progress of aflatoxin research including producing organisms, occurrence, biosynthesis and molecular detection of aflatoxins.

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Secondary metabolism is mainly a characteristic of filamentous fungi. The diversity and complexity of secondary metabolites is astounding, and species of *Aspergillus* are rich in genes for secondary metabolism (Nierman et al. 2005; Kobayashi et al. 2007; Rokas et al. 2007). Secondary metabolites are usually not required for growth of the organism in culture, but do contribute to the fitness of the organism in its natural environment. Secondary metabolites have an impact on our daily life either as toxins or as beneficial compounds. Beneficial secondary metabolites made by species of *Aspergillus* include food additives such as kojic acid or citric acid, antibiotics such as penicillin, and cholesterol reducing drugs such as lovastatin (Endo et al. 1976; Adrio and Demain 2003). In contrast, the repertoire of fungal secondary metabolites also includes harmful products known as mycotoxins.

Aflatoxins are the most thoroughly studied mycotoxins, which are produced by species assigned to the *Aspergillus* genus. They were discovered when the toxicity of animal feeds containing contaminated peanut meal led to the death of more than 100,000 turkeys from acute liver necrosis in the early sixties (Turkey-X disease; Blout 1961; Sargeant et al. 1961; van der Zijden et al. 1962). *Aspergillus flavus* was identified as the producing fungus, and aflatoxins were named after the toxic agent. Aflatoxins have both toxic and carcinogenic properties, posing serious threats to both animal and human health (Bennett and Klich 2003). Comprehensive studies have shown that aflatoxin is a risk factor for human hepatocellular carcinoma, especially in Asia and sub-Saharan Africa (Groopman et al. 2005). Several deaths were also attributed to acute aflatoxicosis (Nyikal et al. 2004). Because of its toxicity, over 100 countries restrict the content of aflatoxins in the food and feed supplies (van Egmond et al. 2007).

Aflatoxins are a group of structurally related difuranocoumarins that were named as aflatoxins B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. Aflatoxin B_1 (Fig. 1) is the most potent natural carcinogen known (Squire 1981, IARC 2012), and is usually the major aflatoxin produced by toxigenic strains. Apart from those mentioned above, over a dozen of other structural analogs including aflatoxins P_1, Q_1, B_{2a} and G_{2a} have been described as mammalian biotransformation products of the major metabolites, while aflatoxin D_1 was detected in ammoniated maize, and aflatoxin B_3 as a metabolite of *A. flavus* (Cole and Schweikert 2003, Varga et al. 2009). Aflatoxin M_1, a hydroxylated metabolite is found primarily in animal tissues and fluids (milk and urine) as a metabolic product of aflatoxin B_1 (Varga et al. 2009; Fig. 1).

In this review, an overview of recent data on aflatoxins will be presented including the range of aflatoxin producing fungi,

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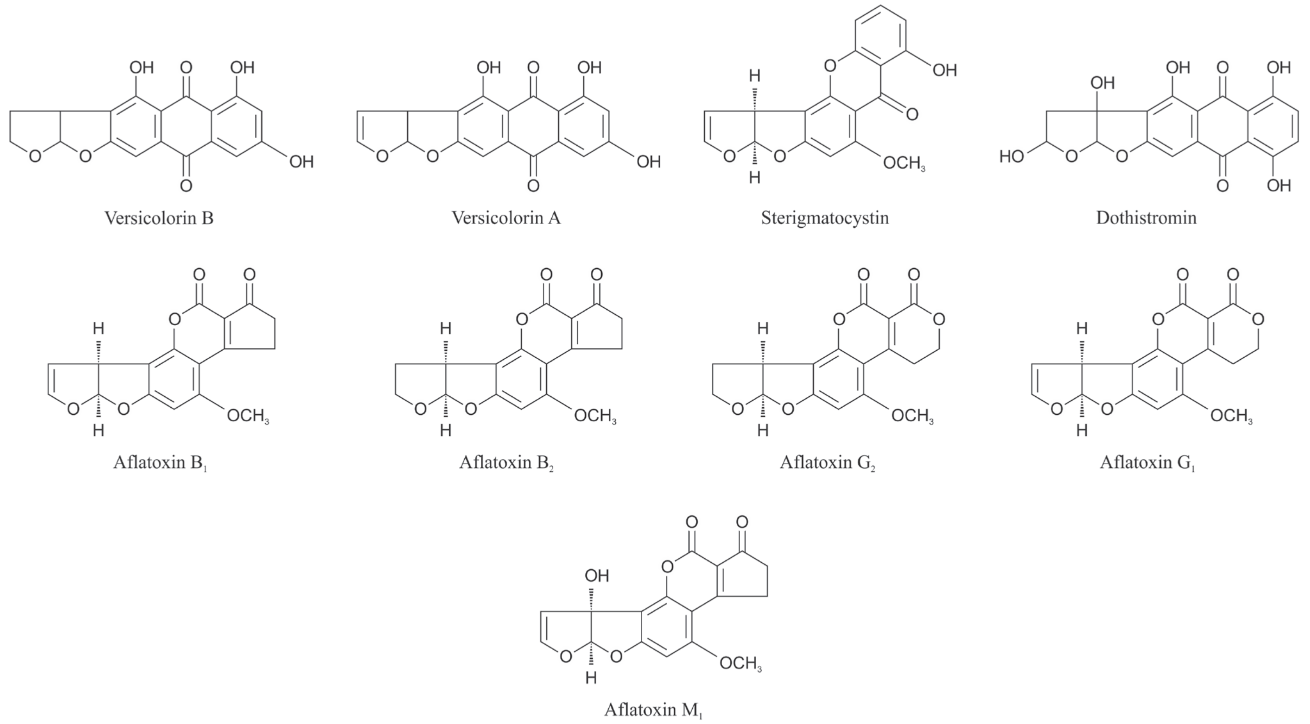

Versicolorin B Versicolorin A Sterigmatocystin Dothistromin

Aflatoxin B_1 Aflatoxin B_2 Aflatoxin G_2 Aflatoxin G_1

Aflatoxin M_1

Figure 1. Structures of the most important aflatoxins and their structural relatives.

occurrence of aflatoxins and producers in various matrices, and biosynthesis and molecular detection of aflatoxins.

Aflatoxin producers

A thorough review has been published recently on the re-evaluation of aflatoxin production in fungi (Varga et al. 2009). At that time, 13 species have been found to be able to produce aflatoxins, all belonging to the *Aspergillus* genus. Since then, 7 more species have been found to be able to produce these compounds including *A. pseudonomius, A. pseudocaelatus* (Varga et al. 2011), *A. togoensis* (Rank et al. 2011), *A. mottae, A. sergii, A. transmontanensis* (Soares et al. 2012) and *A. novoparasiticus* (Gonçalves et al. 2012). These data indicate that aflatoxins are produced by at least 20 species assigned to three sections of the genus *Aspergillus*: sections *Flavi, Nidulantes* and *Ochraceorosei* (Varga et al. 2009; Fig. 2, Table 1). Some aflatoxin producing species have been described as *Emericella* species (one of the sexual stages of the *Aspergillus* genus). However, according to the Amsterdam declaration on fungal nomenclature, only one name can be applied for a fungus (Hawksworth et al. 2011). Under the current rules of the International Code of Nomenclature for algae, fungi, and plants (Hawksworth 2011b; Melbourne Code, McNeill et al. 2012) and the discussions held by the International Commission on *Penillium* and *Aspergillus* (ICPA; http://www.aspergilluspenicillium.org/index.php/single-name-nomenclature/88-single-names/105-aspergillus-options), the *Aspergillus* name was chosen as the valid one for these species (Hawksworth 2011a; Samson et al. unpublished data).

Only B-type aflatoxins are produced by most species, although species related to *A. parasiticus* and *A. nomius* are usually able to produce G-type aflatoxins too (Table 1). Ex-type isolates of *A. oryzae*, *A. fasciculatus*, *A. kambarensis*, *A. effusus* and *A. flavus* var. *columnaris* were treated as synonyms of *A. flavus*, ex-type isolates of *A. toxicarius* and of *A. chungii* (NRRL 4868) were considered not distinct from *A. parasiticus* (Soares et al. 2012), and *A. zhaoqingensis* has been synonymised with *A. nomius* (Varga et al. 2011). Although, aflatoxin production was claimed for several other species and fungal genera (and actually even for bacteria), none of these observations could have been confirmed (Varga et al. 2009). Recently, a *Fusarium kyushuense* isolate was also claimed to produce aflatoxins, but this report also could not be confirmed (Schmidt-Heydt et al. 2009; Varga et al. 2009).

A structurally related compound, the carcinogenic sterigmatocystin is an intermediate of the aflatoxin biosynthesis, and may be important as it can be produced in rather large amounts on cheese and occasionally in cereals (Pitt and Hocking 2010; Samson et al. 2010). Sterigmatocystin has been reported in several phylogenetically and phenotypically different genera (Rank et al. 2011). The major source of sterigmatocystin in foods and indoor environments is *Aspergillus versicolor* and its relatives (Samson et al. 2010).

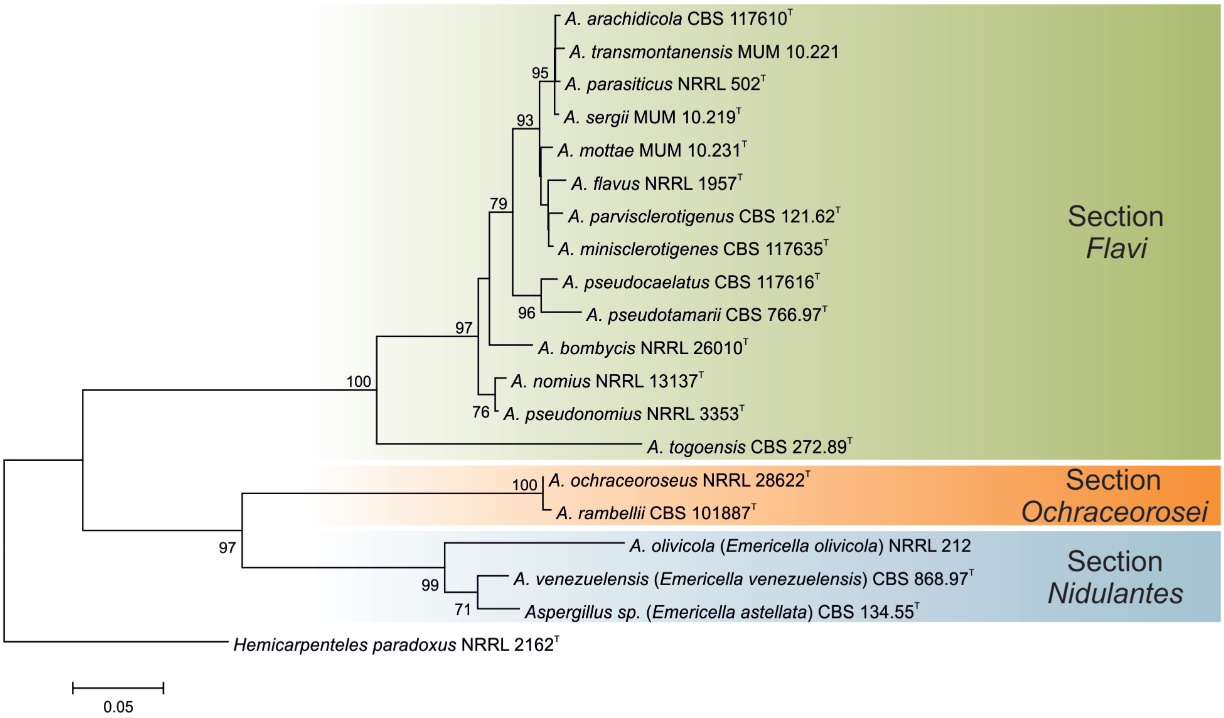

Figure 2. Phylogenetic tree of aflatoxin producing fungi based on partial calmodulin sequence data.

Production of this mycotoxin was confirmed in 31 *Aspergillus*, five *Chaetomium* species and in *Botryotrichum pillulifera, Bipolaris sorokiana* and *Humicola nordinii* under the growth conditions tested using multiple detection methods (Rank et al. 2011). Sterigmatocystin production was also confirmed in *Aspergillus inflatus* (=*Penicillium inflatum;* Rank et al. 2011), which species belongs to *Aspergillus* section *Cremei* according to multilocus phylogenetic studies (Varga et al. unpublished results). More recently, Jurjević et al. (2012, 2013) described 9 new species assigned to section *Versicolores* which are also able to produce this compound. Sterigmatocystin production was also confirmed in *Podospora anserina* (Matasyoh et al. 2011), and the gene cluster responsible for the biosynthesis of sterigmatocystin was also identified (Slot and Rokas 2011). Apart from sterigmatocystin, the immediate precursor of aflatoxin, O-methylsterigmatocystin was also found in *Chaetomium cellulolyticum, Chaetomium longicolleum, Chaetomium malaysiense* and *Chaetomium virescens* (Rank et al. 2011). Besides, the ex-type strain of the newly described species *A. bertholletius* was also found to produce O-methylsterigmatocystin, indicating that the genome of this species also carries the aflatoxin biosynthetic gene cluster (Taniwaki et al. 2012). Although sterigmatocystin is a precursor of aflatoxins, only *Aspergillus ochraceoroseus, A. rambellii* (Frisvad et al. 1999; Klich et al. 2000), and some species belonging to section *Nidulantes* accumulate both sterigmatocystin and aflatoxins (Frisvad et al. 2004; Frisvad and Samson 2004). Members of *Aspergillus* section *Flavi*, which includes the major aflatoxin producers, efficiently convert sterigmatocystin through 3-methoxysterigmatocystin to aflatoxins (Frisvad et al. 1999). An exception in this section is *A. togoensis*, which is able to produce both aflatoxins and sterigmatocystin (Wicklow et al. 1989; Rank et al. 2011).

Another metabolite structurally related to aflatoxins is dothistromin produced by *Dothistroma septosporum*, an important forest pathogen causing red band needle blight disease of pine trees (Bradshaw 2004). Dothistromin is similar in structure to versicolorin B, a precursor of aflatoxin biosynthesis. Full genome sequencing of *D. septosporum* made it possible to identify the genes taking part in the biosynthesis of this compound (Bradshaw et al. 2013). Interestingly, in contrast with other secondary metabolite biosynthesis genes which form gene clusters, most of the genes taking part in dothistromin biosynthesis were found to be spread over six separate regions on chromosome 12 of the pathogen (Bradshaw et al. 2013). The coordinated control of this dispersed set of secondary metabolite genes is achieved by the transcription factor *AflR* (Chettri et al. 2013).

Occurrence of aflatoxin producing fungi and aflatoxins in various habitats

Aflatoxins are primarily produced by *Aspergillus flavus* and *A. parasiticus* on agricultural commodities including cereals

Table 1. *Aspergillus* species able to produce aflatoxins and other mycotoxins.

Species	Occurrence	Type of aflatoxin produced	Other mycotoxins	References
Aspergillus section *Flavi*				
A. arachidicola	Argentina, Brazil	Aflatoxins B_1, B_2 & G_1, G_2	kojic acid, aspergillic acid	Pildain et al. 2008, Calderari et al. 2013
A. bombycis	Japan, Indonesia, Brazil	Aflatoxins B_1, B_2 & G_1, G_2	kojic acid, aspergillic acid	Peterson et al. 2001, Calderari et al. 2013, Okano et al. 2012
A. flavus	Worldwide	Aflatoxins B_1 & B_2	cyclopiazonic acid, kojic acid, aspergillic acid	Varga et al. 2009
A. minisclerotigenes	Argentina, USA, Australia, Nigeria, Portugal, Benin, Argentina, Morocco, Algeria, (Kenya?)	Aflatoxins B_1, B_2 & G_1, G_2	cyclopiazonic acid, kojic acid, aspergillic acid	Pildain et al. 2008, Soares et al. 2012, Moore et al. 2013, Guezlane-Tebibel et al. 2012, El Mahgubi et al. 2013, (Probst et al. 2012)
A. nomius	USA, Japan, Thailand, India, Brazil, Hungary, Serbia	Aflatoxins B_1, B_2 & G_1, G_2	kojic acid, aspergillic acid, tenuazonic acid	Kurtzman et al. 1987, Olsen et al. 2008, Manikandan et al. 2009, Calderari et al. 2013, Okano et al. 2012, unpublished observations
A. novoparasiticus	Colombia, Brazil	Aflatoxins B_1, B_2 & G_1, G_2	kojic acid	Gonçalves et al. 2012
A. parasiticus	USA, Japan, Australia, Brazil, India, South America, Uganda, Portugal, Italy, Serbia	Aflatoxins B_1, B_2 & G_1, G_2	kojic acid, aspergillic acid	Varga et al. 2009, Soares et al. 2012, Baquião et al. 2013, unpublished observations
A. parvisclerotigenus	Nigeria	Aflatoxins B_1, B_2 & G_1, G_2	cyclopiazonic acid, kojic acid	Geiser et al. 2000, Frisvad et al. 2005
A. pseudocaelatus	Argentina	Aflatoxins B_1, B_2 & G_1, G_2	cyclopiazonic acid, kojic acid	Varga et al. 2011
A. pseudonomius	USA	Aflatoxin B_1	kojic acid	Varga et al. 2011
A. pseudotamarii	Japan, Argentina, Brazil, India	Aflatoxin B_1, B2 & G1, G2	cyclopiazonic acid, kojic acid	Ito et al. 2001, Baranyi et al. 2013, Calderari et al. 2013, Massi et al. submitted
A. togoensis	Central Africa	Aflatoxin B_1	sterigmatocystin	Wicklow et al. 1989, Rank et al. 2011
A. transmontanensis	Portugal	Aflatoxins B_1, B_2 & G_1, G_2	aspergillic acid	Soares et al. 2012
A. mottae	Portugal	Aflatoxins B_1, B_2 & G_1, G_2	cyclopiazonic acid, aspergillic acid	Soares et al. 2012
A. sergii	Portugal	Aflatoxins B_1, B_2 & G_1, G_2	cyclopiazonic acid, aspergillic acid	Soares et al. 2012
Aspergillus section *Ochraceorosei*				
A. ochraceoroseus	Ivory Coast	Aflatoxins B_1 & B_2	sterigmatocystin	Frisvad et al. 1999
A. rambellii	Ivory Coast	Aflatoxins B_1 & B_2	sterigmatocystin	Frisvad et al. 2005
Aspergillus section *Nidulantes*				
A. astellatus (=Emericella astellata)	Ecuador	Aflatoxin B_1	sterigmatocystin, terrein	Frisvad et al. 2004
A. olivicola (=Emericella olivicola)	Italy	Aflatoxin B_1	sterigmatocystin, terrein	Zalar et al. 2008
A. venezuelensis (=Emericella venezuelensis)	Venezuela	Aflatoxin B_1	sterigmatocystin, terrein	Frisvad and Samson 2004

(wheat, maize, rice), cotton, peanut, tree nuts, pepper, spices and others (Varga et al. 2009). Aflatoxins were also detected and *Aspergillus flavus* was identified from water from a cold water storage tank by Paterson et al. (1997). More recently, the fungal flora of tap water from an Iranian university hospital was investigated and the results of this study showed that hospital water should be considered as a potential reservoir of fungi particularly *Aspergillus* including *A. flavus* (Hedayati et al. 2011). *A. flavus* is also frequently isolated from indoor air, particularly in subtropical and tropical areas (Hedayati et al. 2007, 2010; Sepahvand et al. 2013). Recently, this species was also identified in large quantities in indoor air in Croatia and Hungary (Varga et al., unpublished results).

The most important aflatoxin producer, *A. flavus* can cause both pre- and postharvest contamination of various agricultural products. Although, the native habitat of this species is

Table 2. Genes taking part in aflatoxin biosynthesis.

Gene	(synonym)	Enzyme or product	Step in aflatoxin biosynthesis pathway
aflA	*(fas-2)*	Fatty acid synthase α	malonyl-CoA → condensed polyketide noranthrone
aflB	*(fas-1)*	Fatty acid synthase β	malonyl-CoA → condensed polyketide noranthrone
aflC	*(pksA)*	Polyketide synthase	malonyl-CoA → condensed polyketide noranthrone
hypC		Anthrone oxidase	noranthrone → norsolonic acid
aflD	*(nor-1)*	Reductase	norsolonic acid (NOR)→ averantin (AVN)
aflE	*(norA)*	NOR-reductase	norsolonic acid (NOR)→ averantin (AVN)
aflF	*(norB)*	Dehydrogenase	norsolonic acid (NOR)→ averantin (AVN)
aflG	*(avnA)*	Cytochrome P450 monooxigenase	averantin (AVN)→ hydroxyaverantin (HAVN)
aflH	*(adhA)*	Alcohol dehydrogenase	hydroxyaverantin (HAVN)→averufin (AVR)
aflI	*(avfA)*	Averufin monooxygenase	averufin (AVR)→ versiconal hemiacetal aceteate (VHA)
aflJ	*(estA)*	Cytosole esterase enzyme	versiconal hemiacetal aceteate (VHA)→ versiconal (VAL)
aflK	*vbs*	Versicolorine B synthase	versiconal (VAL)→ versicolorin B
aflL	*verB*	Cytochrome P450 monooxigenase/ desaturase	versicolorin B → versicolorin A, versicolorin B →demethyldihydrosterigmatocystin (DMDHST)
aflM	*ver-1*	Ketoreductase enzyme	versicolorin A → demethylsterigmatocystin (DMST)
aflN	*verA*	Cytochrome P450 monooxigenase	versicolorin A → demethylsterigmatocystin (DMST)
aflO	*dmtA (mt-1) / omtB*	O-methyltransferase I/ O-methyltransferase B	demethylsterigmatocystin (DMST) → sterigmatocystin (ST) dihydrodemethylsterigmatocystin (DHDMST) → dihydrosterigmatocystin (DHST)
aflP	*omtA*	O-methyltransferase II/ O-methyltransferase A	sterigmatocystin (ST) → O-methylsterigmatocystin (OMST) dihydrosterigmatocystin (DHST) → dihydro-O-methylsterigmatocystin (DHOMST)
aflQ	*ordA*	Monooxygenase	O-methylsterigmatocystin (OMST) → aflatoxin B_1 and G_1 dihydro-O-methylsterigmatocystin (DHOMST) → aflatoxin B_2 and G_2
aflR	*aflR*	Transcription activator	Pathway regulator
aflS	*aflJ*	Transcription enhancer	Pathway regulator
aflT	*aflT*	ABC transporter protein	Unassigned
aflU	*cypA*	Cytochrome P450 monooxigenase	Unassigned
aflV	*cypX*	Cytochrome P450 monooxigenase	Unassigned
aflW	*moxY*	Monooxygenase	Unassigned
aflX	*ordB*	Monooxygenase	Unassigned
aflY	*hypA*	Hypothetical protein	Unassigned

soil and decaying vegetation, it is able to invade many types of organic substrates whenever conditions are favorable for its growth. *A. flavus* is also an important pathogen of various cultivated plants including maize, cotton and peanut, and cause serious yield losses throughout the world. Since aflatoxin production is favored by moisture and high temperature, *A. flavus* is able to produce aflatoxins in warmer, tropical and subtropical climates (Varga et al. 2009). Consequently, aflatoxin contamination of agricultural products in countries with temperate climate, including Central European countries was not treated as a serious health hazard. However, recently several papers have dealt with the effects of climate change on the appearance of aflatoxin producing fungi and aflatoxins in foods (Cotty and Jaime-Garcia 2007; Miraglia et al. 2009; Paterson and Lima 2010; Tirado et al. 2010). Based on these studies, aflatoxin producing fungi and consequently aflatoxins are expected to become more prevalent with climate change in countries with temperate climate. Indeed, several recent reports have indicated the occurrence of aflatoxin producing fungi and consequently aflatoxin contamination in agricultural commodities in several European countries that did not face with this problem before. In western Romania, Curtui et al. (1998) reported that all the examined maize samples were free from aflatoxins in 1997. However, more recently, Tabuc et al. (2009) have found that about 30% of maize samples collected between 2002 and 2004 in southeastern Romania were contaminated with aflatoxin B_1 (AFB_1), and in 20% of these samples the level of toxin exceeded the European Union limit of 5 μg/kg. In Serbia, Jakić-Dimić et al. (2009) isolated *A. flavus* from 18.7% of the maize samples analyzed, and aflatoxins were also detected in 18.3% of the samples, while Jaksić et al. (2011) detected aflatoxins in 41.2% of the analyzed maize samples in the range of 2-7 μg/kg. Polovinski-Horvatović et al. (2009) observed aflatoxin M_1 in 30.4% of milk samples collected from small farms in Serbia in amounts exceeding the allowable legislation of the European Union. Similarly, Torkar and Vengust (2007) detected aflatoxin M_1 above the EU limit in 10% of the examined milk samples in Slovenia. Halt (1994) detected aflatoxins in 9.4% of Croatian flour samples, and isolated *A. flavus* from 38% of the flour samples in 2004 (Halt et al. 2004). Although Haberle (1988) could not detect aflatoxins in Croatian milk samples, Bilandzić et al. (2010)

could detect aflatoxin M_1 above the EU limit in some milk samples collected in Croatia. Regarding Hungary, Richard et al. (1992) examined the mycotoxin producing abilities of 22 isolates collected from various sources in Hungary, and none of the isolates were found to produce aflatoxins. However, more recently, Borbély et al. (2010) have examined mycotoxin levels in cereal samples and mixed feed samples collected in eastern Hungary, and detected AFB_1 levels above the EU limit in 4.8% of the samples. Dobolyi et al. (2011, 2013) identified aflatoxin producing *A. flavus* isolates in several maize fields in Hungary. Aflatoxin contamination of maize (2003) and milk (2007, 2011, 2012) originating from Hungary, Serbia, Romania and Slovenia have also been detected recently in the frame of the Rapid Alert System for Food and Feed of the European Union (https://webgate.ec.europa.eu/rasff-window/portal/). In recent surveys, *A. flavus* was also identified in various agricultural products including maize, wheat and barley in Hungary (Tóth et al. 2012).

Due to the extreme weather conditions in 2012 in Central Europe, aflatoxin contamination of maize and milk caused serious problems in several countries including Serbia, Romania and Croatia (http://en.wikipedia.org/wiki/2013_aflatoxin_contamination). Aflatoxins were also detected in maize kernels in Hungary after harvest in 2012 (Tóth et al. 2013).

Apart from *A. flavus*, other aflatoxin producers have also been observed in Central Europe. *A. nomius* was detected for the first time in the region in cheese samples in Hungary, and in maize in Serbia (Varga et al., unpublished observations). Since *A. nomius* is also able to produce G-type aflatoxins, these data explain their detection in some Serbian maize samples (Kos et al. 2013).

Aflatoxin producers have also been found to be able to cause human infections. *Aspergillus flavus* is considered as the second most important cause of invasive aspergillosis, and also frequently identified in other human infections (Hedayati et al. 2007). Other aflatoxin producing species identified as causes of human diseases include *A. nomius* which was identified from human onychomycosis and keratitis cases (Manikandan et al. 2009), and *A. pseudotamarii* identified from a keratitis case in India (Baranyi et al. 2013). Aflatoxin producing abilities of *A. nomius* have also been proven under *ex vivo* conditions (Klich et al. 2009; Baranyi et al. 2013, unpublished results).

Aflatoxin biosynthesis

Molecular analysis of aflatoxin production in *A. flavus* and *A. parasiticus* led to the identification of an about a 75 kb DNA cluster consisting of two specific transcriptional regulators (*aflR* and *aflS*), and at least 30 co-regulated downstream metabolic genes in the aflatoxin biosynthetic pathway (Liu and Chu 1998; Bhatnagar et al. 2003; Yu et al. 2004; Georgianna and Payne 2009; Ehrlich et al. 2012). Sterigmatocystin is a penultimate precursor of aflatoxin biosynthesis and also a toxic and carcinogenic substance produced by many species belonging mainly to sections *Versicolores* and *Nidulantes* of the *Aspergillus* genus. Sterigmatocystin production also occurs in the phylogenetically unrelated genera *Monocillium*, *Chaetomium*, *Humicola* and *Bipolaris* (Varga et al. 2009; Rank et al. 2011). Two genes, *aflR* and *aflS*, located divergently adjacent to each other within the aflatoxin cluster are involved in the regulation of aflatoxin or sterigmatocystin gene expression. The gene *aflR* encodes a sequence-specific zinc-finger DNA-binding protein (Zn(II)2Cys6), which is required for transcriptional activation of most, if not all, of the structural genes (Chang et al. 1993, 1995, 1999; Payne et al. 1993; Woloshuk et al. 1994; Yu et al. 1996; Flaherty and Payne 1997; Ehrlich et al. 1998; Price et al. 2006).

Aflatoxin biosynthesis is also regulated by *aflS* (formerly *aflJ*), a gene that resides next to *aflR*. The genes *aflS* and *aflR* are divergently transcribed, and they have independent promoters (Georgianna and Payne 2009). The intergenic region between them, however, is short and it is possible that they share binding sites for transcription factors or other regulatory elements (Ehrlich and Cotty, 2002). The precise role of *AflS* in aflatoxin biosynthesis remains unclear (Georgianna and Payne 2009).

The biosynthesis of aflatoxins occurs through a series of highly organized oxidation-reduction reactions (Dutton 1988; Bhatnagar et al. 1992; Minto and Townsend 1997). Aflatoxin biosynthesis starts with conversion of hexanoyl-CoA and 7 malonyl-CoAs to a condensed polyketide noranthrone by the products of two fatty acid synthase genes, *aflA* and *aflB* (whose original names were: *fas-1* and *fas-2*) and a polyketide synthase gene, *aflC* (*pksA*) (Cary et al. 2000). *HypC*, an open reading frame in the region between the *aflC* (*pksA*) and *aflD* (*nor-1*) genes in the aflatoxin biosynthesis gene cluster, encodes a 17-kDa anthrone oxidase which is involved in the catalytic conversion of noranthrone to norsolonic acid (NOR) (Ehrlich 2009). NOR is the first stable metabolite which can be isolated. *AflD* (*nor-1*), *aflE* (*norA*) and *aflF* (*norB*) have an important role in the reduction from NOR to averantin (AVN). NOR is converted to AVN by reductase-, and dehydrogenase enzymes, and this reaction is reversible depending on NADP(H) or NAD(H) (Bennett and Christensen 1983; Dutton 1988; Yabe et al. 1991a; Bhatnagar et al. 1992). The next catalytic step is the conversion of AVN to hydroxyaverantin (HAVN) by a cytochrome P450 monooxigenase enzyme that is encoded by the gene *aflG* (*avnA*) (Yu et al. 1997). Yu and his colleagues (1997) have demonstrated in their gene disruption and substance feeding studies, that HAVN and possibly an additional compound are the intermediers during conversion of AVN to averufin (AVR). The alcohol dehydrogenase encoded by *aflH* (*adhA*) (Chang et al. 2000) can catalyse the conversion from HAVN to AVR.

Some of the catalytic steps in the conversion of AVR to versicolorin B (VERB) have not yet been assigned to a spe-

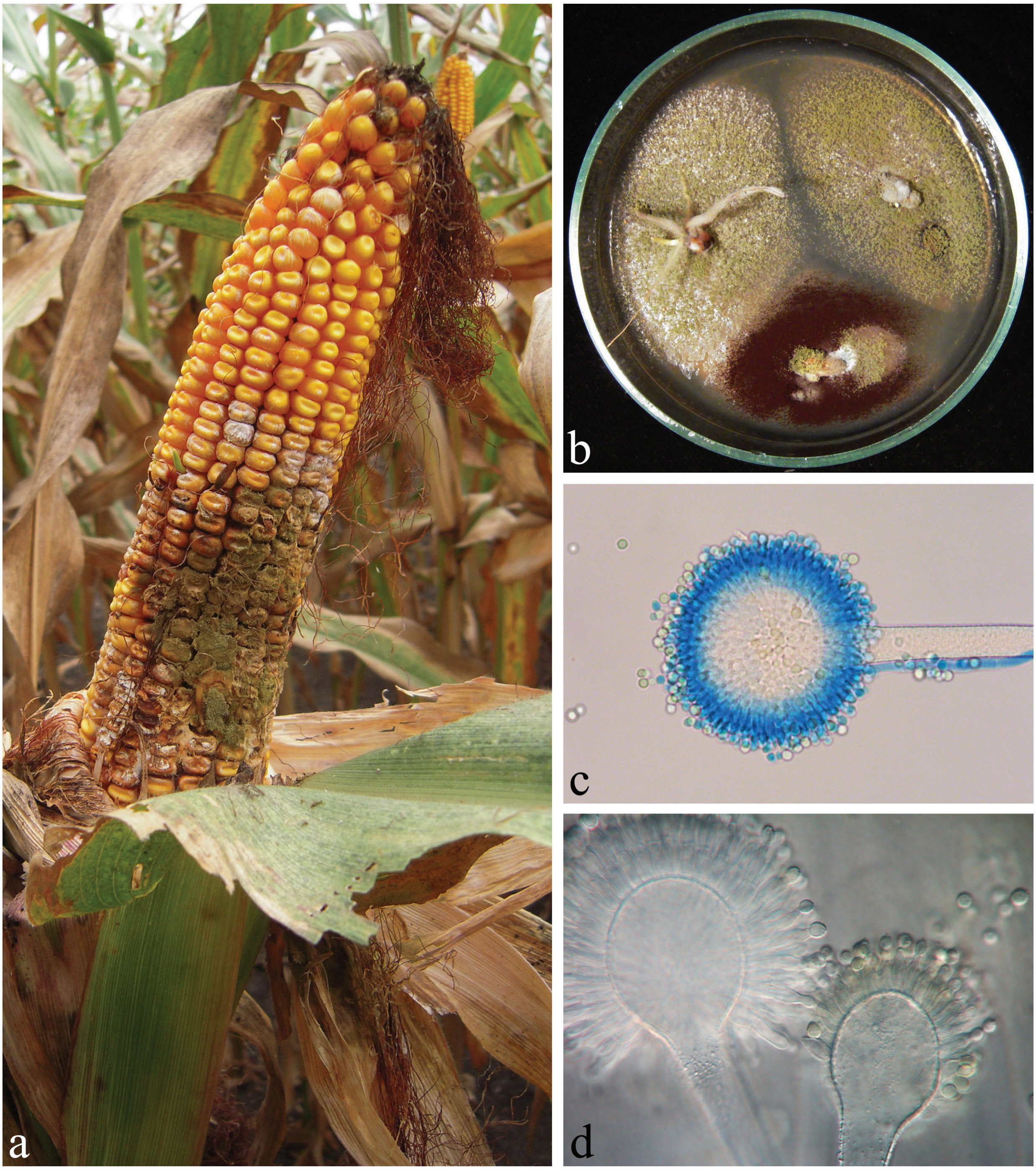

Figure 3. *Aspergillus flavus*. a. Occurrence of *A. flavus* on a maize cob. b. Colonies of *A. flavus* growing on malt extract agar from wheat grain. c-d. *A. flavus* conidial heads.

cific gene in the cluster. Three genes are possible candidates for individual steps: *aflV* (*cypX*), *aflW* (*moxY*), and *aflI* (*avfA*) (Bhatnagar et al. 2003). AVR is converted to hydroxyversicolorone (HVN) by a microsome enzyme in the presence of NADPH (averufin monooxygenase; Yabe et al. 2003). The gene which encodes this enzyme is *aflI* (*avfA*) (Yu et al. 2000a). The *aflV* (*cypX*) and *aflW* (*moxY*) genes (Yu et al. 2000b), also have an important role in the conversion of AVR to versiconal hemiacetal acetate (VHA). The gene *aflV* (*cypX*) encodes a P450 monooxygenase enzyme and *aflW* (*moxY*) encodes a monooxygenase enzyme (Keller et al. 2000).

Hydroxyversicolorone (HVN) is converted to VHA by a VHA synthase enzyme that requires NADPH as a cofactor. The gene which encodes this enzyme has not been identified yet (Yabe et al. 2003). A cytosolic esterase enzyme encoded by the gene *aflJ* (*estA*) is involved in the conversion of VHA to versiconal (VAL).

The conversion of VHA to VERB is the key step in aflatoxin formation since it closes the bisfuran ring of aflatoxin. Silva et al. (1996), Silva and Townsend (1996), and McGuire (1996) cloned and demonstrated the function of the VERB syntase gene (*vbs*) in the conversion of VHA to VERB in *A. parasiticus*. The new name of *vbs* gene is *aflK* (Yu et al. 2004).

The formation of versicolorin A (VERA) from VERB is a branch point in aflatoxin biosynthetic pathway (Bathnagar et al. 1991; Bathnagar et al. 1993; Yabe and Hamasaki 1993). Similarly to AFB$_2$ and AFG2, VERB contains a tetrahydrobisfuran ring in its structure; and, like AFB1 and AFG1, VERA contains a dihydrobisfuran ring. The branching step between B- and G-type aflatoxins is the desaturation reaction from VERB to VERA (Yabe et al. 1991b). The *aflL* (*verB*) gene encodes a cytochrome P450 monooxygenase/ desaturase which is presumed to be involved in the conversion of VERB to VERA in aflatoxin biosynthesis. The gene responsible for the conversion directly from VERB to demethyldihydrosterigmatocystin (DMDHST) and then to AFB$_2$ and AFG$_2$ has not yet been defined. It is possible that *aflL* (*verB*) participates in conversion of both VERB to VERA and VERB to DMDHST (Yu et al. 2004).

The dihydrobisfuran ring in VERA and the tetrahydrobisfuran ring in VERB are maintained through the next steps. The intermediates after these versicolorins are demethylsterigmatocystin (DMST) for VERA and dihydrodemethylsterigmatocystin (DHDMST) for VERB (Yabe et al. 1989). *AflM* (*ver-1*) and *aflN* (*verA*) are required for the conversion of versicolorin A (VERA) to demethylsterigmatocystin (DMST), because the *aflM* (*ver-1*) gene encodes a ketoreductase enzyme (Skory et al. 1992) and *aflN* (*verA*) gene encodes a cytochrome P-450 monooxygenase enzyme (Matsushima et al. 1994). The exact function of *aflN* (*verA*) has not yet been identified (Yu et al. 2004). The conversion of VERA to DMST requires more then one enzymatic activity (Yabe and Nakajima 2004).

DMST and DHDMST contain two free hydroxyl groups, 7-OH and 6-OH. Two distinct O-methyltransferase activities were demonstrated by Yabe et al. (1989) in *A. parasiticus*. O-methyltransferase I catalyzes the transfer of the methyl groups from S-adenosylmethionine (SAM) to the hydroxyl groups of DMST and DHDMST in order to produce sterigmatocystin (ST) and dihydrosterigmatocystine (DHST) (Yabe and Nakajima 2004). The gene for this O-methyltransferase in *A. parasiticus* was cloned by Motomura et al. (1999) and was named *dmtA* or *mt-I* for O-methyltransferase I. The same gene was concurrently cloned by Yu et al. (2000a) in *A. parasiticus*, *A. flavus*, and *A. sojae*. This gene was named *omtB*. The new name of *dmtA* or *omtB* gene is *aflO*.

O-methyltransferase II enzyme is also involved in aflatoxin biosynthesis (Yabe et al. 1989). The rule of O-methyltransferase II is the conversation of ST to O-methylsterigmatocystine (OMST) and DMST to dihydro-O-methylsterigmatocystine (DHOMST) by transferring a methyl group of SAM to 7-OH of ST and DMST (Yabe and Nakajima 2004). O-methyltransferase II was purified by Keller et al. (1993) and its gene, *aflP* (*omtA*), was isolated based on the amino acid sequence of the purified enzyme (Yu et al. 1993). The absence of an *aflP* (*omtA*) homolog in *A. nidulans* is responsible for ST as the final product in this fungus.

The final step in the formation of aflatoxins is the conversion of OMST or DHOMST to aflatoxins B$_1$, B$_2$, G$_1$ and G$_2$, requiring the presence of a NADPH-dependent monooxygenase encoded by *aflQ* (*ordA*) (Prieto and Woloshuk 1997; Yu et al. 1998). The formation of the G toxins involves an additional step, possibly involving the enzyme encoded by *aflX* (*ordB*) (Yu et al. 1998; Yabe et al. 1999). Another gene, *aflT*, encodes an ABC transporter protein that may be necessary for aflatoxin efflux from the cells. Former studies (Yu et al. 1998) are suggested that additional enzymes are required for the synthesis of G-group aflatoxins. It is clear that *aflU* (*cypA*) encodes a cytochrome P450 monooxygenase for the formation of G-group aflatoxins (Ehrlich et al. 2004). Most recently, the *nadA* gene was also found to play a role in AFG$_1$/AFG$_2$ formation (Yu 2012). Cai et al. (2008) disrupted the *nadA* gene and reported that NadA is a cytosolic enzyme for the conversion from a new aflatoxin intermediate named NADA, which is between OMST and AFG$_1$. *A. flavus* produces only AFB$_1$ and AFB$_2$, whereas *A. parasiticus* produces all four major aflatoxins, AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$. Only the G-group aflatoxin producer, *A. parasiticus*, has intact *nadA* and *aflF* (*norB)* genes (Yu 2012). Preliminary data suggests that *aflF* (*norB)* encodes another enzyme predominantly involved in AFG$_1$/AFG$_2$ formation (Ehrlich 2008).

Molecular detection of aflatoxin producing fungi

Early attempts tried to confirm aflatoxin production in fungi using 1-3 genes (Shapira et al. 1996), however, these studies could not get reliable results. A multiplex reverse transcription-polymerase chain reaction (RT-PCR) protocol was elaborated by Degola et al. (2007). It was developed to discriminate aflatoxin-producing from aflatoxin-nonproducing strains of *A. flavus*. Five genes of the aflatoxin gene cluster of *A. flavus*, two regulatory (*aflR and aflS*) and three structural (*aflD, aflO, aflQ*, which synonyms are: *nor-1, omtB, ordA*), were targeted with specific primers to highlight their expression in mycelia cultivated under including conditions for aflatoxins production (Levin 2012).

Three different systems have been used for detection of aflatoxin producing isolates of these fungi targeting genes involved in the biosynthesis of aflatoxins: 1. a multiplex PCR assay targeting the *aflD* (*nor-1*), *aflR*, *aflP* (*omt-1*) genes, (Shapira et al. 1996), 2. PCR assays targeting the *aflP* (*omt-1*), *aflD* (*nor-1*), *aflM* (*ver-1*) genes individually (Färber et al. 1997) and 3. PCR assays amplifying individual sequences of the *aflRS*, *aflJ* and *aflO* (*omtB*) genes (Rahimi et al. 2008). Real-time quantitative PCR (qPCR) moreover provides a tool for accurate and sensitive quantification of target DNA (Mulé et al. 2006; González-Salgado et al. 2009; Rodríguez et al. 2011), that could be applied to quantify aflatoxins producing molds. In addition, qPCR has greatly simplified the procedure relative to conventional culturing techniques, with the continuous monitoring of samples through amplification which allows their easy identification using either the fluorescence of non-specific dyes, such as SYBR Green, which can also give a signal for primer-dimers and non-specific amplified products (Kubista et al. 2006), or a sequence specific hydrolysis probe (TaqMan) (Rodríguez et al. 2012).

A microarray based technique has also been developed recently which was used succesfully to study the effect of various factors on aflatoxin production in *A. flavus* (Schmidt-Heydt et al. 2009; Abdel-Hadi et al. 2012).

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